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The Prion-like Protein Doppel (Dpl) Interacts with the Human Receptor for Activated C-Kinase 1 (RACK1) Protein

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Abstract. *Background: Doppel (Dpl) is a homologue of the prion protein (PrP^C). In contrast to PrP^C, Dpl is dispensable for prion disease, but appears to have an essential function in male spermatogenesis. Recently, Dpl has been found to be aberrantly expressed in astrocytic and leukaemic tumor specimens, showing a peculiar cytosolic cellular localization. The aim of this study was to clarify some of the putative Dpl interacting proteins. Materials and Methods: A yeast two hybrid system was employed and the results were verified by co-immunoprecipitation using transfected cells. Results: Several potential Dpl-binding candidates were identified and, among them, the receptor for activated C-kinase (RACK1) protein was further investigated. RACK1 deletion mutants showed that some of its WD containing domains were directly involved in the binding with Dpl. Our data showed that Dpl interacts with RACK1 by means of its structured globular carboxyl-terminal region. Conclusion: This new Dpl interacting partner might suggest functional hypotheses about the role of this protein in an astrocytoma context where Dpl was found ectopically expressed.*

Doppel (Dpl) is an N-glycosylated, glycosylphosphatidylinositol (GPI) membrane-anchored prion-like protein encoded by the prion gene (*PRND*) gene, which has been found downstream of the *PRNP* in several species, including human, mouse, cattle, sheep and goat (1-3). Dpl is highly expressed in the testis, especially in Sertoli cells and in spermatozoa, and its involvement in male fertility has been recently proposed (4, 5). NMR studies of Dpl have revealed a high structural similarity with the prion protein (PrP^C) (6, 7), which supported the possibility that the two proteins share similar functions *in vivo*. Despite a multitude of studies, the cellular functions of Dpl and PrP^C are still unknown. However, current data suggested that Dpl, unlike PrP^C, is probably not required for the pathogenesis of prion diseases (8, 9) and is not converted into a PrP^{Sc}-like isoform (10, 11). Importantly, Dpl can cause Purkinje cell death and ataxia when over-expressed in *Prnp*-ablated mice (12). These mice are rescued from neuronal degeneration by the re-introduction of a *Prnp* transgene, arguing that the absence of PrP^C is necessary for Dpl to induce cell death (13) and that PrP^C and Dpl may compete for a common ligand (14). This possible PrP^C-Dpl interplay has attracted research towards the identification of common interacting proteins in order to highlight their cellular functions. In particular, several efforts have been made in order to investigate the function of PrP^C, identifying many protein interactants, although several appear to be located in cellular compartments that are not compatible to sustain interactions (15). Pivotal studies of Dpl interactants started with the evidence of common biochemical and topological features between Dpl and PrP^C and moved towards the identification of common protein partners. To date, only one PrP^C interacting protein, namely the 37 kDa/67 kDa laminin receptor (LRP) has been reported to interact also with Dpl (16); besides, our group has recently reported the absence of Dpl interaction with two cytoplasmic PrP^C interacting proteins, namely GFAP and Grb2 (17), while others have reported the failure of Dpl-Dpl and Dpl-PrP^C interactions (18). Due to the incompleteness of these data, it would be useful to perform a wider screening of Dpl interacting proteins. Thus, in this contribution a yeast two hybrid assay is described that suggests the binding of Dpl to the receptor for activated C-kinase 1 (RACK1) protein. Our results may provide new insights into the identification of Dpl cellular functions, particularly in some types of cancer, such as the astrocytomas, where we recently described an aberrant accumulation of Dpl in the cellular cytoplasm (19).

Abbreviations: AD, activation domain; BD, binding domain; DMEM, Dulbecco's modified Eagle's medium; ONPG, 2-nitrophenyl β-D-galactopyranoside; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; X-Gal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside; WD, tryptophan-aspartic acid; GPI, glucosyl-phosphatidylinositol; LRP, laminin receptor, GFAP, glial fibrillary acidic protein; Grb2, growth factor receptor-bound protein2.

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Table I. *The table shows all the oligonucleotides designed and employed in this work. The bases flanking the gene-specific sequences are in bold; restriction sites (underlined) and endonucleases are indicated. PCR thermal profile was: 94˚C for 3 min, 31 cycles of 94˚C for 20 sec, 62˚C for 30 sec, 72˚C for 1 min; final extension at 72˚C for 10 min.*

Name	Sequence (5'-3')	Restriction enzyme
Yeast Two Hybrid Assay:		
YDpl26E-U	**CCG GAA TTC** ACG AGG GGC ATC AAG CAC A	*Eco*RI
YDpl152B-L	**CGG GAT CC**T TAG CCC CTC TCC AAC CAA AAC	*Bam*HI
RACK-U	**TTC CAT ATG** ATG ACT GAG CAG ATG ACC CTT	*Nde*I
RACK-L	**CGA GCT CG**G CGT GTG CCA ATG GTC AC	*Sac*I
Rwd4-U	**TTC CAT ATG** GTG TGC AAA TAC ACT GTC CAG	*Nde*I
Rwd5-U	**TTC CAT ATG** AAC TGC AAG CTG AAG ACC AAC	*Nde*I
Rwd6-U	**TTC CAT ATG** AAC GAA GGC AAA CAC CTT TAC AC	*Nde*I
Rwd7-U	**TTC CAT ATG** CTG AAG CAA GAA GTT ATC AGT AC	*Nde*I
Rwd5-L	**CGA GCT CG**G AGA TCC CAT AAC ATG GCC T	*Sac*I
Rwd4-L	**CGA GCT CG**A GCC AGG TTC CAT ACC TTG A	*Sac*I
Rwd3-L	**CGA GCT CG**A CCC AGG GTA TTC CAT AGC T	*Sac*I
Rwd2-L	**CGA GCT CG**T GTG AGA TCC CAG AGG CG	*Sac*I
Rwd1-L	**CGA GCT CG**A TCC CTG GTC AGT TTC CAC A	*Sac*I
Co-immunoprecipitation:		
Dpl26-U	**GGA ATT C**CC ACG AGG GGC ATC AAG CAC A	*Eco*RI
Dpl152-L	**ATT TGC GGC CGC** TTA GCC CCT CTC CAA CCA AAA C	*Not*I
Dpl53-U	**CCG CTC GAG CG**G AGA ACC GCC CGG GAG	*Xho*I
Dpl53-L	**ATT TGC GGC CGC** CTC AGC CAC CTG GGC C	*Not*I
RACK1-U	**GGG GTA CC**A TGA CTG AGC AGA TGA CCC TT	*Kpn*I
RACK1-L	**ATT TGC GGC CGC** CTA GCG TGT GCC AAT GGT C	*Not*I

Materials and Methods

Yeast two hybrid assay. The "BD Matchmaker™ Library Construction & Screening Kit" (Clontech, Palo Alto, CA, USA) was employed for the yeast two hybrid assay. The bait plasmid containing the *PRND* coding region, encoding the 26-152 amino acids fragment, was amplified from human genomic DNA using YDpl26E-U and YDpl152B-L primers (Table I). PCR fragments were sub-cloned into the pGBKT7 plasmid, thus, generating the pDpl(26-152)-bait vector.

Prey plasmids were derived from a cDNA library, retrotranscribed from 2 μg of total RNA, isolated from a pool of 12 glioblastoma multiforme resections (19). Four hundred ng cDNA were amplified and co-transformed with 3 μg of *Sma*I-linearized pGADT7-Rec AD Cloning Vector (Clontech) into the *S. cerevisiae* yeast strain AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200,gal4Δ, gal80Δ, LYS2 :: GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$ *-GAL2*$_{TATA}$*-ADE2, URA3 :: MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ, MEL1*), following the manufacturer's instructions.

After an *in vivo* recombination-mediated cloning, transformants were plated on a -Leucine single dropout (SD) medium (Clontech), recovered and transformed again into the AH109 strain with 5 μg of the pDpl(26-152)-bait plasmid. Transformants were plated on -Leucine, -Tryptophan, -Histidine triple dropout (TDO) medium (Clontech). Colonies appeared after 5-6 days of incubation at 30˚C and were subsequently streaked on -Leucine, -Tryptophan, -Histidine, -Adenine quadruple dropout (QDO) medium (Clontech). Single colonies were subjected to PCR amplification, using the Matchmaker AD LD-Insert Screening flanking primers, to analyze the sizes of the inserts contained into the prey plasmids. Transformants were assayed for *lac*Z reporter transcription by a β-galactosidase filter assay and the positive clones were subjected to a liquid 2-nitrophenyl β-D-galactopyranoside (ONPG) assay, following the "Yeast Protocol Handbook" (Clontech). Finally, clones were inoculated overnight at 30˚C on TDO liquid medium: the culture pellets were treated with 50 U of lyticase (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 30˚C and the prey plasmids were extracted with the GeneElute™ Plasmid Miniprep kit (Sigma-Aldrich). Sequencing of the DNA inserts was performed with BigDye® Terminator Cycle Sequencing kit v 1.1 (Applied Biosystems, Foster City, CA, USA), subjected to automated sequencer analysis (ABI PRISM® 310 Genetic Analyzer, Applied Biosystems). The sequences were finally analyzed for nucleotide similarities with Genbank database entries using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Different portions of the *RACK1* sequence were amplified from the cDNA library with the primers containing *Nde*I and *Sac*I restriction sites, shown in Table I; the primer pairs used for each construct are shown in Figure 1. PCR products were sequenced, sub-cloned into pGADT7 plasmids and subjected to ONPG liquid assays, in the presence of the pDpl(26-152)-bait plasmid.

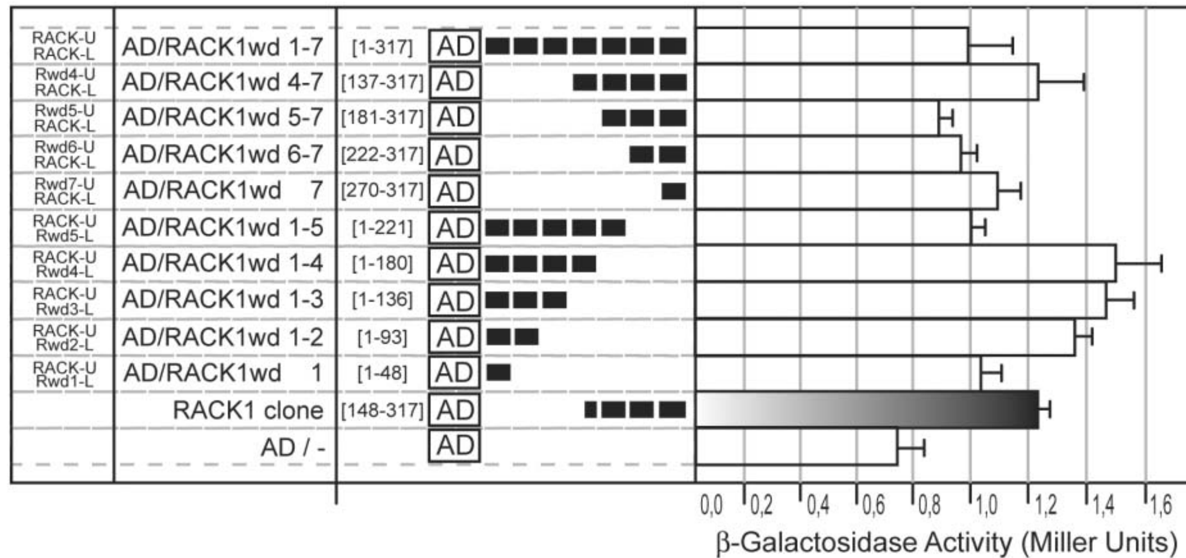

Figure 1. *ONPG liquid assays of protein extracts from cells containing the bait construct, BD/Dpl(26-152), and the RACK1 deletion mutants (AD/RACK1Δ). The figure shows, from the left, the employed primer pairs used to obtain mutants, the name of the AD/RACK1Δ fusion proteins produced, the schematic representation of the deleted proteins with the indication of RACK1 residues (between square brackets) and the β-galactosidase activity. Δ, RACK1 deletions.*

Cell cultures and transfections. HeLa cells (ECACC, Salisbury, UK) were maintained at 37˚C, 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% fetal bovine serum, L-glutamine (1%), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Transient transfections were performed in 60 mm Petri dishes using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Western blot analysis. For immunoblot analysis of yeast extracts, 3 ml overnight culture pellets were boiled for 15 min in 30 μl of Laemmli sample buffer (2% sodium dodecyl sulfate (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 then 15 μl were subjected to SDS polyacrilamide gel electrophoresis (SDS-PAGE) (12% gel). For the immunoblot analysis of HeLa cells extracts, 20 μg of total proteins were boiled for 5 min in Laemmli sample buffer and electrophoresed. Proteins were transferred onto HybondTM-C Extra (Amersham Biosciences, Little Chalfont, UK) and membranes were blocked with 2% non-fat milk phosphate-buffered saline (PBS) containing 0.1% Tween™ 20 (v/v). For immunodetection, the following primary antibodies were employed: rabbit polyclonal anti-human Dpl (kindly provided by Prof. Valentini), monoclonal anti-c-Myc (Clontech), both used at 1:10,000 dilution and rabbit polyclonal anti-HA (3:10,000, Clontech). The blots were then treated with specific peroxidase-coupled secondary antibodies (1:10,000, Amersham Biosciences) and the protein signals were revealed by an "ECL Advance™ Western Blotting Detection Kit" (Amersham Biosciences).

Co-immunoprecipitation. The Dpl(26-152) fragment and the entire RACK1 coding region were amplified from the above mentioned cDNA library using the Dpl26-U, Dpl152-L and RACK1-U, RACK1-L primer pairs, respectively (Table I). PCR products were sub-cloned into pCMV-HA and pCMV-Myc plasmids (Clontech), thus, originating the pHA/Dpl(26-152), pMyc/RACK1, pHA/RACK1 and pMyc/Dpl(26-152) plasmids; all the constructs were verified by sequencing.

Co-immunoprecipitation experiments were performed using HeLa total protein extracts, previously transfected with pHA/Dpl(26-152) and pMyc/RACK1 or with pHA/RACK1 and pMyc/Dpl(26-152) construct pairs. Twenty-four hours after transfection, cells were rinsed twice with ice-cold PBS followed by the addition of ice-cold lysis buffer (1% Triton® X-100, 100 mM NaCl, 50 mM Tris-Cl pH 8.0) and incubated for 30 min in ice. Cell debris were removed by centrifugation at 13,000 rpm for 12 min and supernatants were recovered, quantified by Bradford assay and supplemented with Complete Mini protease inhibitor cocktail (Roche, Basel, Switzerland). One hundred μg of proteins were incubated with 4 μg of anti-HA antibody for 15 h at 4˚C with gentle agitation. Then, 40 μl packed pre-washed protein A sepharose beads ("nProtein A 4FastFlow™", Amersham Biosciences) were added to each protein extract and incubated for 1 h and 30 min 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 10 μl of Laemmli sample buffer prior to SDS-PAGE and immunoblot analysis.

The Dpl(26-53) and Dpl(53-152) fragments, used in the deletion mapping of the Dpl-RACK1 interaction, were produced, as described above, with the primer pairs reported in Table I: Dpl26-U, Dpl53-L and Dpl53-U, Dpl152-L for Dpl(26-53) and Dpl(53-152) fragments, respectively. These products were inserted into the pCMV-Myc vector, thus, yielding the pMyc/Dpl(26-53) and pMyc/Dpl(53-152) expression constructs. These constructs were transfected and co-immunoprecipitated, as described above, against the HA/RACK1 fusion protein.

Results

The yeast two hybrid "Matchmaker™ Library Construction & Screening Kit" was employed in order to identify novel doppel protein interactants. To this purpose, a human cDNA library derived from retrotranscription of total RNA from 12 glioblastoma multiforme specimens was produced. The cDNA library was analyzed by a 1.2% agarose gel electrophoresis, revealing a smear ranging from 0.1 to 4.0 kb (data not shown). The library was co-transformed with the pGADT7-Rec plasmid *Sma*I-linearized into *Saccharomyces cerevisiae* yeast strain AH109 selecting for growth on SD-Leucine plates. After a 5- to 6-day incubation at 30˚C, about 10^5 clones were collected and transformed with the pDpl(26-152)-bait vector. Next, 130 clones were grown on TDO plates and, after a more stringent selection on QDO, the clones were reduced to 52. The sizes of the inserts cloned into the prey plasmids were evaluated by PCR using insert flanking primers; PCR products ranged from 0.3 to 2.0 kb (data not shown). The prey plasmids were then extracted from each yeast clone and the sequences were determined and compared to GenBank databases. Of all the clones examined, 41 matched known genes, as listed in Table II. Three clones were identical to the human *RACK1* gene (GenBank acc.n. BC032006, from 528 to 1060 nucleotides). The specificity of interaction of the products coded by the DNAs contained in the prey plasmids were initially verified by the re-transformation with the bait plasmid into AH109 and by growth on selective medium. As an additional control, the clones obtained after QDO selection were subjected to a β-galactosidase filter assay: all the clones turned blue in the presence of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Negative control yeast clones expressing either an empty prey plasmid and the pDpl(26-152)-bait construct or the empty prey and bait plasmids, did not confer the blue phenotype. Among the identified putative Dpl interactants, the RACK1 candidate protein was investigated further. The results of the β-galactosidase filter assay on the RACK1 clone that turned blue in the presence of X-gal similarly to the positive control (BD/p53 and AD/T-antigen) are shown in Figure 2A; in contrast, the negative control constructs combinations, *i.e.,* pDpl(26-152) and AD/-, gave a white colour phenotype to the yeast colony.

Total protein extraction was then performed for the RACK1 clone, separated by SDS-PAGE and visualized through a Western blot and immunoblotting analysis. As shown in Figure 2B, the BD/Dpl(26-152) and AD/RACK1 proteins were expressed in yeast cells, producing single bands of about 31 and 36 kDa apparent masses, as expected.

The RACK1 protein presented a peculiar secondary structure composed of seven repeated motifs, named WD domains, characterized by tryptophan (W)-aspartic acid (D) amino acid content. On the basis of this structure, nine different deletion constructs were generated into the pGADT7 prey plasmid, *i.e.,* AD/RACK1Δ constructs (Figure 1). An additional RACK1 construct, containing the entire coding region (AD-RACK1wd1-7) was generated. These constructs were analyzed for RACK1-Dpl protein interaction using a liquid ONPG assay, in the presence of the pDpl(26-152)-bait plasmid. The RACK1 clone isolated through the yeast two hybrid assay showed an activity of 1.2 Miller Units; as expected, the AD-RACKwd4-7 construct showed a similar β-galactosidase activity. Of note, the AD-RACK1wd1-4, 1-3, 1-2 constructs showed the highest β-galactosidase activities, while the remaining constructs, namely AD-RACKwd5-7, 6-7, 7, 1, 1-5 and 1-7, exhibited lower signals.

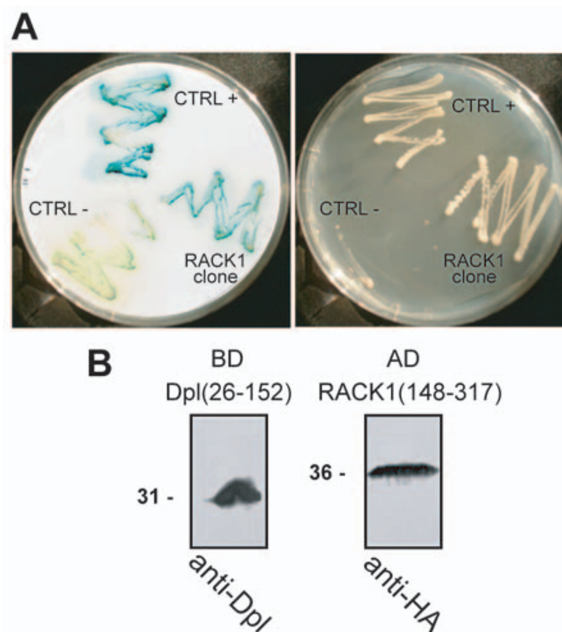

Figure 2. *RACK1 yeast clone characterization through β-galactosidase filter assay and selective growth on TDO (A) and immunoblotting analysis (B). (A) Filter assay, on the left, showing a blue reactivity of the RACK1 yeast clone comparable to CTRL+; the growth assay, on the right, performed on selective TDO plate, showing a growth of RACK1 clone equal to CTRL+. (B) RACK1 yeast clone lysate was visualized by immunoblot analysis, employing anti-Dpl and anti-HA antibodies, respectively. Apparent molecular masses are expressed in kDa. CTRL+: positive control, pGBKT7-p53/pGADT7-Rec-T antigen; CTRL-: negative control, pGBKT-Dpl(26-152)/pGADT7-Rec.*

Co-immunoprecipitation experiments were designed cloning a portion of the *PRND* coding sequence, corresponding to the mature Dpl protein (from 26 to 152 residues), and the entire *RACK1* coding region into the pCMV-HA and pCMV-Myc expression plasmids, respectively. The constructs were transfected into HeLa cells and the expressed HA-tagged

Table II. *Candidate Dpl-interacting proteins identified with the yeast two hybrid screening. For each gene are indicated: GenBank accession number, protein cellular localization and interacting proteins. Protein interactants information is derived from The Biomolecular Interaction Database (http://www.bind.ca/Action).*

Gene name and accession number (GenBank)	Cellular localization	Protein interactants
2,4 dienoyl CoA reductase 1, mitochondrial (DECR1) NM_001359	mitochondrion	-
Acylphosphate 2 (ACYP2) NM_138448	endoplasmic reticulum	-
Aquaporin 1 (AQP1) NM_198098	plasma membrane	-
ATPase, H+ transporting, lysosomal V0 subunit d isoform 1 (ATP6V0D1) NM_004691	lysosome	-
Beta 1,4-mannosyltransferase NM_001015050	endoplasmic reticulum	-
Breast cancer anti-estrogen resistance 1 BC062556	cytoplasm	-
CAAX box 1 (CXX1) NM_003928	plasma membrane	-
CD81 antigen (target of antiproliferative Ab1) (CD81) NM_004356	plasma membrane	-
Centrosome spindle pole associated protein (CSPP) AJ583433	cytoplasm	-
CLL-associated antigen KW-12 AF432212	cytoplasm	-
Collagen type I, alpha 2 (COL1A2) BC042586	extracellular	-
Complement component 1, q subcomponent, alpha polypeptide (C1QA) NM_015991	extracellular	-
DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4) NM_007034	extracellular	-
Dycarbonyl/L-xylulose reductase (P34H) BC001470	plasma membrane	-
Fasciculation and elongation protein zeta 1(FEZ1) BC009545	cytoplasm	JC virus agnoprotein, microtubule, NBR1, CIB.
Ferritin, heavy polypeptide 1 (FTH1) NM_002032	cytoplasm	-
Fragile histidine triad gene (FHIT) BC032336	nucleus, plasma membrane	Tubulin, UBC9.
Fuse binding protein interacting repressor (FIR) BC035435	nucleus	-
FXYD domain containing ion transport regulator 6 (FXYD6) NM_022003	plasma membrane	-
G protein alpha subunit (GNAS) BC008855	cytoplasm	-
Glicoprotein M6B (GPM6B) NM_001001996	plasma membrane	-
Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) BC020308	cytoplasm	Rpb7, Rab2, Sca1.
Receptor for activated C-kinase 1 (RACK1) BC032006	cytoplasm	A73 (Epstein-Barr virus protein), Androgen receptor, AGTRAP, acetylcholinesterase, β-dynamin, β-integrin, β-spectrin, β-tubulin, BZLF1 (Epstein-Barr virus protein), DAT, E1A (Adenovirus protein), E1A oncoprotein, eIF6, FAN, G β1γ1, GABA type A receptor, GM-CSF receptors, IGF-1 receptor, IL-3, IL-5, JNK, Ki-1/57, M1 (influenza virus protein), mRNP, Nef (HIV-1 protein), NHERF1, NMDAR, NR2B, p120GAP, p19, p73, p63α, P85, PDE4D5, PKCb, PLCγ, Plectin, PtPn catalytic region, Src Kinases, STAT-1, type 1 interferon receptor.
Histocompatibility (minor) 13 (HM13) NM_030789	endoplasmic reticulum	-
Interferon, gamma inducible protein 30 (IFI30) BC031020	lysosome	-
Karyopherin alpha 1 (importin alpha 5, KPNA1) BC002374	nucleus	Stat.
NADH dehydrogenase (ubiquinone) FeS protein 6 (NDUFS6) BC038664	mitochondrion	-
NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1) BC008146	mitochondrion	-
Nucleotide binding protein (NBP) AF208536	cytoplasm	-
Phosphofructokinase platelet (PFKP) BC002536	-	-
Polymerase, alpha 2 BC018813	nucleus	-
Proliferation-inducing protein 16 (PIG16) AY305873	-	-
Protein 4 transactivated by hepatitis B virus X (XTP4) AF490253	-	-
Ribosomal protein L15 (RPL15) NM_002948	cytoplasm	-
Similar to CRK-associated substrate AK124844	-	-
Squamous cell carcinoma antigen recognized by T cell (SART) BC018607	nucleus	-
Translation elongation factor 1 alpha 1 (EEF1A1) BC082268	cytoplasm	eEF1Ba, eEF1Bb.
Trifunctional protein, alpha subunit (HADHA) NM_000182	mitochondrion	-
Ubiquitin C (UBC) BC014880	cytoplasm	-
Ubiquitin-conjugating enzyme (E2E3) BC092407	cytoplasm	-
Vacuolar protein sorting 35 (VPS35) NM_018206	cytoplasm	-

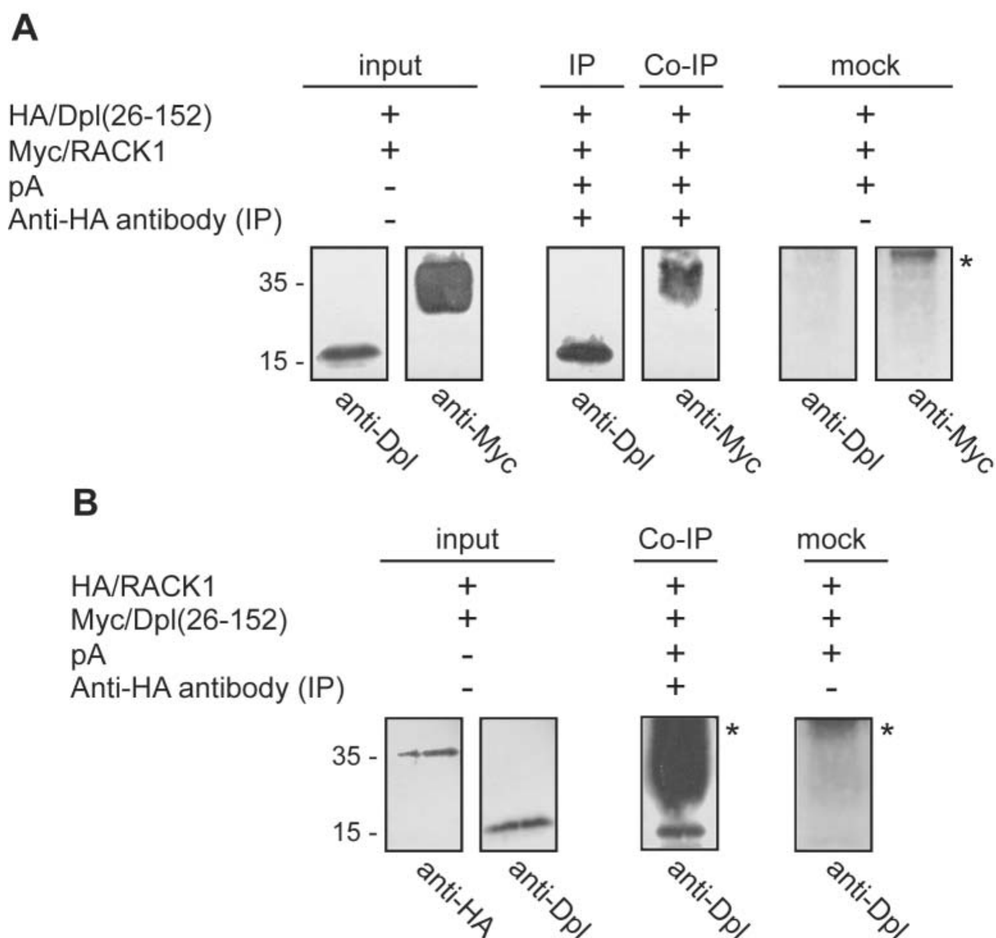

Figure 3. *Co-immunoprecipitation experiments of HA/Dpl(26-152) and Myc/RACK1 (A) or HA/RACK1 and Myc/Dpl(26-152) (B) fusion proteins. (A) The formation of the HA/Dpl(26-152)-Myc/RACK1 complex was assayed with anti-HA antibody for immunoprecipitation and anti-c-Myc antibody for detection: electrophoretic band corresponding to the co-immunoprecipitated Myc/RACK1 construct appeared at 35 kDa in the Co-IP lane. (B) The interaction Dpl(26-152)-RACK1 was demonstrated by inverting the tags. At the bottom of each lane are shown the primary antibodies employed. pA: protein A sepharose, input: protein lysate, IP: immunoprecipitate, CoIP: co-immunoprecipitate, wb: Western blot, mock: negative control, asterisk indicates pA and/or antibody heavy chain.*

fusion proteins were immunoprecipitated with anti-HA tag antibody. The immunoprecipitated proteins were subjected to SDS-PAGE and the co-immunoprecipitated proteins were revealed by Western blotting with anti-c-Myc antibody. In Figure 3A Myc/RACK1 was found to co-precipitate with HA/Dpl(26-152), and the corresponding band matched that of the control lysate (input); on the contrary, negative control did not reveal any band. These results confirmed the interaction of the fusion proteins, HA/Dpl(26-152) and Myc/RACK1, previously revealed in yeast, also in mammalian cells. To further confirm the interaction, the inverted constructs were assayed, *i.e.,* HA/RACK1 and Myc/Dpl(26-152), demonstrating once again the co-immunoprecipitated complex (Figure 3B).

In order to map more finely the interaction between Dpl and RACK1, two portions of Dpl, namely Dpl(26-53) and Dpl(53-152), corresponding to the unfolded and structured globular protein, respectively, were fused to the c-Myc tag and assayed in co-immunoprecipitation experiments with the HA/RACK1 construct. The two Dpl constructs were both expressed in transfected HeLa cells, as shown in Figure 4; however, co-immunoprecipitation of Dpl with RACK1 was only obtained in the case of the Myc/Dpl(53-152) construct.

Discussion

Doppel is a GPI-anchored protein with structural similarities with the prion protein. Dpl is predominantly expressed in the testis and Dpl ablated mice are sterile due to impaired sperm cells production (4). The physiological role of the protein has not been completely elucidated yet;

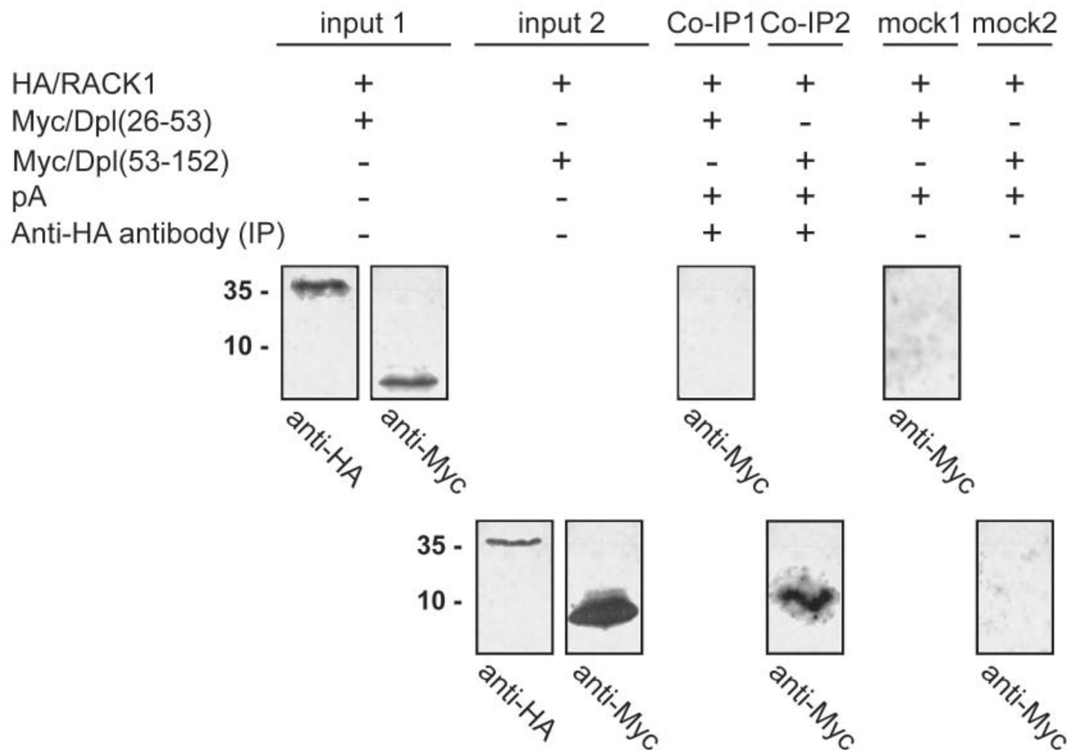

Figure 4. *RACK1 deletion interaction mapping using co-immunoprecipitation assays. The figure summarizes the interaction between RACK1 and Dpl carboxyl-terminal domain, Myc/Dpl(53-152). No electrophoretic band was detected in the Co-IP2 lane, corresponding to the Dpl amino-terminal domain, Myc/Dpl(26-53). The primary antibodies used are shown at the bottom.*

however, its over-expression in PrP^C knock-out mice leads to a neurodegenerative condition with pathological signatures similar to the prion diseases (1).

We have recently described other pathological conditions characterized by an altered expression of doppel. Abnormal expression of *PRND* transcripts in brain-derived tumors, such as astrocytomas and meningiomas, (19), and in acute myeloid leukaemias and myelodysplastic syndromes were detected (20). In tumor-derived cells, Dpl was found in the cytoplasm and showed abnormal glycosylation patterns (Comincini, unpublished data). In a recent study, we tried to address the issue of possible Dpl protein functions within astrocytic tumors by means of protein-interaction studies, reporting the absence of Dpl interaction with two known previously described PrP^C cytoplasmic interacting proteins (GFAP and Grb2) and with PrP^C and Dpl themselves (17). In the present study, owing to the significant expression of Dpl in astrocytic brain tumors, a more extensive screen for potential Dpl interacting candidates was performed. To this purpose, a yeast two hybrid assay was carried out, using a glioblastoma multiforme-derived cDNA expression library as a source of protein interacting candidates. The yeast two hybrid system, originally developed by Fields and Song (21), is a widely used method to detect protein-protein interactions (22). In particular, the system we employed is characterized by an *in vivo* homologous recombination within yeast cells, which allows a rapid preparation of the expression library, as well as a stringent screening of yeast clones containing putative interacting candidates.

The results of the yeast two hybrid screening have supported several novel putative Dpl interacting partners. Noticeably, the previously described Dpl interacting protein, LPR (16), was not selected. After a very stringent selection 52 clones of which 41 contained prey plasmids with DNA inserts homologous to known gene sequences were obtained: unlikely Dpl-candidate interactants were identified because of the subcellular localization of their gene products, such as the mitochondrial (DECR1, NDUFS6, NDUFV1) or nuclear (FIR, KPNA1, polymerase α2, SART). Other proteins were more acceptable candidates, according to their plasma membrane (AQP1, P34H, GPM6B), endoplasmic reticulum (HM13) and cytosolic (FEZ1, RACK1, NBP, E2E3) localization. In particular, Dpl cytosolic potential interaction candidates were focused on owing to the recent cytoplasmic cellular localization of Dpl in astrocytic tumors (19).

Among the yeast selected clones, the *RACK1* sequence was independently isolated three times. RACK1 is a well

characterized cytosolic adaptor molecule, homologous to the hetero-trimeric G protein β-subunit, and is ubiquitously expressed in different tissues of higher mammals and humans. Furthermore, RACK1 shows a high conservation among eukaryotes suggesting that it may contribute to fundamental cellular processes such as cell development, proliferation, movement and adhesion (23). In particular, the central nervous system seems to be an important tissue for RACK1 functioning, as it regulates intracellular Ca^{2+} level and is involved in processes such as learning, memory and synaptic plasticity (24). Interestingly, RACK1 levels were found to be altered in some types of cancer, although it is not clear whether these changes constitute compensatory responses counteracting the cancer phenotype (25). RACK1 has a peculiar secondary structure, composed of seven WD (tryptophan-aspartic acid) domains (26, 23). Similarly, many proteins involved in important cellular functions (RNA-processing, transcription regulation, cytoskeleton assembly, vesicular trafficking and signal transduction) contain WD domains (27). RACK1 was initially described as an intracellular receptor for the activated C-kinase (28) and, until now, several RACK1 interacting partners involved in different transduction pathways have been described (Table II).

The Dpl-RACK1 interaction, revealed by the yeast two hybrid assay, was confirmed by co-immunoprecipitation experiments, using over-expressed HA- and Myc-tagged fusion proteins. Furthermore, co-immunoprecipitation experiments of HA/RACK1 fusion proteins with Dpl deletion mutants, indicated that the structured portion, *i.e.,* Dpl(53-152), was directly involved in the RACK1 interaction. In contrast, Yin and colleagues described the N-terminal unfolded portion of Dpl as an important determinant in the interaction between Dpl and laminin receptor proteins (16). The RACK1 protein structure was also analyzed for the different contribution of its WD domains in the Dpl-interaction and, using WD deletion mutants, it was shown that specific domains differently contribute to the interaction with Dpl.

Finally, an attempt was made to confirm the Dpl-RACK1 interaction by means of a bioinformatics analysis. It is known that RACK1 can interact with different proteins containing PH domains (29). PH domains, originally identified in the Pleckstrin protein, a platelets and leukocytes C kinase substrate, are stretches of 100-120 amino acids present in many proteins involved in cellular signalling, cytoskeletal organization and membrane trafficking (30). Interestingly, a ClustalW analysis (31) (http://www.ebi.ac.uk/clustalw/) between the entire Dpl protein sequence and the two PH domains of Pleckstrin protein (PH-N and PH-C) highlighted conserved amino acids, mainly clustered within the structured Dpl portion; these *in silico* results support our experimental finding of the co-immunoprecipitation between RACK1 and Dpl(53-152). In contrast, the PrP^C protein sequence was not significantly aligned with the PH domain.

In conclusion, a novel interaction of Dpl with the adaptor protein RACK1, involved in different signal transduction cascades was described. This interaction, mainly sustained by the ectopic cytoplasmic localization of Dpl in the astrocytic tumors, might drive Dpl to participate with RACK1 in common molecular pathways. Future studying will target to further highlight the functional nature of Dpl and RACK1 interplay, with particular regard to the astrocytic tumor context, in order to identify a putative role of Dpl in the tumorigenesis and to eventually suggest novel therapeutic targets for the treatment of astrocytomas.

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