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A Functional Network of the Tumor Suppressors APC, hDlg, and PTEN, that Relies on Recognition of Specific PDZ-Domains

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ABSTRACT

APC and PTEN are tumor suppressor proteins that bind through their C-termini to the PDZ domain containing-hDlg scaffolding protein. We have found that co-expression of PTEN and hDlg enhanced the negative regulation of the PI3K/Akt pathway by PTEN, indicating the physiologic importance of these interactions. APC and PTEN share other PDZ domain containing–interacting partners, including the MAGI scaffolding proteins and the MAST family of protein kinases. Mutational analysis revealed that the C-terminal PDZ-binding motifs from APC and PTEN were differentially recognized by distinct PDZ domains. APC bound to the three PDZ domains from hDlg, whereas PTEN mainly bound to PDZ-2/hDlg. This indicates the existence of overlapping, but distinct PDZ-domain recognition patterns by APC and PTEN. Furthermore, a ternary complex formed by APC, PTEN, and hDlg was detected, suggesting that hDlg may serve as a platform to bring in proximity APC and PTEN tumor suppressor activities. In line with this, tumor-related mutations targeting the PDZ-2/hDlg domain diminished its interaction with APC and PTEN. Our results expand the PDZ-domain counterparts for the tumor suppressor APC, show that APC and PTEN share PDZ-domain partners but have individual molecular determinants for specific recognition of PDZ domains, and suggest the participation of the tumor suppressors APC, PTEN, and hDlg in PDZ-domain interaction networks which may be relevant in oncogenesis. J. Cell. Biochem. 113: 2661–2670, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: PDZ DOMAIN; APC; HDLG; PTEN; TUMOR SUPPRESSOR

The adenomatous polyposis coli (APC) and the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) are major tumor suppressor proteins, which exert functions at multiple cell compartments as negative regulators of cell transformation. The *APC* gene is frequently mutated in sporadic carcinomas and adenomas, as well as in the germ-line of familial adenomatous polyposis (FAP, Gardner's syndrome) patients, a disease that manifests with multiple adenomatous polyps in the colon and the rectum, and frequent extraintestinal tumors. APC is a multi-domain high molecular protein whose major function is to regulate cell adhesion and proliferation through its binding to β-catenin, but also exerts β-catenin-independent functions [Aoki and Taketo, 2007; Brocardo and Henderson, 2008; Phelps et al., 2009]. Homozigous deletion of *APC* gene in mice is lethal before gastrulation, whereas heterozygous mice are viable and exhibit a polyposis phenotype similar to FAP patients [Moser et al., 1995; Oshima et al., 1995].

The *PTEN* gene is mutated with high incidence in a wide variety of human cancers, and germ-line *PTEN* mutations are causative of PTEN hamartoma tumor syndrome (PHTS), a disorder with multiple manifestations including multiple hamartomas, macrocephaly, and predisposition to cancer. PTEN is the lipid phosphatase that dephosphorylates the D3 position of phosphatidylinositol (3,4,5)-triphosphate (PIP3), antagonizing the action of the oncogenic phosphatidylinositol 3-kinase (PI3K) and counteracting the pro-survival and cell growth activity of the protein kinase PKB/Akt. In addition, PTEN regulates key cellular functions, including genome stability and gene transcription, independently of its catalytic activity [Salmena et al., 2008; Georgescu, 2010; Leslie et al., 2010].

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Homozigous deletion of *PTEN* gene in mice is also lethal, and heterozygous or haploinsufficient PTEN mice resemble the PHTS phenotype and are prone to develop tumors [Berger and Pandolfi, 2011]. Both APC and PTEN posses at their C-termini a functional type I PDZ-domain binding motif, which regulates subcellular location, stability, and function of these proteins through binding to specific PDZ-domain ligands [Matsumine et al., 1996; Ishidate et al., 2000; Wu et al., 2000ab; Valiente et al., 2005; Bonifant et al., 2007; Mimori-Kiyosue et al., 2007].

The human discs-large protein (hDlg) is an ortholog of Dlg from *Drosophila*, a PDZ-domain containing scaffolding protein with tumor suppressor activity in this organism [Caruana, 2002]. hDlg is an ubiquitous protein that mainly locates at submembranal areas in cell–cell contact sites, and possesses three PDZ domains through which interacts with a variety of signaling proteins, such as NMDA-, AMPA-, and kainato-receptor subunits or the kinases p38γ, MEK2, and PBK [Leonard et al., 1998; Gaudet et al., 2000; Sabio et al., 2005; Wang et al., 2005; Maiga et al., 2011]. The hDlg PDZ-2 domain binds to both PTEN and APC, and the hDlg:APC interaction regulates negatively cell growth [Matsumine et al., 1996; Adey et al., 2000; Ishidate et al., 2000]. Remarkably, several viral oncoproteins bind to the hDlg PDZ domains and target hDlg for degradation, or block its association with specific PDZ binding partners [Kiyono et al., 1997; Lee et al., 1997; Gardiol et al., 1999; Suzuki et al., 1999]. hDlg displays reduced expression in several human cancers, including cervical/cervix neoplasia, colon carcinoma, and malignant fibrous histiocytoma, which correlates with bad prognosis [Watson et al., 2002; Cavatorta et al., 2004; Gardiol et al., 2006]. Mutations in the hDlg PDZ-2 domain have been found in breast cancer samples [Fuja et al., 2004], although no functional information is available regarding these mutations.

In this study, we have analyzed the functional interactions between APC, PTEN, and PDZ-domain containing proteins, including hDlg, and the putative implications of these interactions in human cancer. We have found that APC and PTEN share PDZ-domain binding partners. PTEN and APC displayed distinct binding specificity towards the PDZ domains of hDlg, and cancer mutations targeting the hDlg PDZ-2 domain diminished its binding to those proteins. Furthermore, PTEN, APC and hDlg were able to form ternary complexes, indicating the existence of a network of tumor suppressor interactions facilitated by PDZ-domain binding.

MATERIALS AND METHODS

PLASMIDS, ANTIBODIES, AND WESTERN BLOT

pRK5 HA-PTEN (N-terminal tagging), pGEX-4T PTEN, pGEX-4T PDZ-2/MAGI-2 (residues 591-732), pGEX-4T PDZ-2/MAGI-3 (residues 567–697), pGEX-4T PDZ-2/hDlg (residues 308–411), pGEX-4T PDZ/MAST1 (SAST; residues 958-1065), pGEX-4T PDZ/MAST2 (MAST205; residues 1031–1138), pGEX-4T PDZ/MAST3 (residues 939–1046), and pGEX-4T PDZ/MAST4 (residues 638–751), have been previously described [Andrés-Pons et al., 2005; Valiente et al., 2005]. pRK5 HA-APC C-terminus and pEG202 APC C-terminus (residues 2528-2843; human sequence), were generated by PCR amplification from pCMV HA-APC (provided by Mireia Dunach), and subcloning. pCMV-HA-Akt1 (human sequence) and EGBT-GST-hDlg (rat sequence) were provided by Amancio Carnero and Ana Cuenda, respectively [Sabio et al., 2005]. pJG4-5 PDZ-2/MAGI-2, pJG4-5 PDZ-2/MAGI-3, pJG4-5 PDZ/MAST2, pJG4-5 PDZ/MAST3, pJG4-5 PDZ/MAST4, and pJG4-5 PDZ/MAST1, encoding the corresponding PDZ domains fused at their N-terminus to the B42 transcription activation domain, were obtained by direct subcloning from the pGEX constructs indicated above. pEG202 PTEN, encoding PTEN fused at its N-terminus to the LexA DNA-binding domain, was obtained by subcloning the entire PTEN coding sequence (residues 1–403). PDZ-2/hDlg mutations (G338R, I348V, G338R/I348V, and L329R/G330R [GLGF to GRRF]), were obtained by PCR oligonucleotide site-directed mutagenesis, and subcloned into pGEX-4T and pJG4.5. PTEN and APC single amino acid substitutions were obtained by PCR and subcloned into pEG202. All the mutations were confirmed by DNA sequencing. The antibodies used were anti-pAkt [$pThr^{308} + pSer^{473}$] and anti-Akt (polyclonals; Cell Signaling), anti-PTEN 421B + 425A (monoclonals) [Andrés-Pons et al., 2005], anti-GST [polyclonal; Andrés-Pons et al., 2005], anti-LexA (polyclonal; provided by Wiljan Hendriks), and anti-HA 12CA5 (monoclonal). Secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit (Oncogene) and anti-mouse (Promega) IgG antibodies. Dilution of antibodies was done in NET-gelatine buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, 0.25% gelatine). Quantitation of protein band intensities from immunoblots (Fig. 1) was made using ImageJ software. Values are given as means ± SEM of at least two independent experiments. Differences between groups were analyzed by two-tailed Student's *t*-test. Significance: $^{*}P < 0.05$.

CELL CULTURE, TRANSFECTION, AND CELL LYSIS

HEK293 (human embryonic kidney), COS-7 (simian kidney), and MCF-7 TetOn (human breast carcinoma; [Nunes-Xavier et al., 2010]) cells were grown in Dulbecco's modified Eagle's medium (DMEM; HEK293, COS-7) or Roswell Park Memorial Institute medium (RPMI; MCF-7) 1640 (Invitrogen), respectively, containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin. HEK293 and COS-7 cells were transfected by the calcium phosphate method. To generate MCF-7 TetOn cell lines stably expressing PTEN, cells were transfected with pTREhyg-empty vector or pTREhyg-PTEN, using FuGene (Roche Diagnostics), and PTEN expression was induced by 100 ng/ml Doxycycline (Dox; Sigma–Aldrich). MCF-7 TetOn stable cell lines were grown in the presence of neomycin (200 μg/ml) and hygromycin (100 μg/ml). Cell proliferation/viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, according to the manufacturer's protocol (Roche). Cells were plated at a density of 3,000 cells per well (96-well plates) with complete medium for 24 h. Then cells were incubated for 1–4 days and collected for processing. The absorbance was measured at 580 nm. Data are presented as the average absorbance ±SD, from at least three different experiments. Cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P40; 1 mM phenylmethylsulfonyl fluoride; 1 μg/ml aprotinin; 100 mM NaF; 20 mM $Na_4P_2O_7$; 2 mM Na_3VO_4). The cell lysates were centrifuged (14,000 rpm, 10 min at 4°C), and the

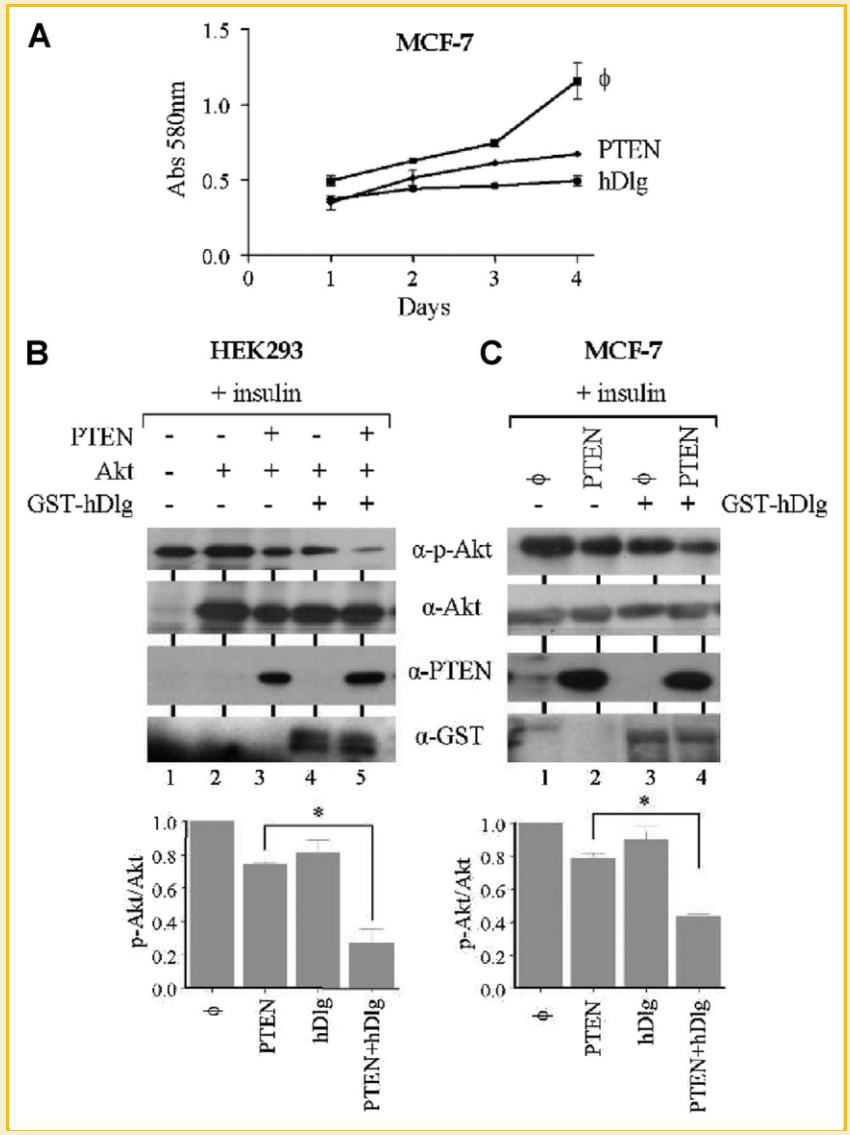

Fig. 1. PTEN and hDlg growth-related activities in cells. A: PTEN and hDlg block cell proliferation/viability. MCF-7 TetOn cell lines, mock (ϕ) or stably expressing PTEN, or mock cells transiently transfected with EGBT-hDlg, were grown during 4 days, and proliferation/viability was measured by the MTT assay. B,C: PTEN activity in the presence of hDlg. In (B), HEK293 cells were co-transfected with the plasmids pRK5-PTEN, EGBT-hDlg, and pCMV-HA-Akt1, as indicated (+), or with empty vector (−, ϕ). 48 h after transfection, the cells (24 h FCS starved) were treated with 100 nM insulin for 20 min and analyzed for p-Akt content by Western blot. In (C), MCF-7 TetOn cell lines, mock (ϕ) or stably expressing PTEN, were transfected with EGBT-hDlg (+) or with empty vector (−). 48 h after transfection, the cells (24 h FCS starved) were treated with 100 nM insulin for 20 min and analyzed for p-Akt content by Western blot. In the lower panels, relative values of the ratio p-Akt/Akt, obtained after quantitation of the bands (from the corresponding upper lanes) from at least two independent experiments, are shown. $^{*}P < 0.05$.

supernatants were used directly for Western blot analysis or for GST pull-down followed by Western blot.

GST FUSION PROTEINS, AND GST PULL-DOWN

The GST fusion proteins containing the different PDZ domains were expressed in bacteria and purified with glutathione–Sepharose using standard procedures. For GST pull-down experiments, HEK293 or COS-7 cells transfected with the expression plasmids encoding HA-tagged recombinant proteins were lysed as indicated above, and the cell lysates were incubated for 2 h with the GST fusion proteins, followed by the addition of glutathione–Sepharose beads and further incubation for 2 h; the incubations were at 4°C and under constant shaking. Samples were washed four times with HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1% glycerol), resolved on 10% SDS–polyacrylamide gels under reducing conditions, and analyzed by Western blot with the anti-HA 12CA5 mAb.

YEAST TWO- AND THREE-HYBRID ANALYSIS

The *Saccharomyces cerevisiae* strains EGY48 (*MATα*, *trp1*, *his3*, *ura3*, *LEU2*::*LexA-op6-LEU2*; for two-hybrid analysis) and YPH499 (*MATa ade2-101 trp1-63 leu2-1 ura3-52 his3*-D*200 lys2-801*; for

three-hybrid analysis) were used. For two-hybrid analysis, cells were transformed with the LacZ reporter plasmid pMW107 (URA3 marker, LexAop4-lacZ) together with pairwise combinations of the pEG202 PTEN or pEG202 APC C-terminus (HIS3 marker), and the pJG4-5 PDZ (TRP1 marker) plasmids. For three-hybrid experiments, cells were transformed with pMW107, pEG202 APC C-terminus, pJG4.5 PTEN, and YCpLG HA-PDZ-1-3/hDlg (LEU2 marker) plasmids. Co-transformants were selected on the appropriate selective medium. As a control, the empty vectors were used. Three independent co-transformant colonies were induced in selective medium supplemented with 2% galactose 1% raffinose; after overnight growth, the cultures were normalized to *A*600 of 1. Cultures were collected by centrifugation, washed and resuspended in buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), and were lysed by adding $CHCl_3$ and SDS. The lysates were incubated in the presence of the β-galactosidase substrate, ONPG (*o*-nitrophenyl β-D-galactopyranoside, 2 mg/ml in buffer Z; 50 μl per sample), and the hydrolysis of ONPG was measured at 405 nm for 3 h at 30°C (99 measurements for each well in a time interval of 1 min) in a Wallac 1420 multiplate reader. Results are shown as bar graphs, where the values represent the slopes of the kinetic curves of enzyme activity versus time. Statistic values are given as means ± SEM of at least three independent experiments. Differences between groups were analyzed by two-tailed Student's *t*-test. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$.

RESULTS

FUNCTIONAL COOPERATION OF PTEN AND hDlg

PTEN binds to multiple PDZ domains through its C-terminal type I PDZ-domain binding motif, including the PDZ-2 domain from the scaffolding proteins MAGI-2/3, the PDZ-2 domain from the tumor suppressor hDlg, and the unique PDZ domain from the protein kinases MAST1/2/3 [Gericke et al., 2006]. The binding between PTEN and hDlg [Adey et al., 2000] suggests the possibility of cooperative anti-oncogenic effects on these two proteins. As shown, constitutive overexpression of PTEN in MCF-7 breast carcinoma cells diminished cell proliferation/viability, and a stronger inhibitory effect was observed upon hDlg overexpression (Fig. 1A). This prompted us to test, by co-expression experiments, the effect of hDlg on the efficiency of PTEN to inhibit the PI3K/Akt pathway. The overexpression of hDlg in HEK293 or MCF7-PTEN cells enhanced the efficiency of PTEN to block the activation of Akt after insulin treatment, as measured by Akt phosphoactive-content (Fig. 1B, lane 3 vs. lane 5; and Fig. 1C, lane 2 vs. lane 4). This demonstrates a positive role for hDlg in PTEN tumor suppressor function and indicates that PTEN:hDlg interactions may be relevant in tumorigenesis. Thus, additional studies to further characterize such interactions were performed, and compared with the binding of a third tumor suppressor, adenomatous polyposis coli (APC), to PDZ domains.

APC BINDS TO PDZ-DOMAIN PTEN PARTNERS

The APC C-terminal region contains a functional type I PDZ-domain binding motif that also recognizes the PDZ-2 domain from hDlg [Matsumine et al., 1996], making possible that APC could bind to other PDZ-domain PTEN partners. To test this possibility, GST pull-down experiments were performed using GST-PDZ fusion proteins purified from bacteria, and the HA-tagged C-terminal domain of APC (APC C-terminus; residues 2,528–2,843) expressed in mammalian cells. HA-APC bound by GST pull-down to PDZ-2/hDlg, PDZ-2/MAGI-2, PDZ-2/MAGI-3, PDZ/MAST1, PDZ/MAST2, PDZ/MAST3, but not to PDZ/MAST4 (Fig. 2A). Mutation of the APC C-terminal PDZ-domain binding motif (APC V2843A mutation) abrogated its binding to PDZ domains (Fig. 2B). Yeast two-hybrid analysis was also performed using as bait the C-terminal domain of APC. APC wild type bound to PDZ-2/hDlg, PDZ-2/MAGI-2, PDZ-2/MAGI-3, PDZ/MAST1, PDZ/MAST2, PDZ/MAST3, but not to PDZ/MAST4, whereas APC V2843A did not bind any of these PDZ domains (Fig. 2C). In conclusion, the PDZ-domain binding profile of APC was coincident with than observed for PTEN [Valiente et al., 2005], indicating that APC and PTEN share PDZ-domain partners. However, the PDZ-domain binding motifs from APC and PTEN differ in their −1 position (APC: -Thr^{2841}Ser^{2842}Val^{2843}, -TSV; PTEN: -Thr^{401}Lys^{402}Val^{403}, -TKV) (Fig. 3A). Thus, the contribution of Ser^{2842} in APC and Lys^{402} in PTEN to the interaction of APC and PTEN with the PDZ domains was analyzed quantitatively by yeast two-hybrid experiments, using APC-Ser^{2842}- and PTEN-Lys^{402}-mutations and PDZ/MAST2 and PDZ-2/MAGI-2 (Fig. 3B). Interestingly, these mutations affected differentially to the binding of the distinct PDZ domains to APC and PTEN, as well as to the binding of the same PDZ domain to these two tumor suppressors. Thus, APC S2842C bound weakly to PDZ/MAST2, but not to PDZ-2/MAGI-2, as compared with binding of APC wild type. Conversely, PTEN K402C displayed diminished binding to PDZ-2/MAGI-2, but not to PDZ/MAST2. Mutations APC S2842W and PTEN K402W diminished the binding to PDZ/MAST2, but not to PDZ-2/MAGI-2, whereas mutations APC S2842A and PTEN K402A did not affect, or increased, the binding to the PDZ domains. Note that PTEN K402Q mutation, mimicking the acetylation of the PTEN Lys^{402} residue, increased the binding of PTEN to the PDZ domains, in agreement with recent findings showing a positive regulation of PTEN Lys^{402} acetylation on this binding [Ikenoue et al., 2008]. As a control, the lack of binding of the PDZ domains to the APC V2843A or the PTEN V403A mutations is shown (Fig. 3B). These results indicate that the PDZ-domain binding motifs from APC and PTEN are distinctly recognized by their common PDZ-domain partners.

APC AND PTEN DIFFERENTIALLY ASSOCIATE WITH hDlg PDZ DOMAINS TO ASSEMBLY IN TERNARY COMPLEXES

The binding of APC and PTEN to the three distinct hDlg PDZ domains was further investigated. GST pull-down experiments using the individual hDlg PDZ domains and recombinant HA-PTEN and HA-APC 2528-2843 showed that PTEN mainly associated to PDZ-2/hDlg, whereas APC associated to PDZ-1, PDZ-2, and PDZ-3/hDlg (Fig. 4A). The binding of APC to the three hDlg PDZ domains was confirmed by quantitative yeast two hybrid analysis. As shown, APC bound stronger to PDZ-2 and PDZ-3/hDlg domains. Non-additive binding was observed using the PDZ-1-2 and PDZ-1-3/hDlg domain combinations, when compared with the binding to the individual PDZ domains (Fig. 4B). In the case of PTEN:hDlg

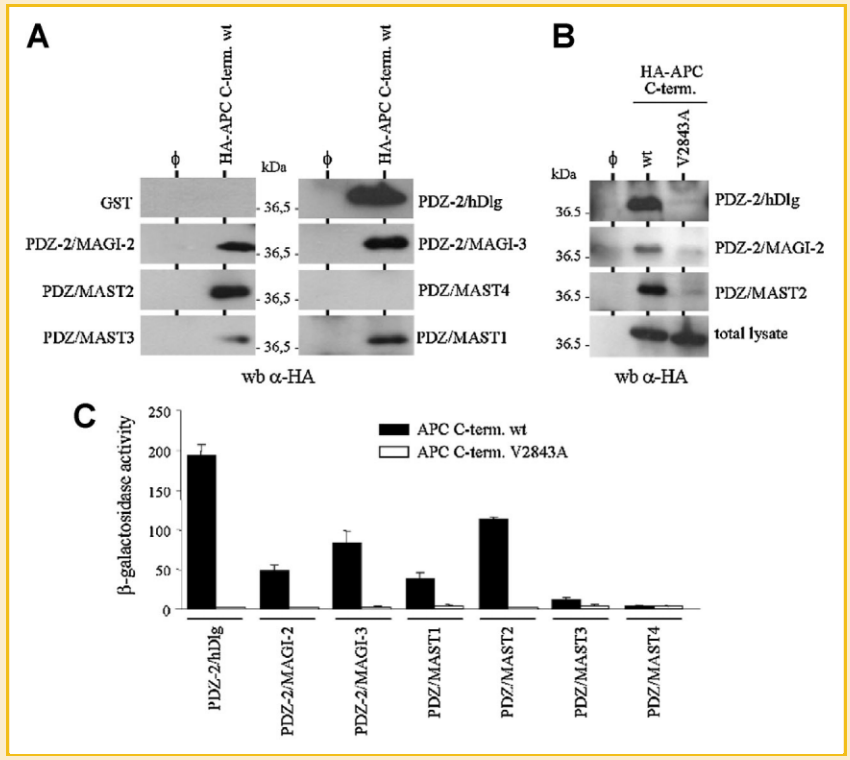

Fig. 2. Binding of PDZ domains to APC. A,B: Binding of APC to PDZ domains by GST pull-down. COS-7 cells were transfected with pRK5 alone (ϕ) or pRK5 HA-APC C-terminus wild type (wt) or V2843 mutation, and equal amounts of cellular lysates were subjected to pull-down with equivalent amounts (3 μg) of GST or the distinct GST-fusion proteins, as indicated. HA-APC was detected by Western blot (wb) with anti-HA antibody. In the bottom panel of B, total lysate samples (50 μg) were loaded. A representative experiment is shown, out of at least three experiments with similar results. C: Binding of APC to PDZ domains by yeast two-hybrid. Yeast cells were co-transfected with pMW107, pEG202 APC C-terminus (black box: wild type, wt; white box: V2843 mutation), and pJG4.5 PDZ plasmids, as indicated, and induction of β-galactosidase activity was measured. Results are shown as the mean ± SD from three independent experiments.

interaction, the yeast two-hybrid analysis was precluded since the binding between PTEN and hDlg is not readily detected by this technique (our unpublished observations). In summary, APC and PTEN show overlapping but distinct binding specificity for hDlg PDZ domains. These results suggested that APC and PTEN could bind to the same hDlg molecule to form a ternary complex. To test this possibility, GST pull-down experiments were performed using GST-hDlg PDZ-1-3 and cell lysates containing HA-APC and HA-PTEN, alone or in combination. As shown, GST-hDlg PDZ-1-3 pulled down both HA-APC and HA-PTEN from the cell lysates containing a mix of these two proteins (Fig. 4C). The binding was mediated in both cases through the PDZ-binding motifs, since mutation of the C-terminal PDZ-binding motif residues abrogated the interaction in each case. These findings suggest that APC and PTEN can bind simultaneously to PDZ-1-3/hDlg without competing for the binding. To further investigate the putative existence of ternary complexes between APC, PTEN, and hDlg, yeast three-hybrid experiments were designed. In these assays, PDZ1-3/hDlg is expressed in yeast to serve as a bridge between the LexA-APC and B42-PTEN hybrid proteins, which do not bind each other in the absence of PDZ1-3/hDlg (Fig. 4D). As shown, expression of PDZ1-3/hDlg in the presence of both APC and PTEN activated the β-galactosidase reporter, demonstrating the formation of ternary complexes between hDlg, APC, and PTEN.

TUMOR-RELATED MUTATIONS AT PDZ-2/hDlg DIMINISH BINDING TO PTEN AND APC

Mutations at the hDlg human gene (*DLG1*), that target the PDZ-2/hDlg domain, have been detected in mammary ductal carcinoma samples [Fuja et al., 2004]. To asses the effect of these mutations in the binding of PDZ-2/hDlg to PTEN and APC, two mutations were chosen, individual (G338R and I348V) and combined (G338R/I348V), and introduced into the recombinant PDZ-2/hDlg. As shown in Figure 5A, residues Gly^{338} and Ile^{348} are located in exposed residues, suggesting a putative involving in protein-protein interactions. A double mutation in the conserved GLGF loop of PDZ-2/hDlg (L329R/G330R), which is important for PDZ domain stability and protein binding [Doyle et al., 1996], was also engineered. Mutations were tested for binding to APC and PTEN, by both GST pull-down and yeast two-hybrid techniques. In GST pull-down experiments, both APC and PTEN displayed diminished binding to PDZ-2/hDlg bearing the tumor-associated mutations, in comparison with the binding to the wild type protein (Fig. 5B). This was confirmed in the case of APC by quantitative yeast two-hybrid analysis (Fig. 5C). The mutation L329R/G330R displayed the weaker binding to APC and PTEN by GST pull-down, and no binding in the yeast two-hybrid assays. This could be partially due to destabilization of the PDZ domain caused by the L329R/G330R mutation, since the levels of expression of this recombinant protein were

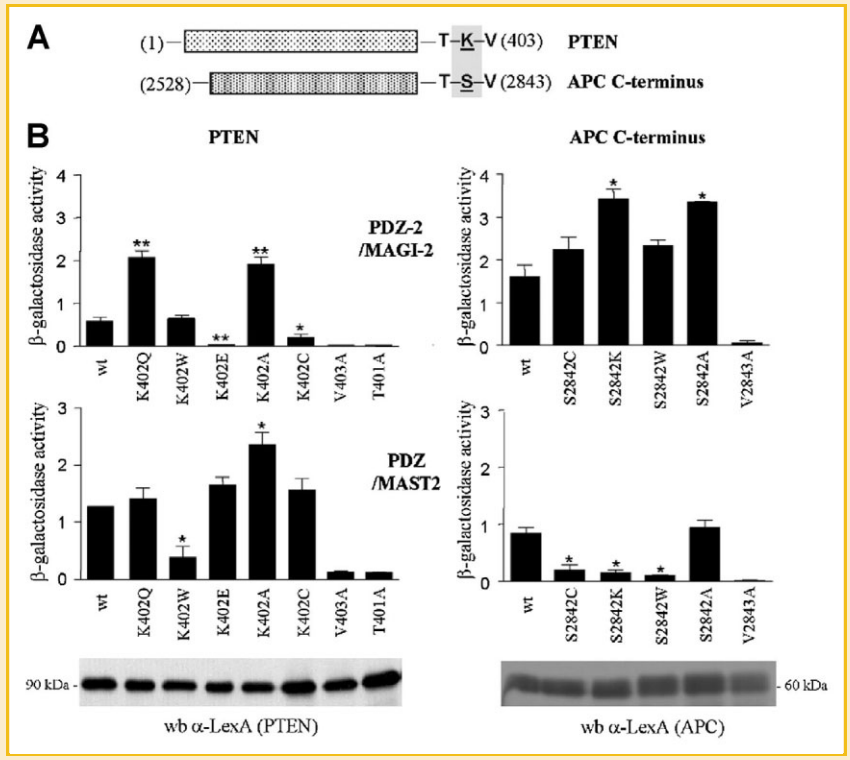

Fig. 3. APC and PTEN share PDZ-domain partners but display different binding properties. A: Depiction of the C-terminal PDZ-binding motifs of the PTEN and APC proteins. Numbers indicate amino acid numbering. The C-terminal residues that configure the PDZ-domain binding motifs are indicated, and the PTEN Lys^{402} and the APC Ser^{2842} residues are underlined. B: Comparative binding of APC and PTEN to PDZ-2/MAGI-2 and PDZ/MAST2 domains. Yeast cells were co-transfected with pMW107, pEG202 APC C-terminus, or pEG202 PTEN (wild type, wt; or mutations), and pJG4.5 PDZ plasmids, as indicated, and induction of β-galactosidase activity was measured. Results are shown as the mean ± SD from three independent experiments. In the lower panels, the expression in the yeast of the different PTEN and APC LexA-hybrid proteins is shown from a representative experiment, as monitored by Western blot (wb) with anti-LexA antibody.

consistently lower in both binding assays. Thus, we cannot rule out that some of the decrease observed in the binding of the tumor-associated PDZ-2/hDlg mutations to PTEN and APC is due to destabilization of these recombinant proteins. These results show that tumor-found mutations located in the PDZ-2/hDlg domain have deleterious consequences for PTEN and APC binding.

DISCUSSION

The binding pattern of PTEN to PDZ domains include multiple scaffolding (MAGI-1/2/3, NHERF1/2, PAR-3, and PSD-95) and signaling (MAST1/2/3) proteins [Gericke et al., 2006; Pinal et al., 2006; Takahashi et al., 2006; Jurado et al., 2010], as well as the tumor suppressor hDlg [Adey et al., 2000]. This binding increases PTEN stability and favors its submembranal localization, which enhances PTEN accessibility to PIP3 substrate [Wu et al., 2000ab; Tolkacheva et al., 2001; Kotelevets et al., 2005; Valiente et al., 2005]. We have shown here that overexpression of PTEN or hDlg inhibits the growth of MCF-7 cells, and that hDlg cooperates with PTEN in the inactivation of the PI3K/Akt pathway, substantiating the hDlg tumor suppressor role and the possibility that PTEN:hDlg interaction may be important in oncogenesis. Furthermore, we have found that APC, another tumor suppressor with high clinical relevance in colorectal cancer [Phelps et al., 2009], share PDZ-domain partners, including hDlg, with PTEN. Our results using APC and PTEN mutations indicate that these two tumor suppressors are distinctly recognized by their common PDZ-domain partners, making possible the existence of distinct mechanisms to specifically regulate the APC:PDZ and PTEN:PDZ interactions. For instance, acetylation of Lys^{402} at the PTEN PDZ-binding motif favors PTEN/PDZ interactions [Ikenoue et al., 2008], but this residue is substituted by a Ser in APC (see Fig. 3A). Also, phosphorylation of Thr^{401} has been proposed to regulate PTEN binding to PDZ domains [Adey et al., 2000].

We have found that APC and PTEN show overlapping but distinct binding avidity and specificity for PDZ/hDlg domains. APC bound strongly to the three individual PDZ/hDlg domains, but non-additive binding was observed towards the PDZ-1-2 and PDZ-1-3/hDlg domain combinations, suggesting that the tandem disposition of the PDZ/hDlg domains may affect the simultaneous binding of APC to more than one PDZ domain (Fig. 4B). At this regard, it has been proposed that the PDZ-1 and PDZ-2/hDlg domains may interact, which could affect specific ligand binding [Marfatia et al., 1996], and simultaneous binding to the three PDZ/hDlg domains has been observed for the E6 protein from human papillomavirus [Chi et al., 2011]. Matsumine et al. [1996] reported the binding of APC to PDZ-2/hDlg, but not to PDZ-1/hDlg or PDZ-3/hDlg. In addition, the binding of a APC C-terminal peptide (APC C-terminal 11 residues) to

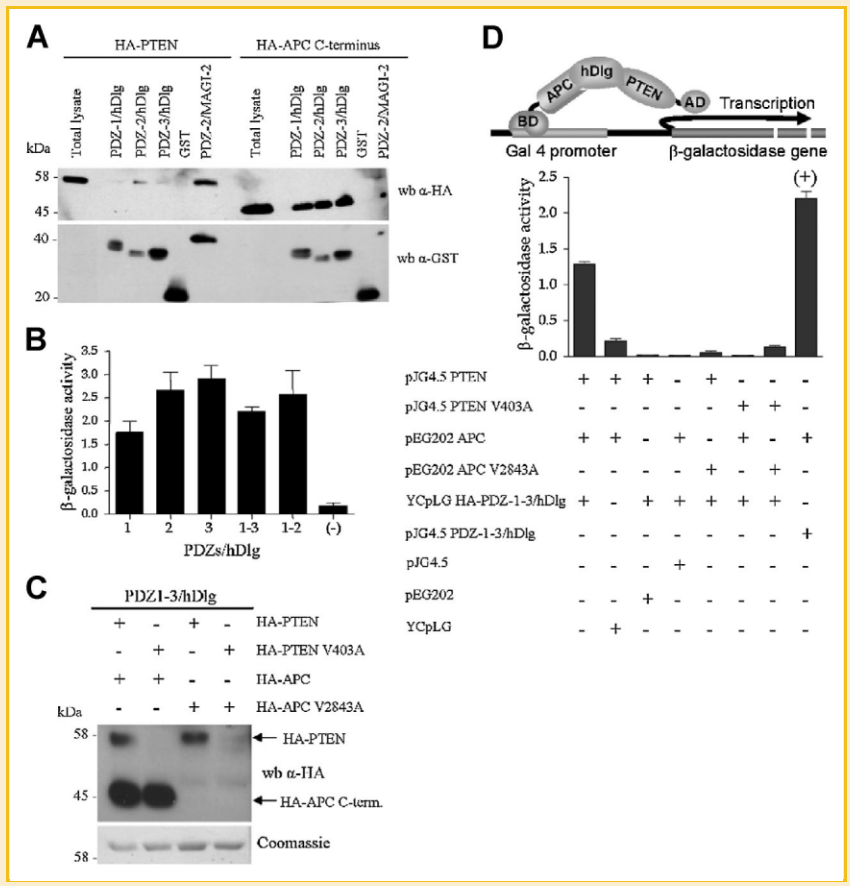

Fig. 4. APC and PTEN bind to hDlg PDZ domains with distinct efficiency and specificity. A: Binding of APC and PTEN to hDlg PDZ domains by GST pull-down. HEK293 cells were transfected with pRK5 HA-APC C-terminus or pRK5 HA-PTEN, and equal amounts of cell lysates were subjected to pull-down with equivalent amounts (3 μg) of GST or the distinct GST-PDZ/hDlg fusion proteins, as indicated. GST-PDZ-2/MAGI-2 was included for comparison. HA-APC and HA-PTEN were detected by Western blot (wb) with anti-HA antibody (upper panel). In the lower panel, the GST fusion proteins used in the assay are shown, after Western blot with anti-GST antibody. A representative experiment is shown, out of at least three experiments with similar results. B: Binding of APC to PDZ/hDlg domains by yeast two-hybrid. Yeast cells were co-transfected with pMW107, pEG202 APC C-terminus and pJG4.5 PDZ/hDlg-domains (individual or in combinations), as indicated, and induction of β-galactosidase activity was measured. The combination pMW107, pEG202 APC C-terminus, and pJG4.5 PDZ-2/hDlg V2843A was used as a negative control (−). C: Co-precipitation of HA-APC and HA-PTEN with PDZ-1-3/hDlg by GST pull-down. HEK293 cells were separately transfected with pRK5 HA-APC C-terminus or pRK5 HA-PTEN, wild type or mutations. Equal amounts of cell lysates were mixed, as indicated, and subjected to pull-down with GST-PDZ-1-3/hDlg (3 μg). HA-APC and HA-PTEN were detected by Western blot with anti-HA antibody (upper panel). In the lower panel, the GST-PDZ-1-3/hDlg fusion proteins used in the assay are shown, after Coomasie staining. A representative experiment is shown, out of at least three experiments with similar results. D: Detection of a ternary complex between hDlg, APC, and PTEN, by yeast three-hybrid analysis. In the top, a schematic depiction of the three hybrid yeast system used is shown; BD, LexA binding domain; AD, B42 activation domain. Yeast cells were co-transfected with pMW107, pEG202 empty vector or pEG202 APC C-terminus, pJG4.5 empty vector or pJG4.5 PTEN, and YCpLG empty vector or YCpLG HA-PDZ1-3/hDlg, in different combinations, as indicated, and induction of β-galactosidase activity was measured. The combination pMW107, pEG202 APC C-terminus, and pJG4.5 PDZ-1-3/hDlg, was included as a positive control (+). In (B) and (D), results are shown as the mean ± SD from three independent experiments.

PDZ-1/hDlg and PDZ-2/hDlg, but not to PDZ-3/hDlg, has also been reported [Zhang et al., 2011]. Our findings show binding of APC C-terminus (residues 2528–2843) to the three PDZ/hDlg domains. Thus, it is likely that recognition of APC by the distinct hDlg/PDZ domains depends on both the arrangement of the PDZ domains and on APC determinants outside of its C-terminal PDZ-domain binding motif. Of interest, we have found that PDZ1-3/hDlg interacts simultaneously with APC and PTEN, bringing them together in a ternary complex APC:hDlg:PTEN. This may be relevant in oncogenesis, since alterations of the protein levels or the subcellular location of any of these tumor suppressors could affect the local assembly of the complexes and favor tumorigenesis. Other ternary complexes that involve PTEN and PDZ-domain containing proteins have been reported in mammalian cells, including the complexes PTEN:MAGI1b:β-catenin, PTEN:NHERF1:PDGFR, and PTEN:NHERF1:PHLPP [Kotelevets et al., 2005; Takahashi et al., 2006; Molina et al., 2012], suggesting that the tumor suppressor role of PTEN may rely, at least in part, in its assembly into PDZ-domain mediated multimolecular complexes. Remarkably, LOH has been reported for the tumor suppressor NHERF1 PTEN-binding protein in human primary breast tumors, and a mutation in the PDZ-2 domain of NHERF1 has been found in a human breast cancer cell line, that abrogated NHERF1 binding to ligands [Dai et al., 2004]. In the case of hDlg, we illustrate here that PDZ-2/hDlg tumor-found mutations

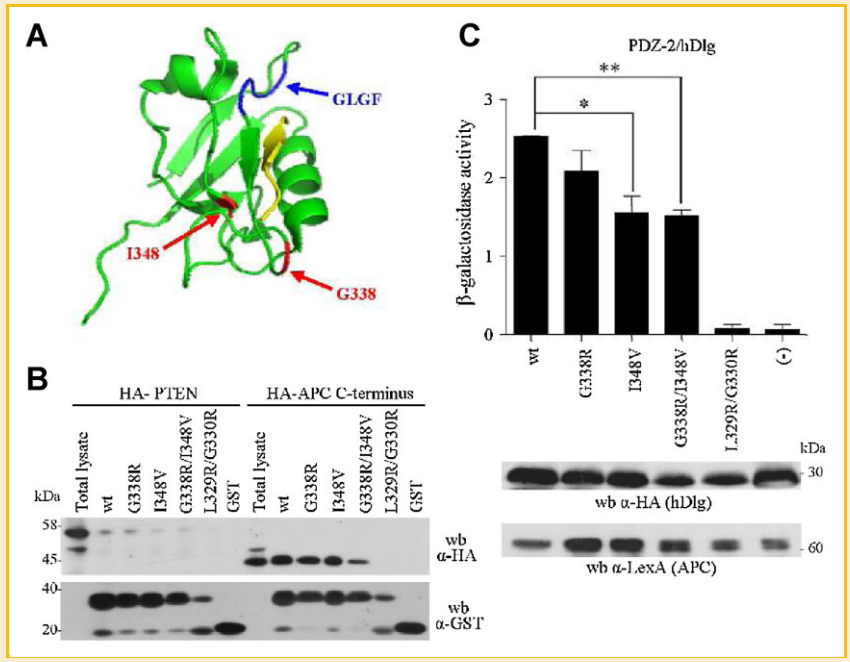

Fig. 5. Binding of APC and PTEN to tumor PDZ-2/hDlg mutations. A: Ribbon depiction of the three-dimensional structure of PDZ-2/hDlg in complex with a C-terminal peptide (yellow) from E6 protein from HPV18 [Zhang et al., 2007]. The spatial location of the residues (Gly^{338} and Ile^{348}) mutated in human tumors is indicated (red), as well as the location of the GLGF motif (blue). B: Binding of APC and PTEN to mutations of PDZ-2/hDlg domains by GST pull-down. HEK293 cells were transfected with pRK5 HA-APC C-terminus or pRK5 HA-PTEN, and equal amounts of cell lysates were subjected to pull-down with equivalent amounts (3 μg) of GST, GST-PDZ-2/hDlg wild type (wt) or mutation (G338R, I348V, G338R/I348V, and L329R/G330R [GLGF to GRRF mutation]) fusion proteins, as indicated. HA-APC and HA-PTEN were detected by Western blot (wb) with anti-HA antibody (upper panel). In the lower panel, the GST fusion proteins used in the assay are shown, after Western blot with anti-GST antibody. A representative experiment is shown, out of at least three experiments with similar results. C: Binding of APC to mutations of PDZ-2/hDlg by yeast two-hybrid. Yeast cells were co-transfected with pMW107, pEG202 APC C-terminus, and pJG4.5 PDZ-2/hDlg wild type (wt) or mutations, as indicated, and induction of β-galactosidase activity was measured. The combination pMW107, pEG202 APC C-terminus, and pJG4.5 PDZ-2/hDlg V2843A was used as a negative control (−). In the upper panel, results are shown as the mean ± SD from three independent experiments. $^{**}P < 0.01$; $^{*}P < 0.05$. In the lower panels, the expression of the PDZ-2/hDlg and the APC C-terminus and PTEN hybrid proteins is shown from a representative experiment, as monitored by Western blot with anti-HA and anti-LexA antibodies, respectively. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

are deleterious for its interaction with APC and PTEN. These findings, together with the fact that PDZ-domain containing proteins, including hDlg, MAGIs, and NHERF1 proteins, are important cell signaling regulators targeted for degradation by viral oncoproteins [Javier, 2008; Accardi et al., 2011; Kranjec and Banks, 2011; Subbaiah et al., 2011], outline the relevance in oncogenesis of unbalanced PDZ-mediated scaffolding of tumor suppressors.

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