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Oncogenic function for the *Dlg1* mammalian homolog of the *Drosophila* discs-large tumor suppressor

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The fact that several different human virus oncoproteins, including adenovirus type 9 E4-ORF1, evolved to target the Dlg1 mammalian homolog of the membrane-associated *Drosophila* discs-large tumor suppressor has implicated this cellular factor in human cancer. Despite a general belief that such interactions function solely to inactivate this suspected human tumor suppressor protein, we demonstrate here that E4-ORF1 specifically requires endogenous Dlg1 to provoke oncogenic activation of phosphatidylinositol 3-kinase (PI3K) in cells. Based on our results, we propose a model wherein E4-ORF1 binding to Dlg1 triggers the resulting complex to translocate to the plasma membrane and, at this site, to promote Ras-mediated PI3K activation. These findings establish the first known function for *Dlg1* in virus-mediated cellular transformation and also surprisingly expose a previously unrecognized oncogenic activity encoded by this suspected cellular tumor suppressor gene.

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Introduction

Dlg1, also known as hDlg or SAP97, is a mammalian homolog of the *Drosophila* discs-large (Dlg) tumor suppressor protein (Lue *et al*, 1994; Muller *et al*, 1995). These proteins localize to specialized cell–cell contact membrane sites (i.e., septate, adherens, and synaptic junctions) and are required for both junction formation and proper polarity establishment in cells (Humbert *et al*, 2003; Nguyen *et al*, 2003; Laprise *et al*, 2004). As MAGUK family proteins, they lack catalytic activity and instead consist of multiple protein interaction modules, including three PDZ domains, an SH3 domain, and a guanylate kinase-homology (GK) domain (Gonzalez-Mariscal *et al*, 2000). Accordingly, these polypeptides function as molecular scaffolds to organize their targets into supramolecular signaling complexes and to translocate them to the plasma membrane (Kim and Sheng, 2004).

Evidence supports the assertion that *Drosophila Dlg* and mammalian *Dlg1* encode evolutionarily conserved tumor suppressor proteins. *Drosophila Dlg* mutations cause cellular overgrowth in the larval brain and imaginal discs, where cells exhibit a neoplastic morphology (Woods *et al*, 1996). Upon transplantation into *wild-type* (*wt*) flies, *Dlg*-mutant imaginal discs produce cancerous outgrowths resembling metastatic tumors (Woodhouse *et al*, 1998), perhaps reflecting the capacity of Dlg to prevent abnormal cellular invasion into adjacent tissues (Goode and Perrimon, 1997). Furthermore, transgenic expression of *Dlg1* by *Drosophila Dlg* mutants rescues their cellular overgrowth phenotype (Thomas *et al*, 1997), demonstrating conservation of the tumor suppressor function. *Dlg1* likewise prevents unscheduled cellular proliferation in ocular lenses of mice (Nguyen *et al*, 2003), sustains mutations accompanied by a loss of heterozygosity in human breast carcinomas (Fuja *et al*, 2004), and exhibits reduced expression in invasive human cervical carcinomas (Cavatorta *et al*, 2004; Lin *et al*, 2004).

Reports showing that three different human virus oncoproteins independently evolved to target Dlg1 in cells provide the most compelling evidence implicating this cellular factor in human cancer. Human adenovirus type 9 (Ad9) elicits mammary tumors in experimental animals (Javier *et al*, 1991), and high-risk human papillomavirus (HPV) and human T-cell leukemia virus type 1 (HTLV-1) are etiological agents for cervical carcinoma and adult T-cell leukemia, respectively (Barmak *et al*, 2003; Heise, 2003). Strikingly, the otherwise unrelated oncogenic determinants of these tumor viruses (Ad9 E4-ORF1, HPV E6, and HTLV-1 Tax) similarly possess a carboxyl-terminal PDZ domain-binding motif (PBM) that mediates binding to several different cellular PDZ proteins (Lee *et al*, 1997; Rousset *et al*, 1998; Mantovani and Banks, 2001), including Dlg1, which is the only PDZ-protein target common to all three viral proteins. Additionally, the PBM plays a key role in cellular transformation induced by these oncoproteins (Lee *et al*, 1997; Watson

et al, 2003; Hirata *et al*, 2004). Evidence specifically implicating endogenous Dlg1 in this process is currently lacking, but it has been reasonably hypothesized that the tumorigenic potentials of these viral proteins stem partly from an ability to inactivate this suspected tumor suppressor protein. Consistent with this idea, HPV E6 promotes degradation of overexpressed Dlg1 (Gardiol *et al*, 1999) and HTLV-1 Tax overcomes cell-cycle arrest caused by Dlg1 overexpression (Suzuki *et al*, 1999), although one caveat is the uncertain physiological relevance of these findings.

The oncogenic potential of Ad9 E4-ORF1 was recently demonstrated to depend on its ability to promote constitutive, growth factor-independent stimulation of cellular phosphatidylinositol 3-kinase (PI3K) (Frese *et al*, 2003). This critical activity of E4-ORF1 requires the PBM, which mediates interactions with Dlg1 (Lee *et al*, 1997) and three other cellular PDZ proteins (MUPP1, MAGI-1, and ZO-2) (Glaunsinger *et al*, 2000, 2001; Lee *et al*, 2000). We report here that specific binding of E4-ORF1 to endogenous Dlg1 promotes Ras-mediated PI3K activation in cells, revealing the first established function for *Dlg1* in viral oncoprotein-mediated cellular transformation. The additional finding that functional as opposed to inactivated Dlg1 mediates E4-ORF1-induced PI3K activation also exposed a surprising oncogenic activity for this suspected tumor suppressor protein.

Results

Mutation of MUPP1, MAGI-1, or Dlg1 fails to reproduce PI3K activation induced by E4-ORF1

Given that *Dlg1* as well as *ZO-2* (Chlenski *et al*, 1999a, b, 2000) are suspected tumor suppressors and that E4-ORF1 sequesters MUPP1, MAGI-1, and ZO-2 in the cytoplasm of cells (Glaunsinger *et al*, 2000, 2001; Lee *et al*, 2000), we postulated that PI3K activation provoked by Ad9 E4-ORF1 may stem from inactivation of one of these cellular targets. If so, disruption of the corresponding cellular gene should recapitulate E4-ORF1-induced PI3K activation in cells.

To explore this idea, we isolated mouse embryo fibroblasts (MEF) from *wt* mice and matched littermates carrying either a large deletion mutation encompassing the entire *MUPP1* gene (Bell *et al*, 1995) or a targeted mutation interrupting either the *MAGI-1* gene (in preparation) or the *Dlg1* gene (Caruana and Bernstein, 2001). The lack of *ZO-2* mutant mice precluded inclusion of the respective mutant MEF in this study; however, ZO-2 seems unlikely to play a role in E4-ORF1-induced PI3K activation given that E4-ORF1 proteins encoded by human Ad3, Ad5, and Ad12 activate PI3K (Frese *et al*, 2003), yet do not bind this single, unique PDZ-protein target of Ad9 E4-ORF1 (Glaunsinger *et al*, 2001).

MUPP1$^{-/-}$ or *MAGI-1*$^{-/-}$ MEF failed to express the respective *wt* gene product, as did *Dlg1*$^{gt/gt}$ MEF, which instead expressed the Dlg/β-geo fusion protein containing the amino-terminal 549 residues of the ~900-residue *wt* Dlg1 polypeptide (Supplementary Figure 1A and B). Following serum deprivation and subsequent stimulation by platelet-derived growth factor (PDGF), all mutant MEF and matched *wt* MEF displayed comparable basal and PDGF-induced levels of activated, phosphorylated protein kinase B (PKB) (Supplementary Figure 1A), a key downstream effector of PI3K. Throughout this study, Thr308- and Ser473-phosphospecific PKB antibodies yielded identical results, so each was used interchangeably. As Dlg/β-geo could feasibly retain some function, including the postulated capacity to suppress PI3K activation, we stably downregulated this protein in *Dlg1*$^{gt/gt}$ MEF using a Dlg1-specific short hairpin RNA (shRNA). Despite substantial, specific downregulation of Dlg/β-geo, Dlg1 shRNA-expressing *Dlg1*$^{gt/gt}$ MEF displayed basal and PDGF-induced levels of activated PKB identical to those of *Dlg1*$^{gt/gt}$ MEF (Supplementary Figure 1C). These observations did not support our hypothesis that E4-ORF1-induced PI3K activation simply results from functional inactivation of MUPP1, MAGI-1, or Dlg1 in cells.

PI3K activation and anchorage-independent growth induced by E4-ORF1 depend on Dlg1

Our negative results prompted consideration of the opposite hypothesis, wherein PI3K activation induced by E4-ORF1 requires one of its cellular PDZ-protein targets. This model predicted instead that *MUPP1*$^{-/-}$, *MAGI-1*$^{-/-}$, or *Dlg1*$^{gt/gt}$ MEF might fail to support E4-ORF1-induced PKB activation. Significantly, during transient E4-ORF1 expression, *Dlg1*$^{gt/gt}$ MEF, but not *MUPP1*$^{-/-}$ or *MAGI-1*$^{-/-}$ MEF, displayed a substantial defect in supporting this activity compared to matched *wt* or heterozygous mutant MEF (Figure 1A). Though prolonged exposures revealed that *Dlg1*$^{gt/gt}$ MEF retained a weak capacity to support E4-ORF1-induced PKB activation, this remnant activity was eliminated by Dlg1 shRNA expression (Figure 1B). Additionally, whereas *Dlg1*$^{+/+}$ MEF stably expressing E4-ORF1 showed constitutive activation of endogenous PKB and grew in soft agar, *Dlg1*$^{gt/gt}$ MEF stably expressing E4-ORF1 lacked these phenotypes (Figure 2A and B), similar to the former MEF treated with the PI3K inhibitor drug LY294002 (LY) (Figure 2C and D). Also notable was that *Dlg*$^{gt/gt}$ MEF and *Dlg*$^{gt/gt}$ MEF expressing the Dlg1 shRNA failed to grow in soft agar (Figure 2B). These findings demonstrated a requirement for functional rather than inactivated Dlg1 in both PI3K activation and oncogenic transformation induced by E4-ORF1 in MEF.

I3-containing Dlg1 isoforms support E4-ORF1-induced PI3K activation

Due to several short insertion elements (e.g., I1, I2, I3) that arise by alternative splicing (see Supplementary Figure 1B), multiple Dlg1 isoforms can be produced in cells (Lue *et al*, 1994; McLaughlin *et al*, 2002). Despite comparable binding of E4-ORF1 to the Dlg1-I2, -I3, -I1I2, and -I1I3 isoforms (Figure 3A), transient expression of green fluorescent protein (GFP)-tagged Dlg1-I3, but not GFP-Dlg1-I2, restored robust E4-ORF1-induced PKB activation in Dlg1 shRNA-expressing *Dlg1*$^{gt/gt}$ MEF (Figure 3B). A nucleotide mismatch between the mouse Dlg1 shRNA and the rat *Dlg1* cDNA used to construct Dlg1 plasmids permitted GFP-Dlg1 expression in these cells.

In *Dlg1*$^{gt/gt}$ MEF, E4-ORF1-induced PKB activation increased markedly even at low undetectable levels of HA epitope-tagged (HA)-Dlg1-I3 expression, peaked at moderate levels, and did not increase appreciably at higher levels (Figure 3C). By contrast, GFP-tagged Dlg1-I2, MUPP1, MAGI-1, ZO-2, or Dlg1-related SAP102 (Thomas *et al*, 1997) lacked any such activity (Figure 3D). GFP-Dlg1-I3 likewise enhanced PKB activation induced by related human Ad3 and Ad5 E4-ORF1 proteins (Frese *et al*, 2003) (Figure 3E), but not

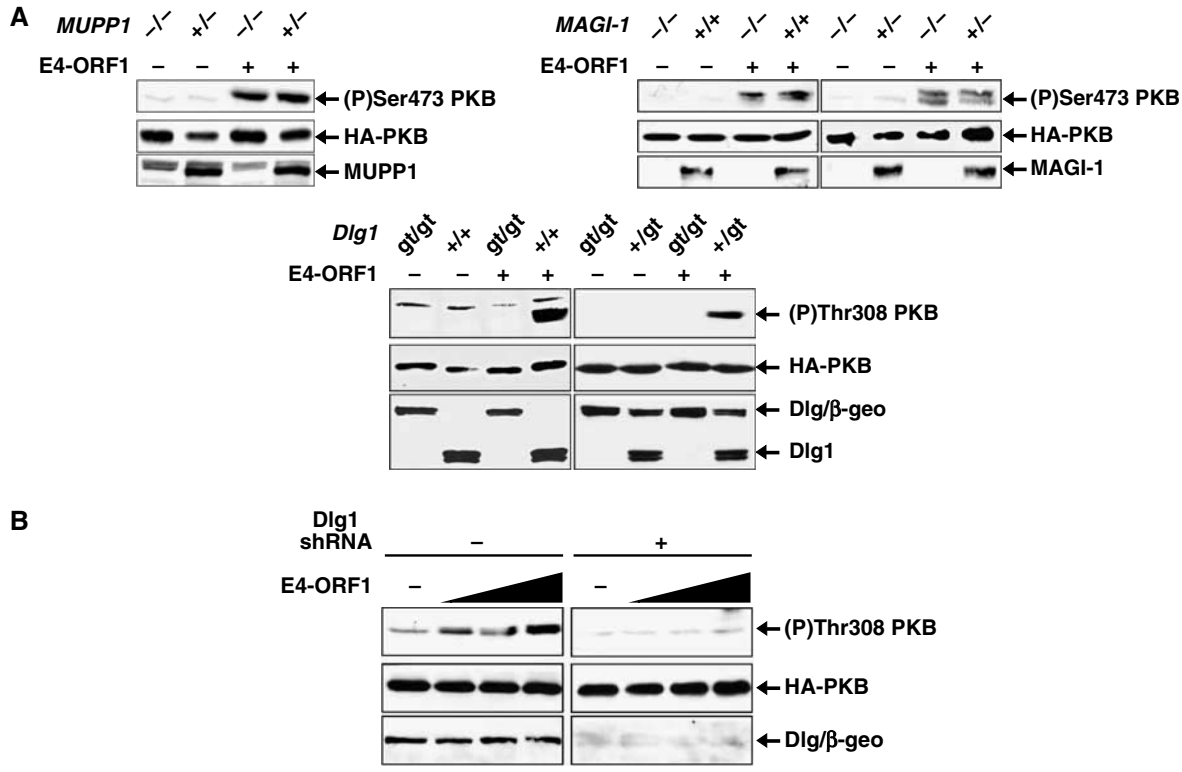

Figure 1 E4-ORF1-induced PKB activation depends on *Dlg1*. (**A**) E4-ORF1-induced PKB activation is deficient in $Dlg1^{gt/gt}$ MEF. Cells on 6-cm dishes were lipofected with pGW1-HA-PKB (0.5, 1, or 1 μg for *MUPP1*, *MAGI-1*, or *Dlg1* MEF, respectively) and either empty pGW1 (−) or pGW1-E4-ORF1 (+) (100, 20, or 50 ng for *MUPP1*, *MAGI-1*, or *Dlg1* MEF, respectively). Cells were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB, (P)Ser473 PKB, HA, MUPP1, MAGI-1, or Dlg1. For this type of experiment, the small quantity of E4-ORF1 plasmid needed to achieve optimal HA-PKB activation did not yield detectable levels of E4-ORF1 expression. (**B**) Dlg1 shRNA expression abrogates weak PKB activation induced by E4-ORF1 in $Dlg1^{gt/gt}$ MEF. On 6-cm dishes, $Dlg1^{gt/gt}$ MEF stably transfected with either empty pSUPER (−) or pSUPER-Dlg1 shRNA (+) were lipofected with pGW1-HA-PKB (0.5 μg) and either empty pGW1 (−) or pGW1-E4-ORF1 (10, 20, or 50 ng). Cells were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB, HA, or Dlg1.

by cellular H-RasV12 or polyomavirus middle T (PyMT) (Figure 3F). Additionally, Dlg1-I1I2 and Dlg1-I1I3 mirrored Dlg1-I2 and Dlg1-I3, respectively, in their capacities to support E4-ORF1-induced PKB activation (e.g., see Figure 4B) (data not shown). Thus, *wt* Dlg1 mediates E4-ORF1-induced PI3K activation in cells, and I3-containing Dlg1 isoforms mainly provide this function. The lack of an I3 element in Dlg/β-geo explains, at least in part, its defect in supporting E4-ORF1-induced PI3K activation.

Multiple Dlg1 domains contribute to E4-ORF1-induced PI3K activation

We next examined the capacity of GFP-Dlg1-I3 deletion mutants (Figure 4A; group 1) to rescue E4-ORF1-induced PI3K activation in $Dlg1^{gt/gt}$ MEF. Like *wt* Dlg1-I3, Dlg1-I3 mutants missing the amino-terminal region (NT), PDZ3, SH3, or GK restored robust E4-ORF1-induced PKB activation (Figure 4B). The modest defect of Dlg1-I3ΔGK probably arises indirectly from its unique, partial nuclear accumulation (Kohu *et al*, 2002) (data not shown). By contrast, Dlg1-I3 mutants missing PDZ1 or PDZ2 exhibited modest or severe activity loss, respectively, whereas Dlg1-I3 mutants missing either all three PDZ domains or PDZ1 + 2, which forms a single conformational unit (Lue *et al*, 1996), lacked any activity (Figure 4B). Given that PDZ1 + 2 was both necessary and sufficient to mediate Dlg1 binding to E4-ORF1 (Figure 4C), the defects of the latter Dlg1 mutants likely reflect nearly complete or complete disruption of this interaction.

To assess possible roles for other Dlg1 domains in E4-ORF1-induced PI3K activation, we examined additional Dlg1 mutants that retain PDZ1 + 2 and I3, but lack certain other elements (Figure 4A; group 2). We excluded mutants with the ΔGK mutation due to their confounding partial or complete nuclear localization. Whereas Dlg1 mutants missing either the unique region between PDZ2 and PDZ3 (U3) or the SH3 domain restored E4-ORF1-induced PKB activation, Dlg1 mutants lacking both elements did not (Figure 4D), suggesting that U3 and SH3 have redundant functions for this activity. In summary, Dlg1 requires PDZ1 + 2, I3, and either U3 or SH3 to support E4-ORF1-induced PI3K activation.

E4-ORF1 causes endogenous Dlg1 to accumulate at the plasma membrane

E4-ORF1 sequesters endogenous MUPP1, MAGI-1, and ZO-2 aberrantly within detergent-insoluble complexes in the cytoplasm of cells, suggesting functional inactivation of these cellular targets. We postulated that E4-ORF1 differentially affects Dlg1 in cells because our results implicated functional rather than inactivated Dlg1 in E4-ORF1-induced PI3K activation. In rat CREF fibroblasts stably expressing E4-ORF1, endogenous ZO-2, but not endogenous Dlg1, was sequestered within detergent-insoluble complexes (Figure 5A) that appeared

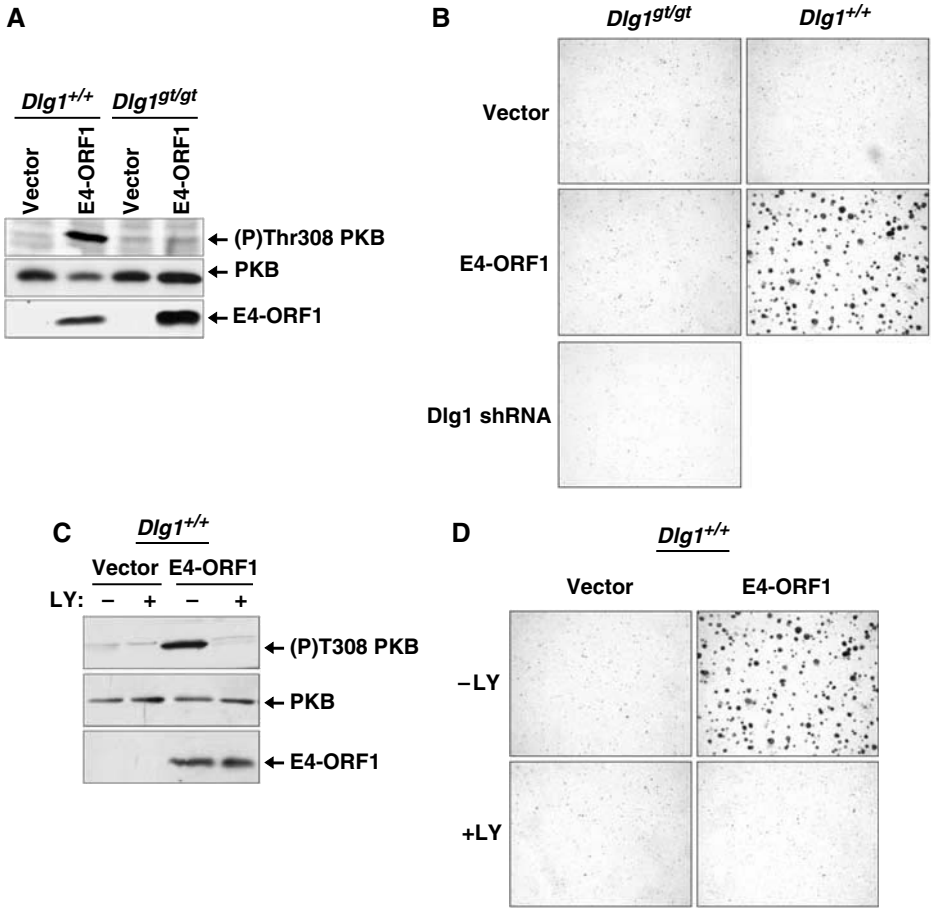

Figure 2 Dlg1 is also required for E4-ORF1 to induce anchorage-independent growth in MEF. (**A**) *Dlg1*$^{gt/gt}$ MEF stably expressing E4-ORF1 fail to accumulate activated, endogenous PKB. *Dlg1*$^{+/+}$ or *Dlg1*$^{gt/gt}$ MEF stably transfected with either empty pBABE (vector) or pBABE-E4-ORF1 (E4-ORF1) were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB, PKB, or E4-ORF1. (**B**) *Dlg1*$^{gt/gt}$ MEF stably expressing E4-ORF1 fail to grow in soft agar. 10^5 viable MEF stably transfected with empty pBABE (vector), pBABE-E4-ORF1 (E4-ORF1), or pSUPER-Dlg1 shRNA (Dlg1 shRNA) were suspended in agar and photographed 21 days later. (**C**) LY294002 (LY) treatment blocks endogenous PKB activation in *Dlg1*$^{+/+}$ MEF stably expressing E4-ORF1. *Dlg1*$^{+/+}$ MEF stably transfected with pBABE (vector) or pBABE-E4-ORF1 (E4-ORF1) were serum starved, and were treated with DMSO vehicle or 10 μM LY for 15 min. Equal amounts of cell extracts were analyzed as described above in A. (**D**) LY treatment inhibits anchorage-independent growth of *Dlg1*$^{+/+}$ MEF stably expressing E4-ORF1. Soft agar assays were conducted as described above in (B), except that the culture medium contained DMSO vehicle (−LY) or 10 μM LY (+LY).

as cytoplasmic punctae in indirect immunofluorescence (IF) assays (Figure 5B). Close inspection of these IF assays revealed that, in ~50% of the E4-ORF1-expressing CREF cells, but in none of the control CREF cells, some Dlg1 accumulated at the plasma membrane (Figure 5B), the known location for E4-ORF1-induced PI3K activation (Frese *et al*, 2003). Moreover, treatment with chlorpromazine, an inhibitor of receptor-mediated endocytosis (Sieczkarski and Whittaker, 2002), increased detection of Dlg1 plasma membrane staining to 95% of E4-ORF1-expressing CREF cells, but to less than 10% of control CREF cells (Figure 5C), implying that E4-ORF1-induced Dlg1 membrane accumulation is a dynamic process continually counterbalanced by endocytosis.

Binding to E4-ORF1 triggers Dlg1-I3 to translocate to the plasma membrane

We also tested whether E4-ORF1 can cause GFP-Dlg1 to accumulate at the plasma membrane of 293T cells. When expressed alone, GFP-Dlg1-I2 and GFP-Dlg1-I3 similarly localized diffusely in the cytoplasm (Figure 5D), as did E4-ORF1, which additionally exhibited characteristic accumulation within cytoplasmic punctae (data not shown) (Weiss *et al*, 1996). Expression of GFP-Dlg1-I3 with E4-ORF1, however, caused both proteins to translocate to the plasma membrane in ~70% of transfected cells (Figure 5D). By contrast, expression of GFP-Dlg1-I2 with E4-ORF1 instead promoted both proteins to concentrate within cytoplasmic punctae in ~70% of transfected cells (Figure 5D). The common requirement for I3 in both PI3K activation and Dlg1 membrane translocation induced by E4-ORF1 served to link these two processes.

Unpublished data indicate that E4-ORF1 consists of two separate domains, the PBM and Domain 2 (D2), the latter of which mediates binding to an undetermined cellular phosphoprotein (pp70). Supporting this model, PBM mutations (e.g., V125A, T123D, and IIIA) eliminate E4-ORF1 binding to PDZ-protein targets, but not to pp70, and, conversely, D2 mutations (e.g., IA, IIA, and IIB) abolish E4-ORF1 binding to pp70, but not to PDZ-protein targets (in preparation) (Lee *et al*, 1997; Weiss and Javier, 1997). Disruption of either domain alone, however, suffices to inhibit E4-ORF1-induced PI3K activation (Frese *et al*, 2003).

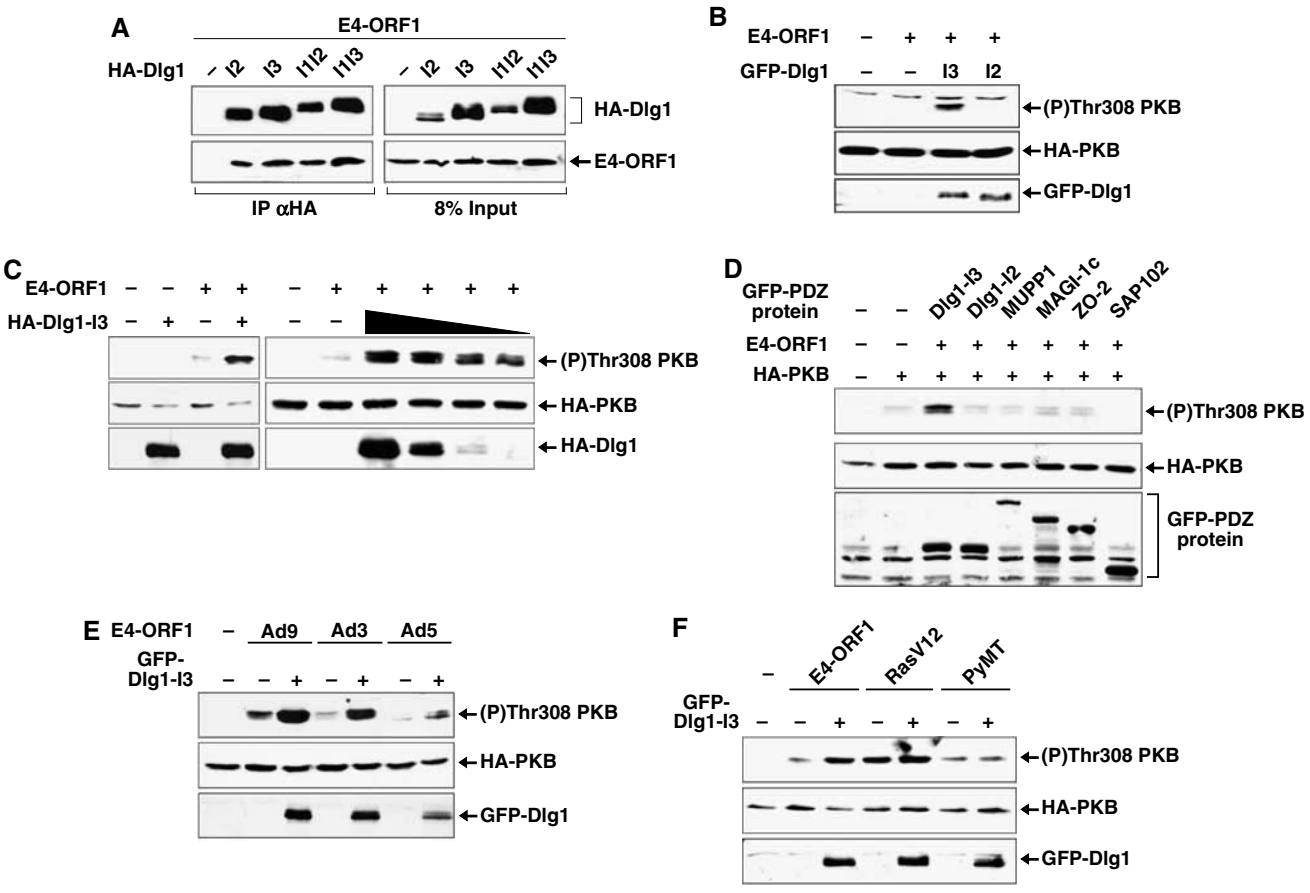

Figure 3 I3-containing Dlg1 isoforms mediate E4-ORF1-induced PKB activation. (**A**) E4-ORF1 binds comparably to four Dlg1 isoforms. COS7 cells on 6-cm dishes were lipofected with pGW1-E4-ORF1 (1.5 μg) and either empty pGW1 (−) or the indicated pGW1-HA-Dlg1 isoform plasmid (1.5 μg). Equal amounts of cell extracts were precipitated with HA antibody. Recovered proteins (left) or cell extracts (right) were blotted with antibody to HA or E4-ORF1. (**B**) Dlg1-I3, but not Dlg1-I2, rescues the defect of Dlg1 shRNA-expressing $Dlg1^{gt/gt}$ MEF in supporting E4-ORF1-induced PKB activation. The latter cells on 6-cm dishes were lipofected with pGW1-HA-PKB (0.5 μg), either empty pGW1 (−) or pGW1-E4-ORF1 (+) (20 ng), and either empty peGFP (−) or the indicated peGFP-Dlg1 isoform plasmid (2.5 μg). Cells were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB, HA, or GFP. (**C**) Low, undetectable levels of Dlg1-I3 expression are sufficient to enhance E4-ORF1-induced PKB activation in $Dlg1^{gt/gt}$ MEF. The latter cells on 6-cm dishes were lipofected with pGW1-HA-PKB (0.8 μg), either empty pGW1 (−) or pGW1-E4-ORF1 (+) (20 ng), and either empty pGW1 (−) or pGW1-HA-Dlg1-I3 (right: 11 ng, 110 ng, 1.1 μg, or 2.2 μg; left: 0.5 μg). Cells were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB or HA. (**D**) Other PDZ proteins fail to augment E4-ORF1-induced PKB activation in $Dlg1^{gt/gt}$ MEF. The latter cells on 6-cm dishes were lipofected with either empty pGW1 (−) or pGW1-HA-PKB (+) (0.4 μg), either empty $pCMV_{BamNeo}$ (−) or $pCMV_{BamNeo}$-E4-ORF1 (+) (0.1 μg), and either empty peYFP (−) or peYFP-Dlg1-I3 (0.3 μg), -Dlg1-I2 (0.3 μg), -MUPP1 (0.3 μg), -MAGI-1c (0.3 μg), or -ZO-2 (0.1 μg), or peGFP-SAP102 (0.3 μg). Cells were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB, HA, or GFP. (**E**) Dlg1-I3 likewise enhances PKB activation induced by *E4-ORF1* encoded by other human adenoviruses. $Dlg1^{gt/gt}$ MEF on 6-cm dishes were lipofected with pGW1-HA-PKB (0.4 μg), either empty $pCMV_{BamNeo}$ (−) or the indicated $pCMV_{BamNeo}$-E4-ORF1 plasmid (Ad9, 0.1 μg; Ad3, 0.6 μg; Ad5, 0.6 μg), and either empty peGFP (−) or peGFP-Dlg1-I3 (+) (0.4 μg). Cells were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB, HA, or SAP97. (**F**) Dlg1-I3 fails to augment PKB activation by other cellular and viral oncoproteins in $Dlg1^{gt/gt}$ MEF. The latter cells on 6-cm dishes were lipofected with pGW1-HA-PKB (0.4 μg), either empty pGW1 (−) or $pCMV_{BamNeo}$-E4-ORF1 (0.1 μg), pGW1-RasV12 (0.2 μg), or pPyMT (0.2 μg), and either empty peGFP (−) or peGFP-Dlg1-I3 (+) (0.2 μg). Cells were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB, HA, or SAP97.

We examined whether the two E4-ORF1 domains also function as a unit to promote GFP-Dlg1-I3 translocation to the plasma membrane. In these assays with 293 T cells, D2 mutant IIB behaved like *wt* E4-ORF1, whereas PBM mutant IIIA lacked any appreciable activity (Figure 5E). Other E4-ORF1 D2 and PBM mutants exhibited phenotypes indistinguishable from those of IIB and IIIA, respectively (data not shown). Results with PBM mutants reinforced the link between E4-ORF1-induced PI3K activation and Dlg1 membrane translocation, and further suggested that the interaction *per se* between E4-ORF1 and Dlg1-I3 suffices to trigger the latter process. Thus, E4-ORF1 binding to Dlg1 PDZ1 + 2 may cause cytoplasmic Dlg1 in the inactive, closed conformation to assume an active, open conformation capable of binding membrane- and cytoskeleton-associated proteins (Wu *et al*, 2000). The dispensability of E4-ORF1 D2 for this process signified specific participation in the subsequent PI3K activation event.

Identical Dlg1 domains determine both PI3K activation and Dlg1 membrane translocation induced by E4-ORF1

We next tested the capacity of E4-ORF1 to promote membrane translocation of all Dlg1 proteins shown in Figure 4A. Identical to *wt* Dlg1, Dlg1 mutants expressed alone in 293 T cells localized diffusely in the cytoplasm (e.g., see Figure 5D) (data not shown). Again, the single exception was Dlg1-

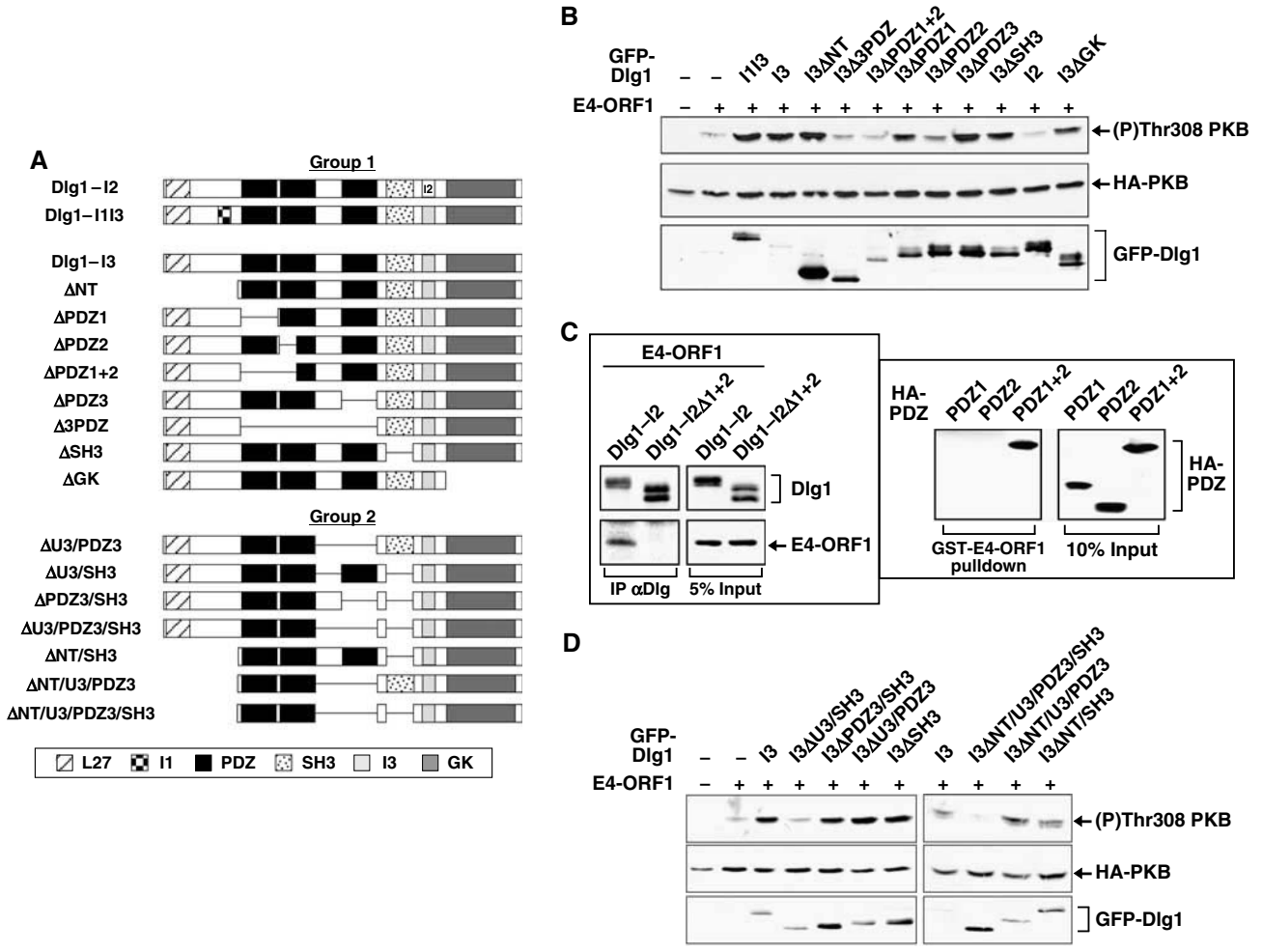

Figure 4 The I3, PDZ1 + 2, and U3 or SH3 elements of Dlg1 are required for E4-ORF1-induced PKB activation. (**A**) Illustration of Dlg1 isoforms and deletion mutants. Mutants below Dlg1-I3 are derived from this isoform. (**B**) The PDZ1 + 2 conformational unit is required for Dlg1-I3 to augment E4-ORF1-induced PKB activation in *Dlg1*$^{gt/gt}$ MEF. The latter cells on 6-cm dishes were lipofected with pGW1-HA-PKB (0.4 μg), either empty pGW1 (−) or pGW1-E4-ORF1 (+) (20 ng), and either empty peGFP (−) or the indicated peGFP-Dlg1 plasmid (0.4 μg). Cells were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB, HA, or GFP. (**C**) The PDZ1 + 2 conformational unit is necessary and sufficient to mediate binding of Dlg1 to E4-ORF1. For the co-precipitation assay (left), 293 T cells on 6-cm dishes were lipofected with pGW1-E4-ORF1 (1 μg) and either pGW1-Dlg1-I2 or pGW1-Dlg1-I2ΔPDZ1 + 2 (1 μg). Equal amounts of cell extracts were precipitated with Dlg1 antibody. Recovered proteins (left) and cell extracts (right) were blotted with antibody to SAP97 or E4-ORF1. For the GST pulldown assay (right), 293 T cells on 6-cm dishes were lipofected with the indicated pGW1-HA-Dlg1-PDZ domain plasmid (1 μg). An equal amount of cell extract was incubated with GST-E4-ORF1 fusion protein (5 μg). Recovered proteins (left) and cell extracts (right) were blotted with HA antibody. (**D**) Either U3 or SH3 is required for Dlg1 to augment E4-ORF1-induced PKB activation in *Dlg1*$^{gt/gt}$ MEF. The latter cells on 6-cm dishes were lipofected with pGW1-HA-PKB (0.4 μg), either empty pGW1 (−) or pGW1-E4-ORF1 (+) (20 ng), and either empty peGFP (−) or the indicated peGFP-Dlg1 plasmid (left, 0.1 μg; right, 0.4 μg). Cells were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB, HA, or GFP.

I3ΔGK, which partially accumulated in the nucleus, a property that likely explained the modest defect of this mutant in E4-ORF1-induced membrane translocation (Table I).

More important, the majority of Dlg1 proteins that could support *wt* levels of E4-ORF1-induced PKB activation were fully competent for E4-ORF1-induced membrane translocation (I1I3, ΔNT, ΔPDZ3, ΔSH3, ΔU3/PDZ3, ΔPDZ3/SH3, ΔNT/SH3, ΔNT/U3/PDZ3), whereas all Dlg1 proteins having little or no capacity to support E4-ORF1-induced PKB activation displayed very weak or no E4-ORF1-induced membrane translocation (I2, ΔPDZ2, ΔPDZ1 + 2, Δ3PDZ, ΔU3/SH3, ΔU3/PDZ3/SH3, ΔNT/U3/PDZ3/SH3) (Table I). These findings revealed an intimate connection between E4-ORF1-induced PI3K activation and Dlg1 membrane translocation, though some slight deviations from this trend were noted. For example, ΔPDZ1 displayed modestly impaired E4-ORF1-induced PKB activation, yet *wt* levels of E4-ORF1-induced membrane translocation, and, conversely, ΔSH3, ΔPDZ3/SH3, and ΔNT/SH3 exhibited *wt* levels of E4-ORF1-induced PKB activation, yet modestly impaired E4-ORF1-induced membrane translocation (Table I). These observations may indicate that PDZ1 and SH3 have some specific role in PI3K activation or Dlg1 membrane translocation, respectively, induced by E4-ORF1.

Ras mediates Dlg1-dependent PI3K activation induced by E4-ORF1

The fact that we consistently failed to detect PI3K associated with either Dlg1 or E4-ORF1 in cells (data not shown) implied that E4-ORF1 indirectly activates PI3K through a cellular

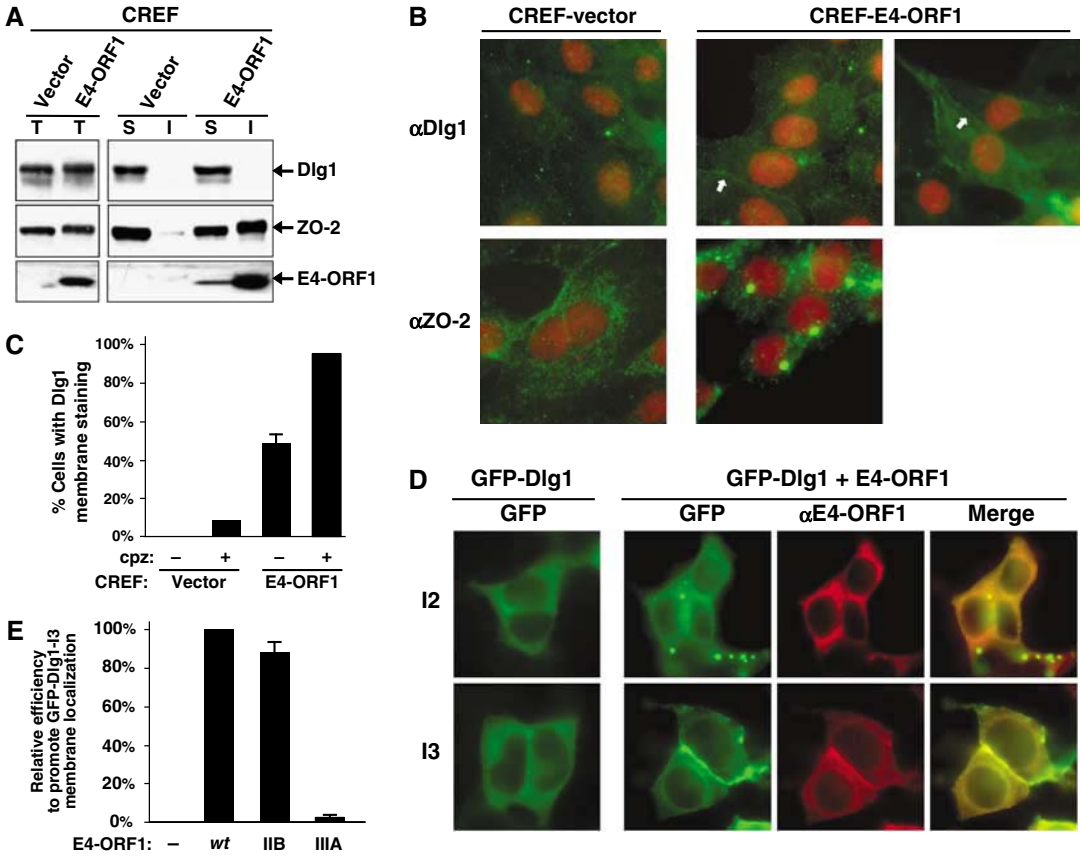

Figure 5 Binding of E4-ORF1 to Dlg1-I3 promotes their translocation to the plasma membrane. (**A**) E4-ORF1 fails to sequester endogenous Dlg1 within detergent-insoluble complexes. Confluent CREF cells stably transfected with empty pBABE (vector) or pBABE-E4-ORF1 (E4-ORF1) were lysed either in sample buffer to isolate total cell extracts (T) or in RIPA buffer to isolate detergent-soluble (S) and detergent-insoluble (I) fractions. Equal amounts of cell extracts were blotted with antibody to SAP97, ZO-2, or E4-ORF1. (**B**) E4-ORF1 promotes endogenous Dlg1 to accumulate at the plasma membrane. Confluent cells described in (A) were subjected to indirect IF assays with antibody to SAP97 or ZO-2 and visualized by fluorescence microscopy. Dlg1 and ZO-2 signals are green, whereas DAPI-stained nuclei are red. The top center and right panels show Dlg1 staining for two independent CREF-E4-ORF1 lines. White arrows denote examples of Dlg1 plasma membrane staining absent in control CREF cells. (**C**) Chlorpromazine (cpz) substantially increases Dlg1 plasma membrane staining in E4-ORF1-expressing cells. Confluent cells described in (A) were untreated or treated with cpz for 30 min and subjected to indirect IF assays with SAP97 antibody. Results represent the average of two independent experiments, where >100 cells were analyzed. (**D**) E4-ORF1/Dlg1-I3 complexes translocate to the plasma membrane. 293 T cells cultured on coverslips in 24-well plates were lipofected with the indicated peGFP-Dlg1 isoform (0.5 μg) and either empty pGW1 or pGW1-E4-ORF1 (0.5 μg). Cells were subjected to indirect IF assays with E4-ORF1 antibody and visualized by fluorescence microscopy. (**E**) E4-ORF1 binding *per se* triggers Dlg1 translocation to the plasma membrane. 293 T cells cultured on coverslips in 24-well plates were lipofected with peGFP-Dlg1-I3 (0.5 μg) and pGW1-E4-ORF1, -IIIA, or -IIB (0.5 μg). Cells were fixed and visualized by fluorescence microscopy. The efficiency of *wt* and mutant E4-ORF1 proteins to promote translocation of cytoplasmic GFP-Dlg1-I3 to the plasma membrane was determined by quantifying the fraction of GFP-positive cells showing GFP membrane staining. The efficiency of *wt* E4-ORF1 was arbitrarily set to 100%, and the efficiency of mutant IIIA or IIB was reported as a percentage of this value. Results represent the average of three independent experiments, where >100 cells were analyzed. The E4-ORF1 PBM or D2 is specifically disrupted in mutant IIIA or IIB, respectively (see text).

intermediate, such as receptor or nonreceptor tyrosine kinases, heterotrimeric G-protein-coupled receptors, or monomeric G-proteins. Due to additional failures at detecting associations between PI3K and tyrosine-phosphorylated proteins in E4-ORF1-expressing cells (Supplementary Figure 2A) and at diminishing E4-ORF1-induced PKB activation with specific inhibitors of receptor tyrosine kinases (genistein) or src kinases (PP2) (data not shown), as well as heterotrimeric G-proteins (Supplementary Figure 2B), our subsequent efforts focused on Ras monomeric G-proteins. We found that expression of dominant-negative mutant H-Ras-N17 substantially diminished E4-ORF1-induced PKB activation in mouse 3T3 cells (Figure 6A), as well as in *Dlg1*$^{gt/gt}$ MEF reconstituted with GFP-Dlg1-I3 (Figure 6B). These results were specific because H-Ras-N17 did not inhibit PKB activation induced by constitutively activated PI3K (Auger *et al*, 2000) (Figure 6A). Moreover, expression of *wt* H-Ras, as well as *wt* N-Ras and K-Ras (data not shown), but not *wt* R-Ras, reproducibly augmented E4-ORF1-induced PKB activation (Figure 6A).

Considering that Ras normally stimulates not only the PI3K pathway but also the Raf/MEK/ERK pathway, the notion that Ras mediates E4-ORF1-induced PI3K activation initially seemed contradictory to the reported absence of activated ERK1/2 in E4-ORF1-expressing CREF cells (Frese *et al*, 2003). In fact, we consistently detected lower levels of activated, phosphorylated ERK1/2 in E4-ORF1-expressing CREF cells than in control CREF cells (Figure 6C). Reports showing that activated PKB can inhibit Raf activation (Zimmermann and Moelling, 1999), however, provided a plausible explanation

Table I Capacities of *wt* and mutant Dlg1 proteins to support E4-ORF1 functions

Dlg1 protein[a]	E4-ORF1 function	
	Dlg1 translocation to the plasma membrane (percentage of cells)[b]	PI3 K activation (strength relative to *wt* Dlg1-I3)[c]
Group 1		
I2	0.3±0.6	−
I1I3	75±3.6	+ + +
I3	71±8.6	+ + +
ΔNT	66±4.6	+ + +
ΔPDZ1	75±2.6	+ +
ΔPDZ2	3.3±2.3	+
ΔPDZ1 + 2	0.0±0.0	−
ΔPDZ3	69±3.5	+ + +
Δ3PDZ	0.0±0.0	−
ΔSH3	45±11	+ + +
ΔGK	44±6.7	+ +
Group 2		
ΔU3/PDZ3	63±2.6	+ + +
ΔU3/SH3	0.0±0.0	−
ΔPDZ3/SH3	36±5.1	+ + +
ΔU3/PDZ3/SH3	0.0±0.0	−
ΔNT/SH3	42±8.4	+ + +
ΔNT/U3/PDZ3	64±7.0	+ + +
ΔNT/U3/PDZ3/SH3	0.0±0.0	−

[a]All mutants below Dlg1-I3 are derived from this isoform.
[b]Average percentage of transfected cells (±standard deviation) that displayed prominent plasma membrane localization in the presence of *wt* E4-ORF1. Results represent three independent experiments, where >100 cells were analyzed.
[c]Except for the mutant ΔU3/PDZ3/SH3 data, which were not shown, results summarize data presented in Figures 4B and D. + + +, *wt* activity; + + modest defect; +, severe defect; −, no activity.

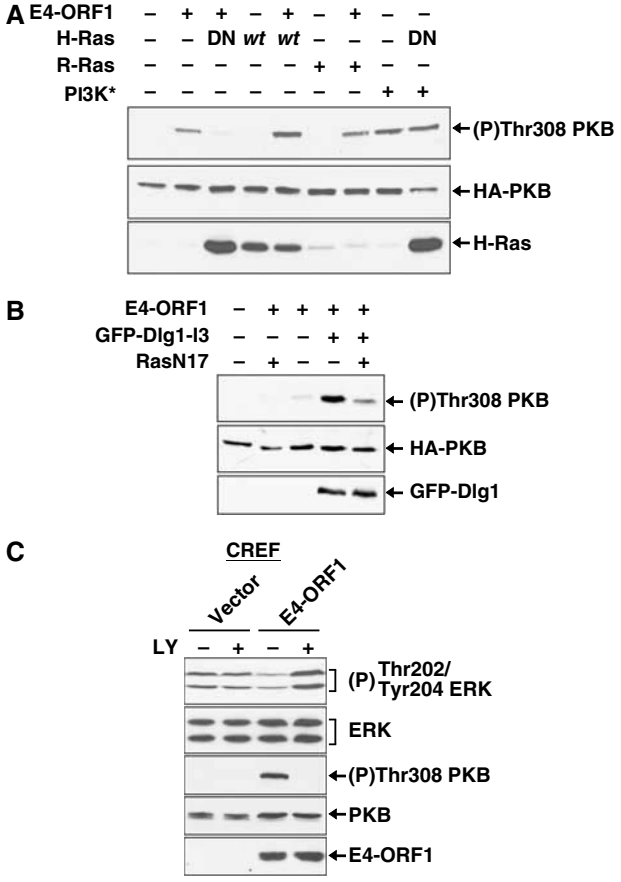

Figure 6 PKB activation by E4-ORF1 requires Ras. (**A**) Dominant-negative (DN) Ras-N17 inhibits E4-ORF1-induced PI3K activation. NIH 3T3 cells on 6-cm dishes were lipofected with pGW1-HA-PKB (0.5 μg) and the indicated combinations of pGW1-E4-ORF1 (25 ng), pGW1-H-Ras-*wt* (100 ng), pGW1-H-Ras-N17 (500 ng), pGW1-R-Ras-*wt* (100 ng), and pCDNA3-myr-p110α coding for constitutively activated PI3K (PI3K*) (25 ng). Cells were serum starved, and equal amounts of cell extracts were blotted with antibody to (P)Thr308 PKB, HA, or H-Ras. (**B**) Ras-N17 also inhibits Dlg1-dependent E4-ORF1-induced PKB activation in *Dlg1*$^{gt/gt}$ MEF. The latter cells on 6-cm dishes were lipofected with pGW1-HA-PKB (0.4 μg), empty pCMV$_{BamNeo}$ (−) or pCMV$_{BamNeo}$-E4-ORF1 (+) (0.1 μg), empty pGW1 (−) or pGW1-RasN17 (+) (1.0 μg), and empty peGFP (−) or peGFP-Dlg1-I3 (+) (0.4 μg). Cells were serum starved, and an equal amount of cell extract was blotted with antibody to (P)Thr308 PKB, HA, or SAP97. (**C**) Treatment with the PI3K inhibitor LY unmasks ERK activation by E4-ORF1 in CREF cells. Confluent CREF cells stably transfected with pBABE (vector) or pBABE-E4-ORF1 (E4-ORF1) were serum starved (−) and treated with 50 μM LY (+) for 1 h. An equal amount of cell extract was blotted with antibody to (P)Thr202/Tyr204 p42/44 MAPK, p42/44 MAPK, (P)Thr308 PKB, PKB, or E4-ORF1.

for these observations. For example, E4-ORF1 could conceivably increase Ras signaling selectively through the PI3K/PKB pathway and thereby promote PKB-mediated suppression of Raf. If so, we predicted that treatment with a PI3K inhibitor to block PKB activation would cause activated ERK1/2 to accumulate above basal levels in E4-ORF1-expressing cells, but not in control cells. The fact that experiments with CREF cells (Figure 6C) and MEF (unpublished data) yielded this exact outcome supported the contention that Ras mediates Dlg1-dependent PI3K activation induced by E4-ORF1.

Model

Based on our cumulative results and published findings on Dlg1, we propose the following model for Dlg1-dependent PI3K activation by the viral E4-ORF1 oncoprotein (Figure 7). (1) In the cytoplasm, the PBM of E4-ORF1 mediates an interaction with Dlg1 PDZ1 + 2, which functions as a molecular switch. With PDZ1 + 2 unoccupied, Dlg1 favors a closed conformation and localizes in the cytoplasm because sterically hindered SH3, I3, and possibly U3 cannot bind membrane- and cytoskeleton-associated complexes at the plasma membrane. (2) With PDZ1 + 2 occupied by E4-ORF1, cytoplasmic Dlg1 in a closed conformation undergoes a dramatic structural change to an open conformation, where SH3, I3, and U3 are exposed and active to engage in protein interactions. (3) By binding to membrane- and cytoskeleton-associated proteins at the plasma membrane, (4) SH3, I3, and U3 recruit cytoplasmic E4-ORF1/Dlg1 complexes to the plasma membrane. (5) E4-ORF1 D2 recruits pp70 into the heterocomplex. (6) The resulting heterocomplex activates Ras, which constitutively localizes at the plasma membrane, by an undetermined E4-ORF1 D2-dependent mechanism that channels most signaling to the PI3K pathway.

Discussion

Tumor viruses represent proven powerful tools to reveal mechanisms commonly underlying the development of human cancers. Given that Dlg1 is targeted not only by

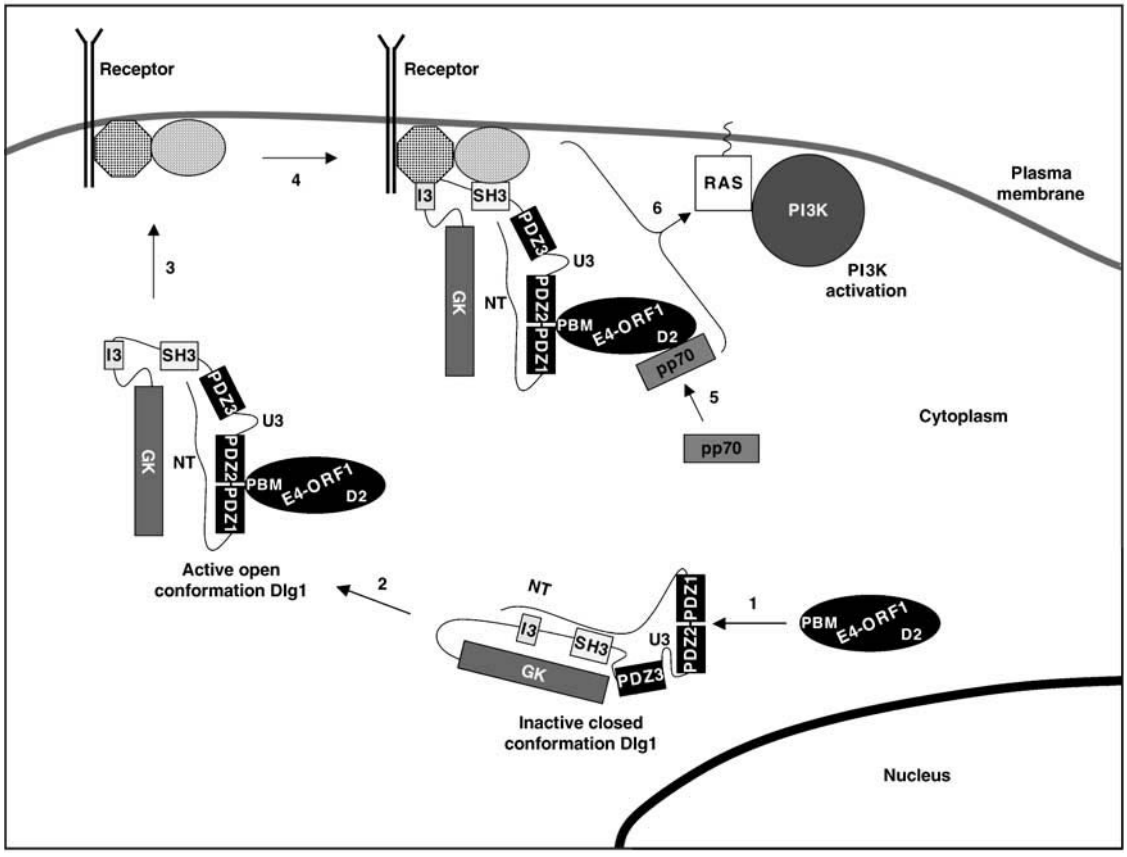

Figure 7 Model for Dlg1-dependent PI3K activation by the adenovirus E4-ORF1 oncoprotein. See text for details.

human adenovirus E4-ORF1 but also by HTLV-1 Tax and HPV E6, deciphering functional consequences for the interaction between Dlg1 and E4-ORF1 may yield valuable insights into molecular events promoting adult T-cell leukemia, cervical carcinoma, and other cancers in people. Significantly, we showed here that binding of E4-ORF1 to Dlg1 specifically promotes oncogenic PI3K activation through a Ras-dependent mechanism. These findings identified the first known function for endogenous Dlg1 in virus-mediated cellular transformation. The absolute requirement for *Dlg1* in E4-ORF1-induced PI3K activation further revealed a surprising proto-oncogene function for this suspected tumor suppressor gene.

It is important to point out that, though unexpected, our finding that *Dlg1* possesses an oncogenic activity does not refute a substantial body of evidence supporting its designation as a candidate tumor suppressor gene. Viral oncoproteins typically disregulate normal functions of their cellular targets, so it may be pertinent that Dlg1 was recently reported to recruit PI3K into a membrane-associated complex with E-cadherin to promote both adherens junction formation and terminal differentiation in enterocytes (Laprise *et al*, 2004). As this activity is predicted to induce an antiproliferative state in cells, it may represent a tumor suppressor function for Dlg1. Therefore, an intriguing possibility is that, whereas Dlg1 normally functions to promote temporally and spatially restricted E-cadherin-dependent PI3K activation during adherens junction formation in cells, Dlg1 modified by E4-ORF1 instead supports constitutive and spatially unrestricted, Ras-dependent PI3K activation at the plasma membrane. This model interestingly hints that E4-ORF1 may simultaneously inactivate a tumor suppressor function and stimulate an oncogenic function of Dlg1.

We discovered nearly identical requirements for PDZ1 + 2, U3, SH3, and I3 in both Dlg1 membrane translocation and PI3K activation induced by E4-ORF1. The fact that Dlg1 translocation to the plasma membrane by definition must precede PI3K activation would seem to dictate that Dlg1 solely contributes to the latter process by trafficking E4-ORF1 to the plasma membrane. Our results cannot, however, rule out the possibility that one or more crucial Dlg1 domains function directly in both Dlg1 membrane translocation and PI3K activation induced by E4-ORF1. Consistent with this idea, despite exhibiting prominent localization at the plasma membrane, an E4-ORF1 PBM mutant tagged with a farnesylation signal failed to activate PI3K in cells (unpublished data).

Our findings showed that PDZ1 + 2 of Dlg1 mediates binding to E4-ORF1 and that this interaction functions as a molecular switch to trigger the resulting heterocomplex to translocate to the plasma membrane, possibly by converting cytoplasmic Dlg1 in an inactive, closed conformation to an active, open conformation, where I3, U3, and SH3 can bind membrane-associated proteins (Figure 7). Supporting this model, localization of Dlg1 to the plasma membrane in both epithelial cells and neurons depends in part on I3 (Hanada *et al*, 2003; Rumbaugh *et al*, 2003), which binds ERM family proteins that connect membrane-associated protein complexes to the cytoskeleton. Furthermore, U3 is reported to recruit Dlg1 into caveolae through an interaction with Caveolin-3 (Folco *et al*, 2004), and SH3 binds CASK (Nix *et al*, 2000) to form a membrane-associated complex. In addition

to their roles in organizing cell–cell membrane junctions, domains equivalent to Dlg1 PDZ2, SH3, and I3 in *Drosophila* Dlg are also essential for its tumor suppressor function (Hough *et al*, 1997). This observation suggests that these domains of Dlg1 may dually regulate its oncogenic and tumor suppressor activities.

The fact that viral oncoproteins often mimic the cytoplasmic domain of activated growth factor receptors may provide insights into the mechanism whereby E4-ORF1 stimulates Dlg1-dependent Ras activation. Such activated cytoplasmic domains bind the Grb2 and Shc adaptor proteins, which, in turn, recruit the Sos guanine nucleotide exchange factor to the plasma membrane to stimulate Ras activity (Hancock, 2003). Future studies will investigate whether these factors also participate in Ras activation induced by E4-ORF1.

It is unclear whether, like E4-ORF1, high-risk HPV E6 and HTLV-1 Tax also activate the oncogenic function of Dlg1. Nonetheless, these viral oncoproteins similarly bind to Dlg1 PDZ1 + 2 (Kiyono *et al*, 1997; Suzuki *et al*, 1999), and our preliminary data suggest that they also promote GFP-Dlg1-I3 translocation to the plasma membrane. Also relevant is a report of PI3K pathway activation in HTLV-1 Tax-transformed cells (Liu *et al*, 2001). In overexpression assays, high-risk HPV E6 promotes degradation of Dlg1 (Gardiol *et al*, 1999), and substantially reduced Dlg1 levels are detected in late-stage invasive HPV-positive cervical carcinomas (Watson *et al*, 2002), consistent with the notion that HPV E6 inactivates Dlg1 in these contexts. Nevertheless, reduced levels of Dlg1 are found neither in HPV-positive premalignant cervical lesions (Watson *et al*, 2002) nor in HPV-infected keratinocytes (Lee and Laimins, 2004), revealing that HPV E6 has differential effects on Dlg1 at early versus late stages of cancer progression. Thus, an intriguing possibility is that HTLV-1 Tax and HPV E6 likewise activate the Dlg1 oncogenic function to provoke disregulated plasma membrane signaling and thereby drive early stages of cancer development.

Materials and methods

Plasmids

pGW1, $pCMV_{Bam3\text{-}Neo}$, pBABE encoding *wt* or mutant *E4-ORF1* cDNAs, as well as pGW1-HA-PKB, were previously described (Weiss *et al*, 1997; Weiss and Javier, 1997; Frese *et al*, 2003). Untagged and HA epitope-tagged *wt* and mutant *Dlg1* cDNAs, derived from *SAP97* cDNAs in pRK174-SAP97-I3 (Muller *et al*, 1995) and pGW1-SAP97-I2 (Kim and Sheng, 1996), were inserted into pGW1, peGFP-C3, or peYFP-C1. The mouse Dlg1 shRNA oligonucleotide (AGGTCCTAAAGGTCTTGGG) was inserted into the pSUPER-retro plasmid (Oligoengine, Seattle). *MUPP1*, *MAGI-1c*, and *ZO-2* cDNAs were inserted into peYFP-C1. *H-Ras* and *R-Ras* cDNAs, generated by PCR amplification from plasmids kindly supplied by Julian Downward and Andrew Chan, were introduced into pGW1. Plasmids were also kindly provided by Silvio Gutkind (pCEFL-m2, pCDNA3-CD8βARKCT), Michael Schneider (pSV-Sport-RasN17), Tom Roberts (pCDNA3-myr-p110α), Robert Kamen (pPyMT1), and Nathalie Sans (peGFP-SAP102).

Cells

Cells were maintained in culture medium (Dulbecco's modified medium supplemented with gentamicin and 6% or 10% fetal bovine serum), which was also supplemented with 20 or 5 μg/ml of puromycin (Sigma) for cells stably transfected with E4-ORF1, PyMT, or Dlg1 shRNA. *MUPP1*, *MAGI-1*, and *Dlg1* mutant MEF were isolated from 13-day embryos of mice missing the *Mpdz* (*MUPP1*) gene (Bell *et al*, 1995) or having the *MAGI-1* gene (in preparation) or the *Dlg1* gene specifically disrupted (Caruana and Bernstein, 2001), respectively.

Transfections and treatments

Transfections were performed using TransIT-LT1 (Mirus) or Lipofectamine (Invitrogen Life). Transient experimental analyses were routinely conducted 48 h post-transfection. In some experiments, cells were serum starved for 16–24 h, during which time they either remained untreated or were treated with 20 ng/ml of PDGF-BB (Invitrogen Life), 10 or 50 μM LY294002 (LY) (Cell Signaling Technologies), 100 μM carbachol (Sigma), 50 ng/ml pertussis toxin (PTx) (Sigma), 10 μg/ml chlorpromazine (cpz), or DMSO vehicle as a control.

Antibodies

Rabbit antisera to MUPP1, MAGI-1, ZO-2, SAP97, and E4-ORF1 were described (Javier, 1994; Lee *et al*, 1997; Glaunsinger *et al*, 2000; Glaunsinger *et al*, 2001). Other antibodies were purchased from Cell Signaling Technologies (Akt, phospho-Akt(Ser473), phospho-Akt(Thr308), p42/44 MAPK, phospho-p42/44 MAPK (Thr202/Tyr204)), Santa Cruz Biotechnologies (phospho-tyrosine PY20), BD Biosciences (H-Ras, Dlg1, GFP), Upstate Biotech (p85 PI3K), Covance (HA 16B12), Southern Biotechnology Associates Inc. (horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG), and Molecular Probes (goat anti-rabbit IgG conjugated to either AlexaFluor 594 or AlexaFluor 488).

Extracts, immunoprecipitations, immunoblots, and pulldowns

Cell extracts were prepared in RIPA buffer supplemented with protease and phosphatase inhibitors as described (Lee *et al*, 1997). For certain experiments, cell extracts in RIPA buffer were fractionated by centrifugation (10 000 *g*, 5 min at 4°C) into detergent-soluble supernatants and detergent-insoluble pellets, and the latter fractions were resuspended in a volume of sample buffer equal to that of the detergent-soluble supernatant. Equal volumes of each fraction were analyzed. GST pulldown, immunoprecipitation, and immunoblot analyses were carried out as described previously (Weiss *et al*, 1996).

Soft agar assays

Soft agar assays were conducted as described previously (Javier, 1994) and were photographed with an Olympus DP11 microscope digital camera mounted on an Olympus CK40 inverted microscope.

Microscopy

Indirect IF assays were performed as described previously (Frese *et al*, 2003). Cells were visualized with a Zeiss Axioplan 2 epifluorescence microscope and photographed with a CoolSnap HQ CCD camera (Photometrics).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

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