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A New Crucial Protein Interaction Element That Targets the Adenovirus E4-ORF1 Oncoprotein to Membrane Vesicles▽

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Human adenovirus type 9 exclusively elicits mammary tumors in experimental animals, and the primary oncogenic determinant of this virus is the *E4-ORF1* oncogene, as opposed to the well-known *E1A* and *E1B* oncogenes. The tumorigenic potential of E4-ORF1, as well as its ability to oncogenically stimulate phosphatidylinositol 3-kinase (PI3K), depends on a carboxyl-terminal PDZ domain-binding motif (PBM) that mediates interactions with several different membrane-associated cellular PDZ proteins, including MUPP1, PATJ, MAGI-1, ZO-2, and Dlg1. Nevertheless, because certain E4-ORF1 mutations that alter neither the sequence nor the function of the PBM abolish E4-ORF1-induced PI3K activation and cellular transformation, we reasoned that E4-ORF1 must possess an additional crucial protein element. In the present study, we identified seven E4-ORF1 amino acid residues that define this new element, designated domain 2, and showed that it mediates binding to a 70-kDa cellular phosphoprotein. We also discovered that domain 2 or the PBM independently promotes E4-ORF1 localization to cytoplasmic membrane vesicles and that this activity of domain 2 depends on E4-ORF1 trimerization. Consistent with the latter observation, molecular-modeling analyses predicted that E4-ORF1 trimerization brings together six out of seven domain 2 residues at each of the three subunit interfaces. These findings importantly demonstrate that PI3K activation and cellular transformation induced by E4-ORF1 require two separate protein interaction elements, domain 2 and the PBM, each of which targets E4-ORF1 to vesicle membranes in cells.

Studies of human adenovirus (Ad) have greatly contributed to our understanding of mechanisms leading to the development of human cancers (65). The 51 different serotypes of human Ad are classified into six subgroups (A through F), and while Ad infection is not linked to human cancers, all subgroup A and B Ads and two subgroup D Ads can elicit tumors in experimentally infected immune-competent rodents (50). Nonetheless, the two subgroup D viruses, Ad type 9 (Ad9) and Ad10, differ strikingly from subgroup A and B Ads by solely eliciting estrogen-dependent mammary tumors, as opposed to undifferentiated sarcomas, in animals (22). In accordance with these two distinct tumorigenic phenotypes, the primary oncogenic determinant of subgroup D Ad9 is the *E4* region-encoded open reading frame 1 (E4-ORF1) protein (23, 54) rather than the *E1* region-encoded E1A and E1B proteins of subgroup A and B Ads (55). Moreover, replacement of the *E1* region in nontumorigenic subgroup C Ad5 with an Ad9 *E4-ORF1* expression cassette confers a tumorigenic phenotype virtually identical to that of Ad9 (54), indicating that *E4-ORF1* likewise controls the oncogenic tropism of Ad9 for mammary gland tissue.

Evidence suggests that Ad *E4-ORF1* genes evolved from an ancestral cellular dUTP pyrophosphatase (dUTPase) gene (63), which encodes an essential enzyme of nucleotide metabolism. This enzyme functions to maintain low dUTP levels in cells, thereby preventing detrimental uracil incorporation into replicating DNA (40). Nevertheless, E4-ORF1 neither possesses this enzymatic activity nor binds or perturbs the function of cellular dUTPase, indicating that these two related proteins have functionally diverged. Results instead suggest that E4-ORF1 exploited the structural framework of the homotrimeric dUTPase enzyme to develop novel cellular growth-promoting activities (63).

The tumorigenic potential of E4-ORF1 depends on a class 1 PDZ domain-binding motif (PBM) having the consensus sequence -(S/T)-X-(V/I/L)-COOH (where X is any amino acid residue) located at its extreme carboxyl terminus (13). This crucial protein interaction element mediates binding to a select group of cellular PDZ proteins, including MUPP1, PATJ, MAGI-1, ZO-2, and Dlg1 (14, 15, 26, 28, 29), most of which are suspected tumor suppressors (7, 15, 33, 56). In general, PDZ proteins function as multivalent scaffolds to organize supramolecular signaling complexes and to localize them to specialized regions of cell-cell contact at the plasma membrane, such as the adherens junction or tight junction (TJ) of epithelial cells (49). Further underscoring the relevance of cellular PDZ proteins to human cancer, the Tax oncoprotein of human T-cell leukemia virus type 1 and the E6 oncoproteins of high-risk human papillomaviruses likewise possess a carboxyl-terminal class 1 PBM that mediates binding to several different cellular PDZ proteins, including one or more of

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those targeted by E4-ORF1 (14, 26, 28, 29). Like the PBM of E4-ORF1, the PBMs of Tax and E6 also contribute to their capacities to transform cells (18, 39, 60).

TJ disruption and a loss of apicobasal polarity are common defects of epithelium-derived cancer cells, and accumulating evidence suggests that such deficiencies directly contribute to carcinogenesis (34). It is therefore notable that the E4-ORF1-interacting PDZ proteins MUPP1, PATJ, MAGI-1, and ZO-2 associate with the TJs of epithelial cells (16, 21, 24, 30) and that both PATJ, an evolutionarily conserved polarity protein (30), and ZO-2 represent key regulators of TJ biogenesis (51, 57). Moreover, in epithelial cells, E4-ORF1 via its PBM prevents proper TJ localization of PATJ and ZO-2 by directly sequestering them in the cytoplasm and, in doing so, disrupts the TJ barrier function and causes a loss of apicobasal polarity (26). This finding supports the idea that E4-ORF1 inactivates the TJ functions of both PATJ and ZO-2 in epithelial cells. The fact that E4-ORF1 similarly sequesters MUPP1, MAGI-1, and ZO-2 in the cytoplasm of fibroblasts (14, 15, 28), which lack TJs, suggests that inactivation of TJ-independent functions of these cellular PDZ proteins, such as the tumor suppressor activity of ZO-2 (15), also contributes to the oncogenic potential of E4-ORF1.

An additional crucial PBM-dependent activity of E4-ORF1 is oncogenic stimulation of cellular phosphatidylinositol 3-kinase (PI3K) (13). PI3K represents a key component of a signaling pathway that regulates cellular metabolism, survival, growth, and proliferation (4, 25) and that is also commonly activated in a wide spectrum of human cancers (36, 46, 47). Recent findings demonstrated a specific requirement for the adherens junction-associated PDZ protein Dlg1 in Ras-dependent E4-ORF1-induced PI3K activation, exposing a previously unrecognized oncogenic function for this cellular tumor suppressor protein (12). In addition, PBM-mediated binding of E4-ORF1 triggers Dlg1 translocation to sites of PI3K activation at the plasma membrane (12, 13), contrasting with the cytoplasmic sequestration of TJ-associated PDZ proteins.

Despite the central importance of the PBM for E4-ORF1 activity, certain E4-ORF1 mutations that alter neither the PBM sequence nor its binding function, including cytoplasmic sequestration of TJ-associated PDZ proteins (26) and plasma membrane translocation of Dlg1 (12), abrogate E4-ORF1-induced PI3K activation and cellular transformation, as well as mammary tumorigenesis in the context of Ad9 (13, 61). These observations indicate that the latter activities of E4-ORF1 depend not only on its PBM, but also on an additional poorly characterized protein element. In this paper, we define this second crucial element, designated domain 2, and show that it functions to mediate both binding to a 70-kDa cellular phosphoprotein and association with membrane vesicles in cells.

MATERIALS AND METHODS

Protein sequences and alignments. Human Ad9 (P89079), Ad12 (AP 000141), Ad3 (ABB17792), and Ad5 (AP 000232) E4-ORF1 protein sequences were acquired from The National Center for Biotechnology Information protein database. The AlignX application module of the Vector NTI 7 software package (InforMax Inc.) was used to generate multiple-sequence alignments.

Mutagenesis and plasmids. Ad9 E4-ORF1 mutations were generated by the recombinant PCR method (19). *E4-ORF1* mutant genes were inserted into plasmid pJ4Ω (66), pGEX-2TK (Pharmacia), or pBABE-*puro* (38) at the BamHI and EcoRI sites and verified by sequencing. The cDNAs coding for six-histidine-tagged human dUTPase (His-dUTPase) and some E4-ORF1 mutants were inserted into plasmid GW1 (British Biotechnology) at the HindIII and EcoRI sites. Plasmids pJ4Ω, GW1, and pGEX-2TK coding for wild-type (wt) E4-ORF1 or mutant E4-ORF1 IA, L89Q, F91S, IIIA, A122D, T123D, L124P, or V125A were described previously (13, 61, 62). Plasmid pME-VSVG, coding for vesicular stomatitis virus G glycoprotein, was a gift of K. Maruyama.

Cells, transfections, and extract preparation. CREF (11), NIH 3T3 (ATCC), TE85 (35), and 293T (42) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% to 10% fetal bovine serum (FBS) and 20 μg/ml gentamicin. Transfections were conducted with either Fugene6 (Roche Molecular Biochemicals), TransIT LT1 (Mirus Bio Corporation), or Lipofectamine (Invitrogen Corporation) as recommended by the manufacturers. Cell extracts in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) supplemented with protease inhibitors and phosphatase inhibitors were prepared as described previously (29). Prior to extract preparation, some cells were either serum starved by incubation in FBS-free DMEM for 16 h or metabolically radiolabeled on a 10-cm dish by incubation with 0.5 mCi of [^{32}P]orthophosphate in phosphate-free DMEM containing 6% dialyzed FBS for 4 h at 37°C. The protein concentrations of cell extracts were determined by the Bradford method (2).

For fractionation assays, cells were lysed in RIPA buffer and then centrifuged (10,000 × *g* for 5 min at 4°C) to isolate detergent-soluble supernatant and detergent-insoluble pellet fractions. The pellet fraction was subsequently solubilized in a volume of sample buffer (65 mM Tris-HCl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue) equal to that of the detergent-soluble supernatant fraction (26). Experiments compared equal volumes of the detergent-soluble supernatant and detergent-insoluble pellet fractions.

Cell line production. Cell lines were generated by either transfection of plasmids using Fugene6 or transduction with retroviral vectors (13, 52). Vesicular stomatitis virus G-pseudotyped murine leukemia retrovirus vectors were produced by cotransfection of 293T cells with plasmids pHIT60, pME-VSVG, and pBABE-*puro* carrying either wt or mutant *E4-ORF1* genes, as described previously (52). Cells transfected or transduced with pBABE-*puro* plasmids were selected and maintained in culture medium supplemented with 10 μg/ml puromycin. Experiments utilized pooled puromycin-resistant cells.

Antibodies. Rabbit polyclonal antiserum to Ad9 E4-ORF1 or ZO-2 were described previously (15, 23). Antibodies to protein kinase B (PKB) and phospho-PKB (Thr308) (Cell Signaling Technologies), Dlg1 (BD Transduction Laboratories), and clathrin heavy chain (Sigma) were purchased, as were rhodamine-conjugated donkey anti-goat immunoglobulin G (IgG) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) and horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (Southern Biotechnology Associates Inc.).

Immunoblot, GST pull-down, and immunoprecipitation assays. Immunoblot analyses were conducted as described previously (13). Glutathione *S*-transferase (GST) pull-down or immunoprecipitation assays were performed as described previously (62), except that cell extracts in RIPA buffer were preincubated with either glutathione-Sepharose beads (Amersham Biosciences) bound to GST or protein A-Sepharose beads (Amersham Biosciences) for 3 h. In some experiments, the recovered proteins were incubated in digestion buffer (10 mM Tris-HCl, pH 8.0, 0.1% SDS, 5 mM EDTA) containing 10 μg/ml proteinase K (Roche Molecular Biochemicals) prior to subsequent analyses.

Focus formation and IF assays. Focus formation and immunofluorescence (IF) assays were carried out as described previously (13, 23). For IF assays, cells cultured on coverslips were fixed for 30 min at room temperature in freshly prepared 4% paraformaldehyde (Polysciences, Inc.) and permeabilized for 5 min at room temperature in 0.5% Triton X-100. The coverslips were mounted on slides with Slowfade Light (Molecular Probes), and cells were visualized with a Zeiss Axioplan 2 epifluorescence microscope and photographed with a CoolSnap HQ charge-coupled device camera (Photometrics).

Size exclusion chromatography. Extracts from E4-ORF1-expressing cells in RIPA buffer (200 μl) were mixed with size markers (200-kDa β-amylase, 66-kDa bovine serum albumin, 43-kDa ovalbumin, 29-kDa carbonic anhydrase, and 12.4-kDa cytochrome *c* [Sigma-Aldrich Co.] and 48.6-kDa recombinant trimeric His-dUTPase expressed in 293T cells) prior to being loaded on a Superose 6 10/300 size exclusion column (Amersham Biosciences) preequilibrated with TBS elution buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% sodium deoxycholate). A Pharmacia LKB Biotechnology fast protein liquid chromatography system was employed to run samples through the column with elution buffer at a flow rate of 0.2 ml/min and to collect 300-μl fractions. Aliquots of each fraction were separated by SDS-polyacrylamide gel electrophoresis

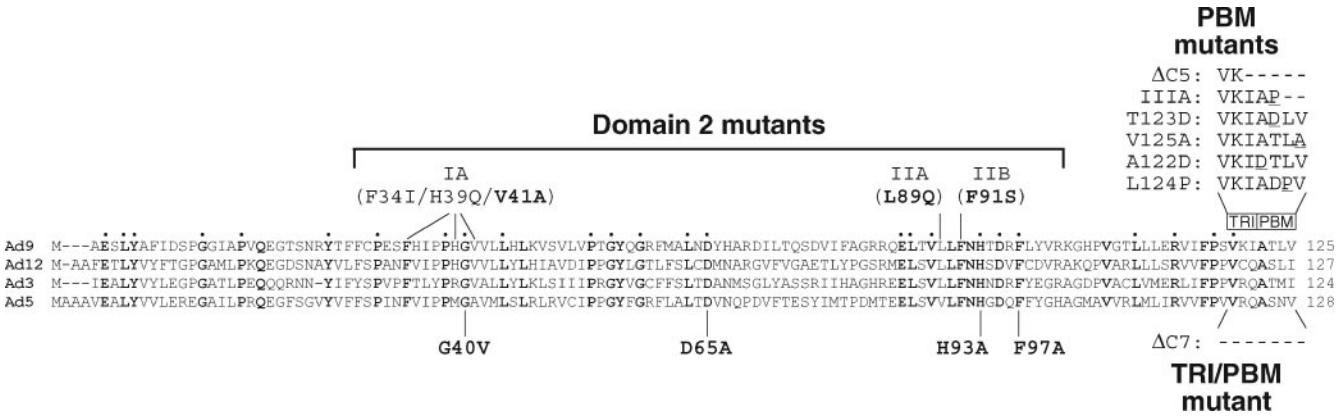

FIG. 1. Mutations in domain 2, TRI, and PBM elements of the Ad9 E4-ORF1 protein. A multiple-sequence alignment of Ad subgroup A to D E4-ORF1 protein sequences reveals 33 conserved amino acid residues (boldface). The filled circles are placed above 25 such Ad9 E4-ORF1 residues mutated in the current study. Also depicted are the locations of residues defining domain 2, TRI (VKI, residues 119 to 121), and core PBM (ATLV, residues 122 to 125) elements of the Ad9 E4-ORF1 polypeptide. The names of all Ad9 E4-ORF1 mutants are also shown, with boldface names indicating the seven mutants that define domain 2. For PBM and TRI/PBM mutants, underlined residues or dashes represent substituted or deleted residues, respectively.

(PAGE) and immunoblotted with E4-ORF1 antibody. Size markers were visualized by staining them with Coomassie brilliant blue, except for His-dUTPase, which was detected by immunoblotting it with the His probe antibody (Santa Cruz).

Molecular modeling. 3D-JIGSAW automatic modeling server software (1) and MacPyMol software (DeLano Scientific LLC) were used to model the E4-ORF1 protein to the crystal structure of trimeric human dUTPase (Protein Data Bank accession number 1Q5H) and to generate surface representations of the E4-ORF1 trimer, respectively.

RESULTS

Mutational analysis of conserved residues in Ad9 E4-ORF1. The existence of an additional crucial protein element, referred to as domain 2, in Ad9 E4-ORF1 is suggested by the unique characteristics of E4-ORF1 triple point mutant IA (F34I/H39Q/V41A) and single point mutants IIA (L89Q) and IIB (F91S) (61). Specifically, these three domain 2 mutants carry mutations situated outside of the PBM sequence (Fig. 1) and accordingly retain wt binding to cellular PDZ proteins (26, 62) yet fail to promote either PI3K activation or cellular transformation (13, 61). To further define domain 2, we sought to isolate additional E4-ORF1 mutants with the same characteristics. As we noted that the domain 2 mutation F91S alters one of 33 conserved amino acid residues among subgroup A through D human Ad E4-ORF1 proteins, all of which stimulate PI3K and transform cells (13, 61, 63), and that all conserved residues except alanine 122 lie outside of the PBM sequence (Fig. 1), our chosen approach for isolation of additional domain 2 mutants was to introduce a point mutation into each conserved residue of the 125-residue Ad9 E4-ORF1 polypeptide.

A previous study had already reported the characterization of Ad9 E4-ORF1 mutants with point mutations in 8 of the 33 conserved residues (61). One of these mutants (N92I) was not stably expressed, and among the remaining seven expression-competent mutants, five (Q19L/Q19G, F34I, L46P, L90V, and V106A) displayed approximately wt cellular transforming activity whereas two (domain 2 mutant F91S and PBM mutant A122D) retained no or very limited cellular transforming activity (Fig. 1) (61). In the present study, we therefore constructed a panel of new Ad9 E4-ORF1 mutants in which each of the remaining 25 conserved residues was changed to an alanine residue, except for conserved glycine residues, which were changed to either a threonine or a valine residue.

Isolation of five new candidate domain 2 mutants. Two basic characteristics of the original domain 2 mutants, as well as PBM mutants, are wt protein expression and severe cellular transformation defects, so we used these two criteria in our initial screening of the 25 new Ad9 E4-ORF1 mutants. In transient-expression assays with CREF fibroblasts, 11 mutants exhibited either severe (P17A, Y26A, L109A, and P117A) or moderate (E3A, L5A, G13T, P31A, G58T, E85A, and L86A) protein expression defects compared to wt E4-ORF1 (Fig. 2A) and therefore were not further investigated. The other 14 mutants displayed wt protein expression, although 9 of them manifested either approximately wt (V88A and D95A with ≥85% of wt focus formation) or appreciable (Y6A, P38A, L44A, P53A, G55T, L63A, and V119A with 19% to 44% of wt focus formation) cellular transforming activity (Fig. 2A to C) and therefore also were not further investigated. By contrast, the remaining five mutants exhibiting wt protein expression satisfied our initial criteria for candidate domain 2 mutants by showing either minimal (F97A and R113A with 1% or 7% of wt focus formation, respectively) or no detectable (G40V, D65A, and H93A) cellular transforming activity (Fig. 2A to C); the latter phenotype matches that of the original domain 2 mutants IA, L89Q, and F91S and PBM mutants IIIA, T123D, and V125A (Fig. 1) (13). Because we subsequently discovered a PBM defect for mutant R113A (S. H. Chung, unpublished data), however, only mutants G40V, D65A, H93A, and F97A were selected for further characterization.

We next wanted to identify which of the three point mutations (F34I/H39Q/V41A) in domain 2 mutant IA (Fig. 1) is primarily responsible for its severe cellular transformation defects; however, the previously reported transformation-proficient phenotype of an Ad9 E4-ORF1 mutant carrying the F34I mutation (61) focused this effort on the H39Q and V41A

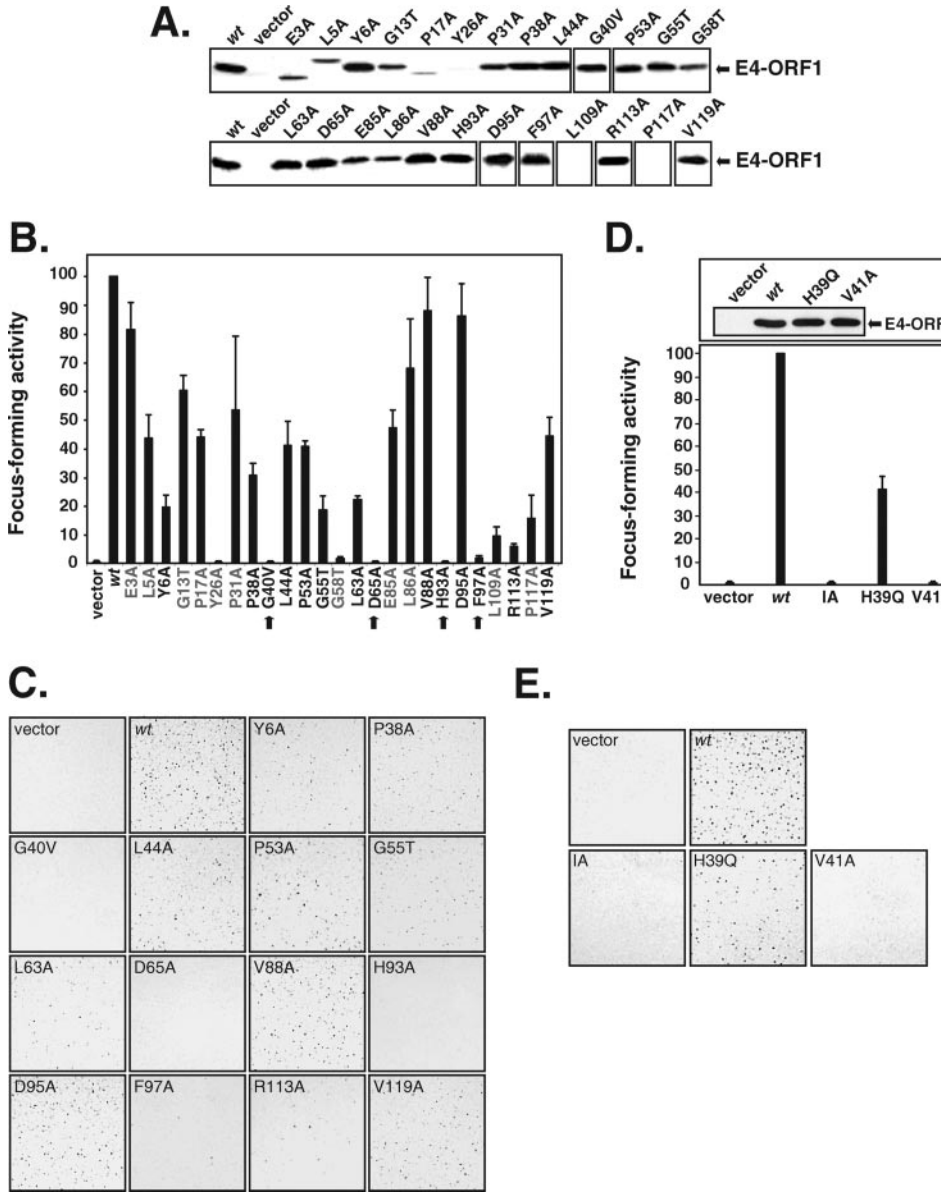

FIG. 2. Protein expression and cellular transformation phenotypes of 25 new Ad9 E4-ORF1 mutants. (A) Transient expression levels of E4-ORF1 mutant proteins. CREF fibroblasts on 6-cm dishes were transfected with 3.5 μg of empty pJ4Ω plasmid (vector) or pJ4Ω plasmid coding for either wt E4-ORF1 (wt) or the indicated mutant E4-ORF1. An equivalent amount of each cell extract (100 μg protein) was immunoblotted with E4-ORF1 antibodies. Two separate controlled experiments are presented in the top and bottom rows, with double vertical lines denoting the locations of deleted irrelevant lanes. Similar results were obtained in four independent experiments. (B) Cellular transforming activities of E4-ORF1 mutants. CREF fibroblasts were transfected with empty pJ4Ω plasmid (vector) or pJ4Ω plasmid coding for either wt E4-ORF1 (wt) or the indicated mutant E4-ORF1, as for panel A. Transformed foci were counted at 3 weeks posttransfection. Focus-forming activity is reported as the percentage of foci generated by each E4-ORF1 mutant relative to wt E4-ORF1, which was set at 100%. Data compiled from three independent experiments include the means plus standard deviations. The names of mutants with or without protein expression defects, as determined by data presented in panel A, are colored gray or black, respectively. The arrows point to candidate domain 2 mutants. (C) Photographs of selected focus formation assays quantified in panel B. Only expression-competent Ad9 E4-ORF1 mutants are shown. (D) V41A is the crucial mutation in mutant IA. Focus formation assays in CREF fibroblasts were carried out, quantified, and presented as described for panel B. Data compiled from two independent experiments show the mean plus the range. The immunoblot assay comparing E4-ORF1 protein levels (top) was conducted as described for panel A. (E) Photographs of focus formation assays quantified in panel D.

mutations. Indeed, the results from focus formation assays demonstrated that, whereas mutant H39Q retains substantial cellular transforming activity, albeit somewhat less than wt E4-ORF1, mutant V41A behaves identically to mutant IA in lacking any detectable cellular transforming activity despite expressing wt levels of protein (Fig. 2D and E).

In summary, our results designated G40V, V41A, D65A, H93A, and F97A candidate domain 2 mutants. The experi-

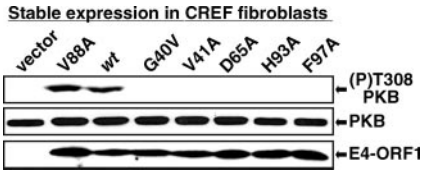

FIG. 3. All five candidate domain 2 mutants fail to activate PKB. PKB activation by stably expressed candidate domain 2 mutants. An equivalent amount of extract (60 μg protein) from serum-starved CREF fibroblasts stably transfected with empty pBABE plasmid (vector) or pBABE plasmid coding for either wt E4-ORF1 (wt) or the indicated mutant E4-ORF1 were immunoblotted with antibodies to phospho-PKB (T308), PKB, or E4-ORF1.

ments detailed below investigated whether these five mutants share additional properties with the original domain 2 mutants.

Candidate domain 2 mutants fail to stimulate the PI3K pathway. Besides lacking cellular transforming activity, another common defect of original domain 2 and PBM mutants is the inability to stimulate the PI3K pathway in cells (13). We therefore analyzed the five candidate domain 2 mutants for their capacities to elevate cellular levels of activated, phosphorylated PKB, a key downstream effector of PI3K (4). Following transient expression in 3T3 fibroblasts (Chung, unpublished) or stable expression in CREF fibroblasts, wt E4-ORF1 substantially increased PKB phosphorylation on both serine residue 473 (Chung, unpublished) and threonine residue 308, whereas all candidate domain 2 mutants lacked this activity, identically to the negative control empty plasmid (vector) (Fig. 3). These results are specific, because the transformation-proficient mutant V88A, which has a mutation adjacent to those of the original domain 2 mutants L89Q and F91S (Fig. 2B and C), behaved identically to wt E4-ORF1 in these assays.

Candidate domain 2 mutants bind to cellular PDZ proteins. The one known characteristic that distinguishes the original domain 2 mutants from PBM mutants is retention of binding to cellular PDZ proteins. Consequently, we screened candidate domain 2 mutants for this activity by subjecting them to GST pull-down assays with extracts of CREF fibroblasts metabolically labeled with [^{32}P]orthophosphate. Consistent with previous results (14, 15, 28, 29, 62), these assays revealed that positive control wt E4-ORF1, mutant V88A, and original domain 2 mutants L89Q and F91S, but not negative control PBM mutant IIIA, bind endogenously expressed, phosphorylated forms of the E4-ORF1-associated cellular PDZ proteins (Fig. 4A and B). Importantly, like the original domain 2 mutants, all five candidate domain 2 mutants retained this activity (Fig. 4A and Fig. 5B and C).

This finding provided us with seven different domain 2 mutants, two original mutants (L89Q and F91S) and five new mutants (G40V, V41A, D65A, H93A, and F97A), which in subsequent experiments were employed to expose functions for domain 2.

Domain 2 defines a new protein interaction element. The domains of DNA tumor virus oncoproteins generally act to mediate interactions with critical cellular regulatory proteins (17), so we hypothesized that domain 2 would perform the same function for E4-ORF1. Indeed, the results of GST pull-down assays presented in Fig. 4A and B additionally revealed that wt E4-ORF1, PBM mutant IIIA, and transformation-proficient mutant V88A, but none of the seven domain 2 mutants, interact with a 70-kDa cellular phosphoprotein (pp70). Similar results were obtained with extracts from a ^{32}P-labeled Ad9-induced mammary tumor cell line (Chung, unpublished). We also substantiated the designation of pp70 as a phosphoprotein by showing its elimination by treatment with proteinase K (Fig. 4B) but not RNase A or DNase I (Chung, unpublished).

Significantly, a cellular protein comigrating with pp70 recovered in GST pull-down assays coimmunoprecipitated with wt E4-ORF1, but not domain 2 mutant F91S stably expressed in human TE85 cells (Fig. 4C). The pp70 binding defect of mutant F91S was specific, because cellular PDZ proteins efficiently coimmunoprecipitated with this mutant. These findings suggest that E4-ORF1 and pp70 form a complex in cells.

Four important conclusions were drawn from the experiments described thus far. First, the common failure of domain 2 mutants to bind pp70 indicates that the seven domain 2 residues collectively define a single protein-interaction element. Second, the reciprocal phenotypes of PBM mutant IIIA versus domain 2 mutants in binding only to either cellular pp70 or cellular PDZ proteins, respectively, demonstrate that domain 2 and the PBM are independent protein interaction elements. Third, both domain 2 and the PBM are required for PI3K activation and cellular transformation induced by E4-ORF1. Fourth, the transformation-deficient phenotype of domain 2 mutants, which cannot bind pp70, coupled with the transformation-proficient phenotype of the control mutant V88A, which can bind pp70 (Fig. 4A), links the pp70 interaction to cellular transformation.

Also noteworthy is the fact that, in the GST pull-down assays presented in Fig. 4B, comparison of the protein binding profiles for PBM mutant IIIA and domain 2-PBM double mutant F91S/IIIA exposed two additional putative domain 2-interacting cellular phosphoproteins migrating at approximately 150 kDa and 190 kDa. While results with domain 2 mutant F91S gave the impression that it unexpectedly binds to the 190-kDa protein, this band actually represents the E4-ORF1-associated PDZ protein MAGI-1, which comigrates with the 190-kDa protein. These observations hint that domain 2 mediates binding not only to pp70, but also possibly to other cellular factors.

Domain 2 mutants form functional homotrimers and monomers in cells. Unpublished results show that the 14-kDa Ad9 E4-ORF1 protein exists as both a trimer and a monomer in cells (S. H. Chung, B. V. V. Prasad, and R. T. Javier, unpublished data). We first measured the capacities of domain 2 mutants to trimerize by subjecting extracts of 293T cells transiently expressing each protein to size exclusion chromatography in the presence of 0.1% deoxycholate (DOC), a detergent that promotes quantitative trimerization of oligomerization-competent E4-ORF1 proteins. We found that all domain 2 mutants, as well as all PBM mutants and the domain 2-PBM double mutant F91S/IIIA, eluted similarly to wt E4-ORF1 at approximately 39 kDa (Fig. 5A), consistent with the mass of a 42-kDa trimer. It is important to note that the monomeric form of E4-ORF1 in cells was not detected in these assays due to the presence of 0.1% DOC. The trimerization competencies of the mutants described above are concordant with the fact that mutations in neither domain 2 nor the PBM affect the E4-ORF1 trimerization (TRI) element (Fig. 1). By contrast, even in the presence of 0.1% DOC, the control

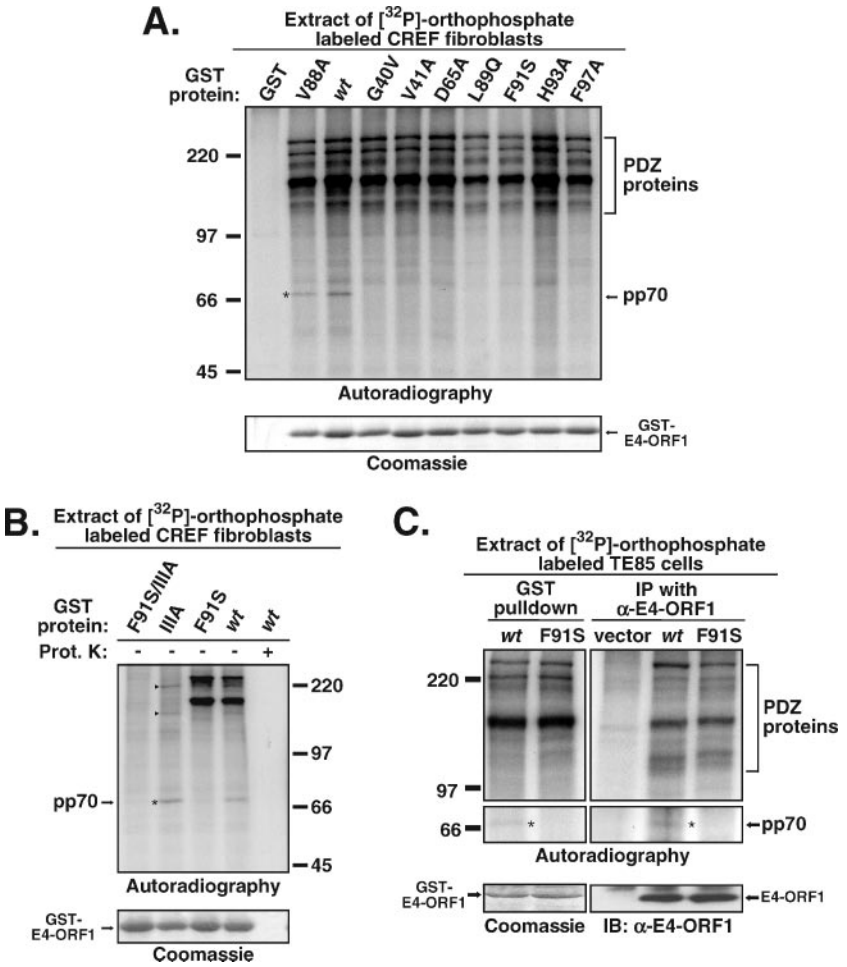

FIG. 4. Domain 2 is a new protein interaction element in Ad9 E4-ORF1. (A) Domain 2 mutants bind to cellular PDZ proteins but not to cellular pp70 in GST pull-down assays. GST pull-down assays conducted with extracts (750 μg protein) of [^{32}P]orthophosphate-labeled CREF fibroblasts were performed as described in Materials and Methods. The recovered proteins were resolved by SDS-PAGE and visualized by autoradiography. An asterisk marks the location of the cellular pp70 band. The control Coomassie-stained gel (bottom) verified the use of equivalent amounts of each GST-E4-ORF1 fusion protein. (B) Cellular pp70 is a phosphoprotein that binds to PBM mutant IIIA but not domain 2 mutant F91S. GST pull-down assays were conducted as described for panel A. Recovered proteins were either mock treated (−) or treated with proteinase K (+) for 30 min at 37°C and then analyzed as described for panel A. The control Coomassie-stained gel (bottom) verified the use of an equivalent amount of each GST-E4-ORF1 fusion protein. Putative domain 2-interacting 150-kDa and 190-kDa proteins are marked with arrowheads. (C) Cellular pp70 coimmunoprecipitates with E4-ORF1. Human TE85 cells or the same cells stably transduced with empty pBABE plasmid (vector) or pBABE plasmid encoding either wt E4-ORF1 (wt) or mutant F91S were metabolically labeled with [^{32}P]orthophosphate. Extracts (800 μg protein) of TE85 cells were subjected to GST pull-down assays with the indicated fusion proteins, whereas extracts (3 mg protein) of the indicated stably transduced TE85 lines were immunoprecipitated with E4-ORF1 antibodies. Proteins recovered by pull-down (left) and immunoprecipitation (IP) (right) were resolved on the same SDS-PAGE gel, and portions of the gel containing either cellular PDZ proteins (top) or pp70 (middle) were visualized by autoradiography. The data are assembled into four separate properly aligned boxes to permit the presentation of optimal exposures. Other portions of the gel were either Coomassie stained to verify the use of equivalent amounts of each GST fusion protein (lower left) or immunoblotted (IB) with E4-ORF1 antibodies to verify comparable immunoprecipitations of E4-ORF1 protein (lower right).

E4-ORF1 mutant ΔC7, with a seven-residue carboxyl-terminal deletion that eliminates both the TRI and PBM elements (Fig. 1), instead eluted at approximately 13 kDa, comparable to the mass of a 14-kDa monomer. The proper trimerization and PDZ protein binding observed for domain 2 mutants importantly argue that their mutations do not cause gross protein misfolding.

The unpublished results mentioned above further indicate that E4-ORF1 trimers and monomers perform distinct functions in cells, where trimers bind to Dlg1 but monomers instead bind to MUPP1, PATJ, MAGI-1, and ZO-2 and sequester them within detergent-insoluble complexes. Thus, the facts that, in CREF fibroblasts, stably expressed wt E4-ORF1 and all domain 2 mutants, but not PBM mutant IIIA, efficiently coimmunoprecipitated endogenous Dlg1 (Fig. 5B) and sequestered endogenous ZO-2 within detergent-insoluble complexes (Fig. 5C) imply that domain 2 mutants produce trimers and monomers that properly bind to PDZ proteins. In addition, these findings extend the results presented in Fig. 4A and B by showing that domain 2 mutants likewise retain a wt capacity to complex with PDZ proteins in cells.

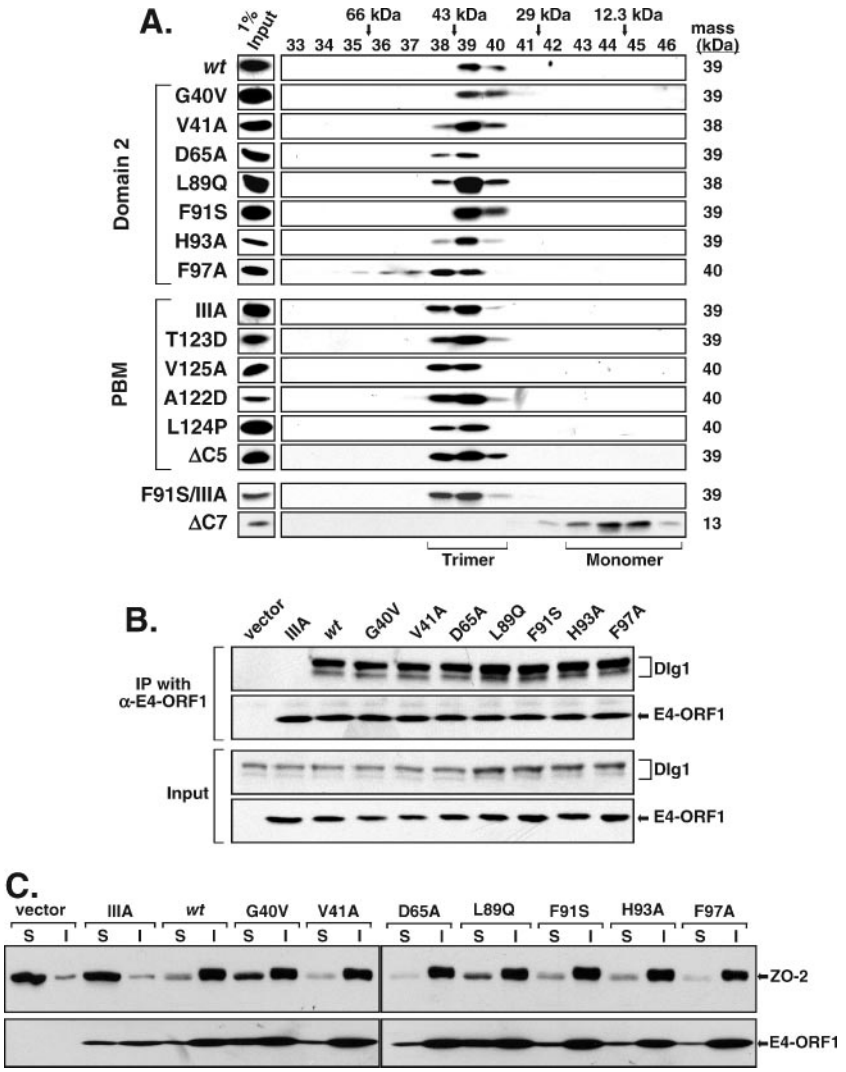

FIG. 5. Domain 2 mutants form functional homotrimers and monomers. (A) Domain 2 mutants properly trimerize. 293T cells on 6-cm dishes were transfected with 3 μg of GW1 plasmid coding for either wt E4-ORF1 (wt) or the indicated mutant E4-ORF1 using TransIT LT1, and cell extracts were subjected to analytical size exclusion chromatography in the presence of 0.1% deoxycholate as described in Materials and Methods. One-tenth of each eluted fraction was immunoblotted with E4-ORF1 antibodies. Only fractions containing E4-ORF1 are shown. The peak elution fraction of each size marker is also indicated. (B) Endogenous Dlg1 coimmunoprecipitates with domain 2 mutants stably expressed in cells. Extracts (250 μg protein) from CREF fibroblasts transduced by empty pBABE retrovirus (vector) or pBABE retrovirus coding for either wt E4-ORF1 (wt) or the indicated mutant E4-ORF1 were immunoprecipitated with E4-ORF1 antibodies. Recovered proteins or total cell extracts (60 μg protein) were immunoblotted with antibodies to E4-ORF1 or Dlg1. (C) Domain 2 mutants aberrantly sequester ZO-2 within detergent-insoluble complexes. The CREF fibroblast lines shown in panel B were fractionated into detergent-soluble supernatant (S) and detergent-insoluble pellet (I) fractions as described in Materials and Methods. The soluble fraction (60 μg protein) and an equivalent amount of the insoluble fraction were subjected to SDS-PAGE and immunoblotted with antibodies to E4-ORF1 or ZO-2.

Domain 2 or the PBM independently targets E4-ORF1 to membrane vesicles. Though some E4-ORF1 localizes to the plasma membrane (13), the majority of the protein associates with numerous small punctae distributed throughout the cytoplasm (13, 64), a staining pattern strikingly similar to that of membrane vesicles. Significantly, the results of IF assays revealed that a substantial fraction of wt-E4-ORF1-containing cytoplasmic punctae colocalized with clathrin-coated membrane vesicles in CREF fibroblasts (Fig. 6A). This finding suggests that the cytoplasmic punctae produced by E4-ORF1 reflect its association with clathrin-coated vesicles and likely other types of membrane vesicles in cells. It is also important to note that all data presented in Fig. 6A to C are representative of approximately 100% of the cells visualized in the IF assays.

Previous results indicate, however, that disruption of either the PBM alone or domain 2 alone does not abolish E4-ORF1 targeting to membrane vesicles (61). In this regard, PBM mutants and original domain 2 mutants, as well as new domain 2 mutants (Chung, unpublished), retain the capacity to localize to cytoplasmic punctae in CREF fibroblasts. We therefore investigated whether disruption of both protein elements in E4-ORF1 would abolish this localization. In CREF fibroblasts, stably expressed domain 2 mutant F91S or PBM mutant IIIA

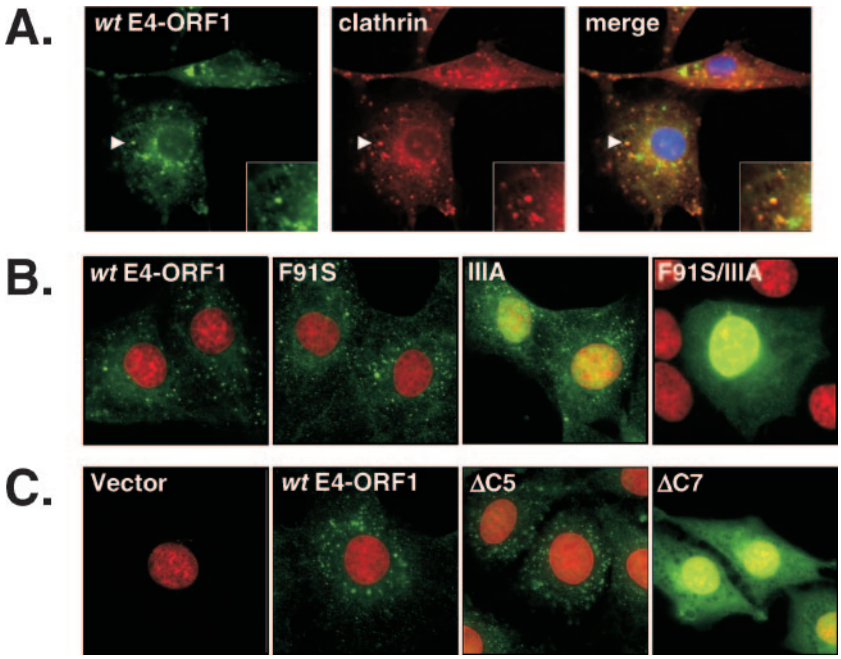

FIG. 6. Domain 2 or the PBM independently targets Ad9 E4-ORF1 to membrane vesicles. (A) E4-ORF1 colocalizes with clathrin-coated vesicles in cells. CREF fibroblasts stably transfected with pBABE plasmid coding for wt E4-ORF1 were stained with E4-ORF1 (green) and clathrin (red) antibodies and then visualized by epifluorescence microscopy, as described in Materials and Methods. The arrow points to a representative vesicle where E4-ORF1 and clathrin colocalize. The area of interest is magnified in the inset. In the merged image, DAPI-stained nuclei are blue. E4-ORF1 membrane staining is not evident under these experimental conditions. (B) Domain 2 mutant F91S and the PBM mutant IIIA, but not domain 2-PBM double mutant F91S/IIIA, retain vesicular staining patterns. CREF fibroblasts stably transfected with pBABE plasmid coding for either wt E4-ORF1 or the indicated mutant E4-ORF1 were stained with E4-ORF1 antibody and visualized by epifluorescence microscopy. DAPI-stained nuclei are pseudocolored red. (C) E4-ORF1 trimers, but not monomers, possess domain 2 activity. CREF fibroblasts transduced with empty pBABE plasmid (vector) or pBABE plasmid coding for the indicated E4-ORF1 mutant were stained with E4-ORF1 antibody and visualized by epifluorescence microscopy. DAPI-stained nuclei are pseudocolored red.

exhibited the expected punctate cytoplasmic staining patterns, similar to that of wt E4-ORF1 (Fig. 6B), although mutant IIIA produced somewhat more diffuse staining throughout the cell, as reported previously (61). More importantly, the stably expressed double mutant F91S/IIIA, as well as G40V/IIIA and D65A/IIIA (Chung, unpublished), failed to produce cytoplasmic punctae and instead exclusively localized diffusely in CREF fibroblasts (Fig. 6B). The prominent diffuse nuclear staining displayed by double mutants presumably reflects passive diffusion into the nucleus, given that control green fluorescent protein localized similarly in these cells (K. K. Frese, unpublished data). These findings indicate that domain 2 or the PBM independently functions to target E4-ORF1 to membrane vesicles.

The fact that E4-ORF1 trimers and monomers interact with distinct subsets of the cellular PDZ protein targets prompted additional experiments to determine whether one of these forms likewise selectively mediates domain 2-dependent vesicle association. With respect to this idea, we found that, whereas the trimerization-competent PBM mutant ΔC5 (Fig. 1 and 5A) displayed an expected punctate cytoplasmic staining pattern in CREF fibroblasts, reflecting domain 2-dependent targeting to membrane vesicles, the trimerization-incompetent TRI-PBM double mutant ΔC7 (Fig. 1 and 5A) instead exclusively localized diffusely throughout the cells (Fig. 6C). We were unable to establish whether this vesicle localization defect of mutant ΔC7 stems from a failure to bind pp70, because GST dimerization complemented the trimerization deficiency of TRI mutants in GST pull-down assays and because reduced stable expression of TRI mutants relative to wt E4-ORF1 in cells precluded interpretable comparisons in coimmunoprecipitation assays (Chung, unpublished). Nevertheless, our results show that trimers, but not monomers, of E4-ORF1 promote its domain 2-dependent association with membrane vesicles in cells.

Predicted clustering of domain 2 residues at subunit interfaces of the E4-ORF1 trimer. Recent evidence suggests that E4-ORF1 adopts a protein fold similar to that of human dUTPase (S. H. Chung, B. V. V. Prasad, and R. T. Javier, unpublished data), which functions as a homotrimer in cells (63). Crystal structure data demonstrate that trimeric dUTPase contains three identical catalytic sites, each consisting of a catalytic cleft formed at the interface between two adjacent subunits capped by a carboxyl-terminal nucleotide-binding P-loop motif supplied by the third subunit (37). As our results show that E4-ORF1 trimers, but not monomers, mediate domain 2-dependent vesicle association (Fig. 6C), we modeled E4-ORF1 on the crystal structure of trimeric human dUTPase to predict the locations of domain 2 residues. Interestingly, this analysis predicted that, in an E4-ORF1 trimer, six out of seven domain 2 residues become clustered at each of the three subunit interfaces, where adjacent subunits supply either residues G40, V41, L89, and F91 or residues D65 and H93 (Fig. 7), analogous to residues that form the dUTPase catalytic cleft

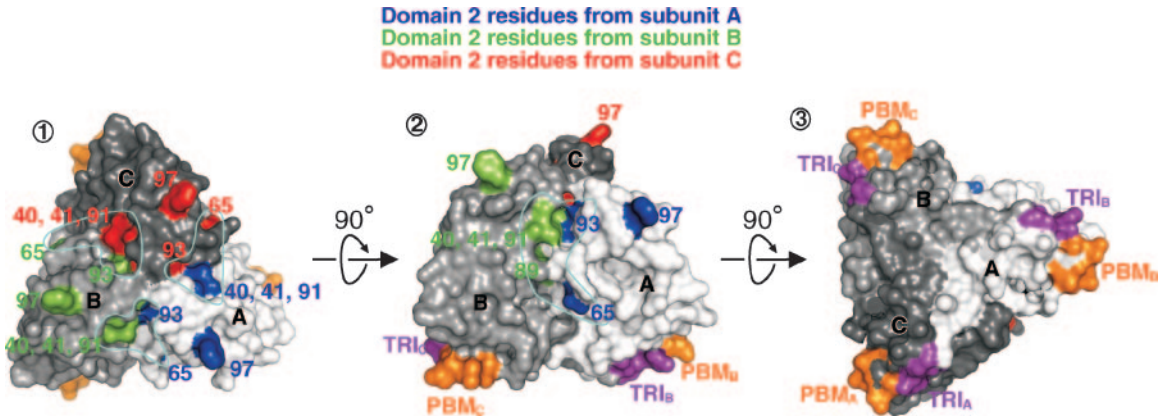

FIG. 7. Predicted clustering of domain 2 residues at subunit interfaces of the E4-ORF1 trimer. Surface representations of the E4-ORF1 trimer are shown from top (1), side (2), and bottom (3) viewpoints, with different monomer subunits colored white (subunit A), light gray (subunit B), or dark gray (subunit C) and their corresponding domain 2 residues (indicated by residue number) colored blue, green, or red, respectively. In the top and side viewpoints, a cyan-colored line circumscribes the six domain 2 residues that group together at one subunit interface. TRI and PBM elements are colored purple and orange, respectively, with subscript A, B, and C indicating their subunit origins.

(37). This analysis also predicted that the E4-ORF1 carboxyl-terminal PBM occupies a separate location analogous to the more extended dUTPase carboxyl-terminal arm containing the P-loop motif (Fig. 7).

DISCUSSION

Previous findings suggested that the oncogenic potential of Ad9 E4-ORF1 depends not only on the PBM, but also on a second protein element, which until now remained poorly characterized. In the present study, we defined this second crucial element, termed domain 2, through the identification of seven point mutations that affect neither the sequence (Fig. 1) nor the function (Fig. 4A and 5B and C) of the PBM yet eliminate the ability of Ad9 E4-ORF1 to activate PI3K (Fig. 3) and to transform cells (Fig. 2). As five of these mutations alter highly conserved amino acid residues (Fig. 1), we postulate that domain 2, like the PBM, represents a crucial protein element common to all human Ad E4-ORF1 proteins.

We also showed that domain 2 residues collectively constitute a single protein interaction element that mediates binding to the unidentified cellular phosphoprotein pp70 (Fig. 4). Other data indicated that domain 2 and the PBM represent two independent protein-interaction elements in the E4-ORF1 protein (Fig. 4) and that the functional defects of domain 2 mutants do not indirectly stem from gross protein misfolding or an inability to form functional trimers and monomers in cells (Fig. 5). More importantly, we exposed an intimate link between domain 2-mediated binding to pp70 and both PI3K activation and cellular transformation induced by E4-ORF1 (Fig. 2 to 4). Thus, identification of pp70 in future studies should aid in the delineation of molecular mechanisms that underlie these key Ad9 E4-ORF1 activities.

The facts that domain 2 residues distribute to three widely separated regions of the linear Ad9 E4-ORF1 polypeptide (Fig. 1) and that E4-ORF1 trimers, but not monomers, possess domain 2 activity (Fig. 6C) hint that E4-ORF1 trimerization brings domain 2 residues together to form a functional protein interaction element. Consistent with this idea, molecular modeling of E4-ORF1 to the crystal structure of related trimeric human dUTPase predicted that six out of seven domain 2 residues cluster together at each of the three subunit interfaces in an E4-ORF1 trimer (Fig. 7). The single outlier was domain 2 residue F97, hinting that the domain 2 protein-binding surface may extend beyond subunit interfaces. Results from this analysis additionally supported a model in which an ancestral dUTPase gene evolved into the adenovirus *E4-ORF1* gene by events that transformed the dUTPase catalytic cleft or carboxyl-terminal P-loop motif into E4-ORF1 domain 2 or the PBM, respectively. This model implies that, analogous to the three catalytic clefts and P-loop motifs present in a dUTPase trimer, three domain 2 and PBM elements similarly exist in an E4-ORF1 trimer. As multiple PBM elements of an E4-ORF1 trimer permit cooperative binding to the PDZ1 +2 domain tandem of Dlg1 (Chung et al., unpublished), multiple domain 2 elements may similarly allow synergistic interactions with pp70.

Exciting new findings have revealed that domain 2 or the PBM is independently capable of mediating E4-ORF1 localization to membrane vesicles (Fig. 6A and B), which interestingly represent key regulators of both protein trafficking and signal transduction in cells (48, 53). Previous results indicated that E4-ORF1-induced PI3K/PKB activation depends on the PBM, due at least in part to its capacity to mediate binding to Dlg1 (12). In the current study, we found that E4-ORF1-induced PI3K/PKB activation additionally requires a functional domain 2 (Fig. 3). Given the requirement for E4-ORF1 trimerization for both Dlg1 binding and domain 2 activity (Fig. 6C), it is tempting to speculate that an E4-ORF1 trimer assembles both Dlg1 and pp70 into a single ternary complex, which traffics on cytoplasmic membrane vesicles to the plasma membrane and, at this site, promotes Ras-dependent PI3K activation.

As disregulated vesicle trafficking in cells can promote uncontrolled cellular proliferation, as well as the development of cancer, our discovery that E4-ORF1 associates with membrane vesicles warrants further consideration. For instance, loss of either the *Tsg101* gene encoding a component of the ESCRT-I

complex or the *RhoB* gene encoding a small GTPase involved in endosomal trafficking triggers cellular transformation (31) or increased susceptibility to DMBA-induced skin carcinogenesis (20), respectively. Furthermore, the exocyst complex is a direct effector for Ral GTPases, which are required for Ras-mediated tumorigenic transformation of human cells (3), and overexpression of the clathrin-associated oncoprotein Hip1 that regulates endocytosis is detected in multiple human epithelial tumors (44). In *Drosophila*, disruptive mutations in genes coding for the endocytic vesicle trafficking regulator Avl or Rab5 or the ESCRT-II component Vps25 provoke cellular overproliferation and neoplastic-like growths (32, 58). Also relevant is the fact that cellular transformation induced by the human papillomavirus type 16 E5 protein is linked to elevated epidermal growth factor (EGF)-induced signaling caused by decreased endosomal trafficking of activated EGF receptors to lysosomes and increased recycling of such receptors to the plasma membrane (9). Thus, an interesting possibility is that E4-ORF1 similarly disregulates vesicle trafficking in cells. Consistent with this idea, cellular PDZ proteins represent key modulators of both receptor trafficking (67) and clathrin-coated pit dynamics (43). Moreover, Dlg1 associates with membrane vesicles, as well as the Hrs protein that regulates endosomal trafficking, and the *Drosophila* Dlg1 homologue controls vesicle trafficking necessary for plasma membrane formation (6, 27). Likewise, MUPP1 accumulates in membrane vesicles of Caco-2 cells unable to express the CAR receptor (8) and MAGI-1 binds the endocytic receptor megalin in kidney cells (41).

Previous findings revealed that E4-ORF1 stimulates the PI3K/PKB pathway by a Ras-dependent mechanism that fails to similarly activate the Ras/MAPK/ERK pathway (12, 13). Interestingly, vesicle trafficking has been reported to differentially affect membrane signaling from these two important pathways. For example, inhibition of endocytosis enhances PKB, but not ERK, activation by the EGF receptor (10) or blocks ERK, but not PKB, activation by either insulin receptor or the RET tyrosine kinase receptor (5, 45). Additionally, overexpression of the transforming protein intersectin, which inhibits clathrin-mediated endocytosis, promotes Ras-dependent activation of PKB, but not ERK, in cells (59). Based on these observations, together with the results presented in this paper, we plan in future studies to investigate whether E4-ORF1 domain 2- and PBM-interacting cellular factors function to regulate vesicle trafficking and whether PI3K activation and cellular transformation induced by E4-ORF1 stem from its ability to perturb this fundamental key process in cells.

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