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A Carboxy-Terminal Region Required by the Adenovirus Type 9 E4 ORF1 Oncoprotein for Transformation Mediates Direct Binding to Cellular Polypeptides

ROBERT S. WEISS AND RONALD T. JAVIER*

Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 77030

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Human adenovirus type 9 (Ad9) is unique among oncogenic adenoviruses in that it elicits exclusively mammary tumors in rats and requires the viral E4 region open reading frame 1 (9ORF1) gene for tumorigenicity. The 9ORF1 oncogenic determinant codes for a 14-kDa transforming protein, and three separate regions of this polypeptide, including one at the extreme C terminus, are necessary for transforming activity. In this study, we investigated whether the 9ORF1 transforming protein interacts with cellular factors. Following incubation with cell extracts, a glutathione *S*-transferase (GST)–9ORF1 fusion protein associated with several cellular phosphoproteins (p220, p180, p160, p155), whereas GST fusion proteins of transformation-defective 9ORF1 C-terminal mutants did not. Similar interactions requiring the 9ORF1 C terminus were revealed with protein-blotting assays, in which a GST-9ORF1 protein probe reacted specifically with cellular polypeptides having gel mobilities resembling those of the 9ORF1-associated cellular phosphoproteins, as well as with additional cellular polypeptides designated p140/p130. In addition, GST fusion proteins containing 9ORF1 C-terminal fragments associated with some of the 9ORF1-associated cellular polypeptides, as did GST fusion proteins of full-length wild-type Ad5 and Ad12 E4 ORF1 transforming proteins. Significantly, the results of coimmunoprecipitation analyses suggested that the same cellular polypeptides also associate with wild-type but not C-terminal-mutant 9ORF1 proteins in vivo. Together, these findings suggest that the 9ORF1 C terminus, which is essential for transformation, participates in specific and direct binding of the 9ORF1 oncoprotein to multiple cellular polypeptides. We propose that interactions with these cellular factors may be responsible, at least in part, for the transforming activity of the 9ORF1 viral oncoprotein.

Human adenovirus type 9 (Ad9) is a member of the subgroup D adenoviruses, which cause primarily eye infections in people (16). Whereas most subgroup D adenoviruses are nononcogenic in rodents, infection of Wistar-Furth rats with Ad9 produces estrogen-dependent mammary tumors in females and no tumors of any type in males (2, 19, 21). A mixture of both benign and malignant mammary tumors arises in the Ad9-infected female animals, but the majority of neoplasms are fibroadenomas (19), the most common benign breast tumor of women (7). Interestingly, the oncogenicity of Ad9 differs considerably from those of the subgroup A and B adenoviruses, which generate primarily undifferentiated sarcomas at the sites of virus injection in both male and female animals (42). These observations suggest that new mechanisms of viral oncogenesis may be revealed by studying the Ad9 tumor model system.

For subgroup A and B adenoviruses, the viral E1 region is both necessary and sufficient for tumorigenesis (42). In contrast, yet consistent with its unique oncogenicity, Ad9 requires the viral early region 4 (E4 region) open reading frame 1 (9ORF1) gene to produce mammary tumors (20, 22). The 9ORF1 gene encodes a 125-amino-acid-residue polypeptide that localizes predominantly to the cytoplasm of cells (48) and, alone, exhibits cellular growth-transforming activity in several different rodent cell lines, including CREF (22, 48), as well as in human cell line TE85 (47). Moreover, in TE85 cells, the related E4 ORF1 genes from subgroup A Ad12 (12ORF1), subgroup B Ad3 (3ORF1), and subgroup C Ad5 (5ORF1) demonstrate transforming potentials similar to that of subgroup D 9ORF1 (47), despite displaying little or no transforming activity in CREF cells (22). The block to transformation for non-subgroup-D E4 ORF1s in CREF cells may be due to general protein expression deficiencies in rodent cell lines for these viral genes (47). Interestingly, although they lack detectable enzymatic activity, the adenovirus E4 ORF1 transforming proteins show sequence and predicted structural similarities to a variety of organismal and viral dUTPase enzymes (47). This finding may indicate that adenovirus E4 ORF1 and dUTPase polypeptides have a common structure or function.

Results with seven different transformation-defective mutant 9ORF1 genes indicate that three separate regions of the 125-residue 9ORF1 polypeptide (region I, residues 34 to 41; region II, residues 89 to 91; region III, residues 122 to 125) are essential for transforming activity (46). Although activities have yet to be ascribed to these protein regions, other DNA virus oncoproteins transform cells through physical interactions with the products of cellular proto-oncogenes or tumor suppressor genes (6, 15, 33). Consequently, we hypothesized that the 9ORF1 protein also complexes with cellular factors and that such interactions would be mediated by one or more of the 9ORF1 protein regions required for transformation. In this study, we report that 9ORF1 C-terminal region III is necessary for direct binding of the 9ORF1 polypeptide to multiple cellular proteins in vitro and in vivo and that this binding may be required for transforming function.

* Corresponding author. Mailing address: Division of Molecular Virology, Room 928D, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030. Phone: (713) 798-3898. Fax: (713) 798-3586. E-mail: rjavier@bcm.tmc.edu.

MATERIALS AND METHODS

Cells. The rat embryo fibroblast CREF (12) and human osteosarcoma-derived TE85 (23) cell lines, as well as G418-selected cell pools derived from these cell

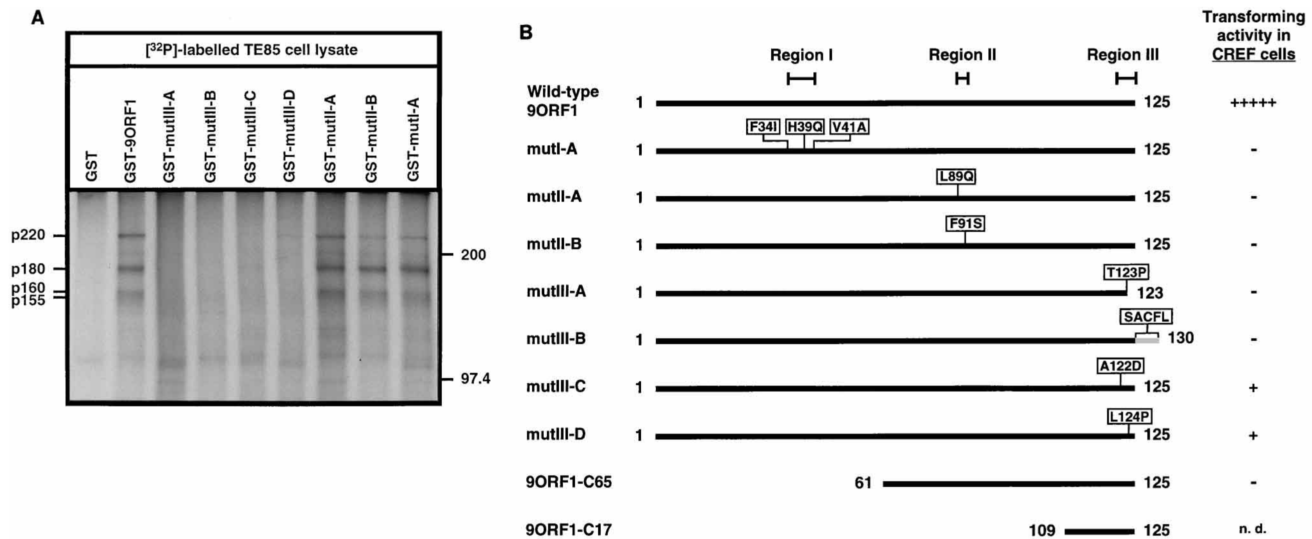

FIG. 1. (A) Cellular phosphoproteins complex with wild-type but not C-terminal region III mutant 9ORF1 proteins in vitro. GST pulldown reactions were performed with the indicated wild-type or mutant 9ORF1 GST fusion protein and 0.8 mg of protein from extracts of [^{32}P]orthophosphate-labelled TE85 cells. Prominent 9ORF1-associated cellular phosphoproteins are indicated on the left, and protein size standards are shown on the right. (B) Diagram of wild-type, mutant, and truncated 9ORF1 proteins and summaries of their transforming phenotypes in CREF cells (46). +++++, strong transforming activity; +, weak transforming activity; −, very weak or no transforming activity; n.d., not determined.

lines (46, 47), were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 20 μg of gentamicin per ml.

Plasmids and GST fusion protein preparation. Wild-type adenovirus E4 ORF1 genes from Ad12 (12ORF1), Ad5 (5ORF1), and Ad9 (9ORF1), as well as mutant 9ORF1 genes, were introduced in-frame with the glutathione *S*-transferase (GST) gene at the *Bam*HI and *Eco*RI sites of plasmids pGEX-2T and pGEX-2TK (46, 47). For construction of the pGEX-2T-9ORF1-C17 plasmid, the 9ORF1 *Kpn*I-*Eco*RI DNA fragment derived from pGEX-2T-9ORF1 was blunt-ended at the *Kpn*I site and inserted directionally into the *Sma*I and *Eco*RI sites of pGEX-2T. GST fusion proteins encoded by these plasmids were expressed in *Escherichia coli* XA90 and purified by standard methods (44). Briefly, bacteria were induced to overexpress the fusion proteins, lysed in buffer containing 1% Triton X-100, and centrifuged at 12,000 × *g* for 15 min at 4°C. The resulting supernatants (S1) contained most of the GST or approximately 10% of the GST-E4 ORF1 fusion proteins expressed in the bacteria. For recovery of additional GST-E4 ORF1 protein, pellets from 1% Triton X-100-lysed bacteria were dissolved in Sarkosyl solubilization buffer (1.5% [wt/vol] *N*-lauroylsarcosine, 25 mM ethanolamine, 1 mM EDTA [pH 8.0]) and centrifuged at 12,000 × *g* for 15 min at 4°C (13). To these supernatants (S2), Triton X-100 and $CaCl_2$ were added to final concentrations of 1% (vol/vol) and 1 mM, respectively. For affinity purification of GST fusion proteins, glutathione-Sepharose beads were incubated with either S1 or S2 supernatant and washed extensively with ice-cold radioimmunoprecipitation assay (RIPA) buffer. Similar results were obtained with S1 and S2 supernatant-derived fusion proteins.

Radiolabelling cells and preparing cell lysates. A total of 5 × 10^6 TE85 cells were radiolabelled by incubation with 0.5 mCi of [^{32}P]orthophosphate in phosphate-free Dulbecco's modified Eagle medium supplemented with 10% dialyzed fetal bovine serum for 4 h at 37°C. For preparation of cell lysates, radiolabelled or unlabelled cells were washed with phosphate-buffered saline (150 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4) and lysed in approximately 5 volumes of ice-cold RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS]) containing protease and phosphatase inhibitors (300 μg of phenylmethylsulfonyl fluoride per ml, 6 μg each of aprotinin and leupeptin per ml, 50 mM NaF, 0.1 mM sodium orthovanadate) (41). Before utilization in subsequent assays, cell lysates were cleared of debris by centrifugation at 10,000 × *g* for 10 min at 4°C. Protein concentrations of cleared cell extracts were determined by the Bradford method (43).

GST pulldown, immunoprecipitation, and protein-blotting assays. GST pulldown reactions (45) were performed by incubation of cell lysates with approximately 5 μg of each affinity-purified GST fusion protein bound to 30 μl of glutathione-Sepharose beads for 2 h at 4°C. Lysates from [^{32}P]orthophosphate-labelled cells, containing approximately 1 mg of protein, were precleared by incubation with 50 μg of GST protein bound to 30 μl of glutathione-Sepharose beads for 1 h at 4°C. Immunoprecipitation reactions were performed by incubating 9ORF1 rabbit polyclonal antiserum and protein A-Sepharose beads with cell lysates for 3 h at 4°C (22). The amount of immunoprecipitated 9ORF1 protein was estimated by immunoblot analysis with 9ORF1 antiserum as previously described (48). In both GST pulldown and immunoprecipitation reactions, the respective beads were washed extensively with ice-cold RIPA buffer and boiled in sample buffer (0.13 M Tris-HCl [pH 6.8], 4% [wt/vol] SDS, 20% [vol/vol] glycerol, 2% [vol/vol] β-mercaptoethanol, 0.003% bromophenol blue) and recovered proteins were separated by SDS–7.5% polyacrylamide gel electrophoresis (PAGE). Relevant portions of protein gels were stained with Coomassie brilliant blue dye to verify the use of equal amounts of each GST fusion protein. For detection of radiolabelled cellular proteins recovered in GST pulldown reactions, protein gels were dried and subjected to autoradiography. Unlabelled cellular proteins recovered from GST pulldown or immunoprecipitation reactions were detected in protein-blotting assays (24). In this procedure, polypeptides were electrotransferred from protein gels to polyvinylidene fluoride membranes, which were blocked in TBST (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.2% [vol/vol] Tween 20) containing 5% nonfat dried milk, incubated in blotting buffer (0.1% nonfat dried milk and 100 μg of unlabelled GST protein per ml in TBST) containing a ^{32}P-labelled protein probe (5 × 10^5 cpm/ml) for 12 h at 4°C, washed extensively with RIPA buffer, and developed by autoradiography.

GST fusion proteins expressed from the pGEX-2TK plasmid, which encodes a consensus protein kinase A phosphorylation site, were used to prepare radiolabelled GST fusion protein probes (24). Approximately 3 μg of GST fusion protein bound to 30 μl of glutathione-Sepharose beads was incubated in kinase reaction mixture (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 12 mM $MgCl_2$, 10 U of purified protein kinase A [Sigma Chemical Co.], 20 μCi of [γ-^{32}P]ATP [6,000 Ci/mmol]) for 30 min on ice, and the reaction was terminated with stop solution (10 mM sodium phosphate [pH 8.0], 10 mM sodium pyrophosphate, 10 mM EDTA, 0.1% [wt/vol] bovine serum albumin). Radiolabelled fusion proteins bound to beads were washed with RIPA buffer and recovered in elution buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% [vol/vol] Nonidet P-40, 40 mM glutathione). Similar specific activities were obtained for wild-type and mutant E4 ORF1 fusion protein probes.

RESULTS

In vitro association between the wild-type 9ORF1 protein and several cellular phosphoproteins requires functional 9ORF1 C-terminal region III sequences. To investigate whether the 9ORF1 protein is capable of interacting with cellular proteins in vitro, we performed GST pulldown reactions (45). For these assays, affinity-purified GST-9ORF1 protein was incubated with lysates from [^{32}P]orthophosphate-labelled human TE85 cells, and cellular factors capable of binding the fusion protein were separated by SDS-PAGE. The results indicated that multiple cellular phosphoproteins complexed with the GST-9ORF1 protein but not with the GST protein alone

(Fig. 1A). The most prominent 9ORF1-associated phosphoprotein bands were designated p220, p180, p160, and p155. It should be noted, however, that each of the indicated bands may represent several distinct protein species and also that additional less prominent bands are evident. That the ^{32}P-labelled cellular factors were phosphoproteins was verified by observing their sensitivity to proteinase K and resistance to RNAse A and DNAse I, as well as by detecting similarly migrating polypeptides in GST-9ORF1 protein pulldown reactions performed with lysates from [^{35}S]methionine- and [^{35}S]cysteine-labelled cells (data not shown).

We recently reported the characterization of seven transformation-defective 9ORF1 mutants, for which mutations map to three separate protein regions (region I, region II, region III) (Fig. 1B) (46). Considering the results described above, it was of interest to determine whether any of these 9ORF1 mutants would fail to bind the cellular phosphoproteins. In these analyses, the 9ORF1 region I and region II mutants (GST-mutI-A, GST-mutII-A, GST-mutII-B) showed wild-type binding phenotypes, whereas the four 9ORF1 C-terminal region III mutants (GST-mutIII-A, GST-mutIII-B, GST-mutIII-C, GST-mutIII-D) were significantly impaired in binding the cellular phosphoproteins (Fig. 1A). GST-mutIII-D, however, did show some reduced capacity to bind p220. Additionally, equivalent GST pulldown experiments using lysates from [^{32}P]orthophosphate-labelled CREF cells yielded results similar to those described above for TE85 cells (data not shown). These findings indicated that 9ORF1 C-terminal region III, but not region I or region II, is required for specific interactions between the 9ORF1 protein and several cellular phosphoproteins in vitro. The fact that transformation-defective 9ORF1 region I and region II mutants were fully competent for these interactions may suggest that, similar to other viral oncoproteins, the 9ORF1 protein is multifunctional and that more than one activity of this viral polypeptide is necessary for transformation.

Direct binding of the wild-type 9ORF1 protein to cellular polypeptides in vitro. As an alternate method for detecting interactions between 9ORF1 and cellular proteins in vitro, protein-blotting assays were also performed (24). For these assays, unlabelled TE85 proteins, recovered from GST pulldown reactions, were separated by SDS-PAGE, transferred to a membrane, and blotted with a radiolabelled GST-9ORF1 protein probe. The results of these experiments showed that GST pulldown reactions performed with GST-9ORF1 but not with GST protein recovered multiple polypeptides which, in a protein-blotting assay, reacted with the GST-9ORF1 probe (Fig. 2A). That none of these polypeptides was detected either in GST-9ORF1 protein pulldown reactions performed without cell lysate or by blotting with a GST-mutIII-A protein probe indicated that these factors were derived from TE85 cells and that reactions with the GST-9ORF1 protein probe were specific (Fig. 2A). Significantly, the gel mobilities for some of the observed cellular polypeptides corresponded well with those of cellular phosphoproteins p220, p180, p160, and p155 (compare Fig. 1A and 2A). In addition, another group of 9ORF1-associated cellular proteins, designated p140/p130, was also uncovered in these protein-blotting assays (Fig. 2A). The failure to detect the p140/p130 proteins in GST-9ORF1 protein pulldown reactions with lysates from [^{32}P]orthophosphate-labelled cells (Fig. 1A) may be due to lower levels of phosphorylation for these polypeptides relative to other 9ORF1-associated proteins. Aside from one additional prominent band, designated p145, CREF cell lysates yielded a similar set of 9ORF1-associated proteins, which reacted with the GST-9ORF1 but not the GST-mutIII-A probe in protein-blotting assays (Fig. 2B). Nonetheless, minor differences in gel mobility were in some cases evident for the corresponding TE85 and CREF cell-derived 9ORF1-associated polypeptides. Based on results with several human and rodent cell lines, these differences appear to be species specific (data not shown). In summary, the results of these protein-blotting assays confirmed that the 9ORF1 protein interacts with multiple cellular proteins in vitro and further suggested that the observed interactions are direct.

Besides possessing gel mobilities similar to those of the 9ORF1-associated cellular phosphoproteins, the TE85 and CREF polypeptides detected in protein-blotting assays also showed similar binding reactivities toward 9ORF1 mutant proteins. By performing GST pulldown reactions and protein blotting the recovered proteins with a GST-9ORF1 probe, we found that the cellular polypeptides bound 9ORF1 region I and region II mutant proteins (GST-mutI-A, GST-mutII-A, GST-mutII-B) equivalently to the wild-type 9ORF1 protein (GST-9ORF1) (data not shown) but did not interact detectably with two of the 9ORF1 region III mutant proteins (GST-mutIII-A, GST-mutIII-B) (Fig. 2A and B). Although the remaining two 9ORF1 region III mutant proteins (GST-mutIII-C, GST-mutIII-D) also failed to complex with some of the cellular proteins, full or reduced binding to a subset of the cellular factors was detected for these mutants (Fig. 2A and B). The fact that these leaky binding phenotypes for GST-mutIII-C and GST-mutIII-D were not fully revealed in GST pulldown reactions performed with lysates from [^{32}P]orthophosphate-labelled cells (Fig. 1A) may indicate that protein blotting is more sensitive for detecting not only p140/p130 but also the other 9ORF1-associated cellular proteins. More important, these results established the 9ORF1 C-terminal region III was required for the 9ORF1 protein to associate directly with these cellular factors. Taken together, these findings suggested that the cellular polypeptides revealed in protein-blotting assays were probably the same as the cellular phosphoproteins detected in GST pulldown reactions.

9ORF1 C-terminal protein fragments containing region III sequences retain the ability to bind some 9ORF1-associated cellular proteins. Considering that 9ORF1 C-terminal region III mutants exhibited defects in associating with cellular proteins in vitro, it seemed possible that C-terminal sequences alone may mediate the observed protein-protein interactions. To test this idea, we utilized the C-terminal 17 (GST-9ORF1-C17) or 65 (GST-9ORF1-C65) amino acid residues of the 9ORF1 protein (Fig. 1B) in GST pulldown reactions and protein blotted the recovered proteins with a GST-9ORF1 probe. We found that the GST-9ORF1-C17 protein bound p155 and p180 but not other 9ORF1-associated cellular proteins (Fig. 3). While similar results were obtained with the GST-9ORF1-C65 protein, longer exposures of the autoradiogram shown in Fig. 3 indicated that this larger C-terminal fragment additionally displayed reduced binding to p220 and p140/130. Therefore, 9ORF1 C-terminal sequences alone were sufficient to mediate interactions with some of the cellular factors.

Interaction of related adenovirus E4 ORF1 transforming proteins with some 9ORF1-associated cellular proteins in vitro. Besides the 9ORF1 protein, other adenovirus E4 ORF1 proteins also demonstrate growth-transforming activity in cells (47), suggesting that these related viral proteins may bind the same or similar cellular factors. This possibility was investigated by performing GST pulldown reactions with Ad5 and Ad12 E4 ORF1 fusion proteins (GST-5ORF1 and GST-12ORF1, respectively) and examining the recovered cellular proteins in protein-blotting assays with a GST-9ORF1 probe. In these experiments, we found that both GST-5ORF1 and GST-12ORF1 proteins complexed with cellular proteins that

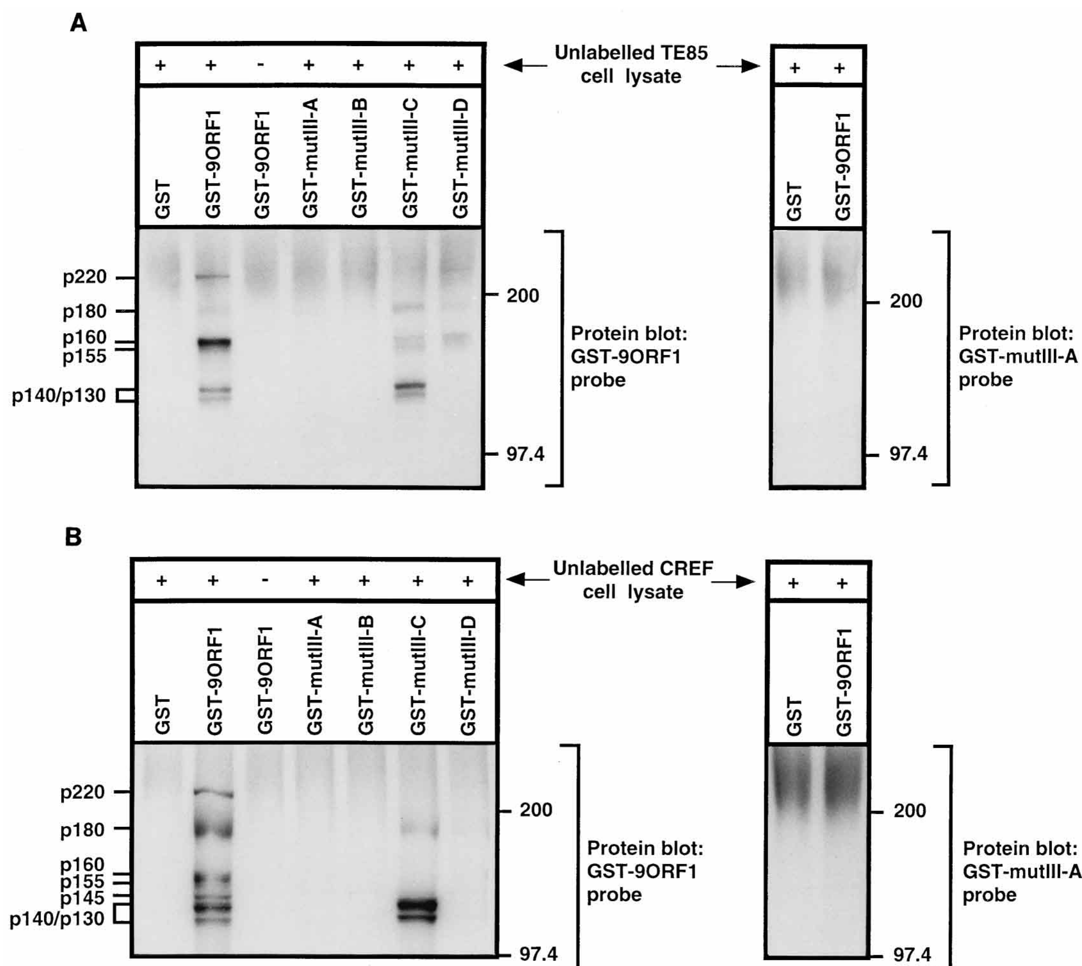

FIG. 2. Requirement for 9ORF1 C-terminal region III for the direct binding of the 9ORF1 protein to membrane-immobilized 9ORF1-associated cellular proteins. GST pulldown reactions were performed with the indicated wild-type or mutant 9ORF1 GST fusion protein and either 0.8 mg of protein from extracts of unlabelled TE85 cells (A) or 3.9 mg of protein from extracts of unlabelled CREF cells (B). Recovered proteins were protein blotted with either a ^{32}P-labelled GST-9ORF1 probe or a ^{32}P-labelled GST-mutIII-A probe. The GST-9ORF1 protein pulldown reactions performed without cell extract (−) were included as controls. Prominent 9ORF1-associated cellular proteins are indicated.

reacted with the GST-9ORF1 probe and that comigrated with p220, p155, and p140/p130 (Fig. 4). Nevertheless, binding of the GST-12ORF1 protein to p180 or of GST-5ORF1 and GST-12ORF1 proteins to p160 was weak or not detected. Similar conclusions could be drawn from the results of equivalent experiments using either GST-5ORF1 or GST-12ORF1 protein as a probe (data not shown). Additionally, in contrast to what was observed for the interaction between p160 and the 9ORF1 protein, cellular factors that uniquely associated with either the 5ORF1 or 12ORF1 protein were not observed in these experiments.

The specific depletion of 9ORF1-associated cellular proteins from cell extracts by incubation with either GST-5ORF1 or GST-12ORF1 protein would provide further evidence that related adenovirus E4 ORF1 proteins bind the same cellular factors. This idea was tested by subjecting a cell extract to several sequential GST-5ORF1 protein pulldown reactions to remove 5ORF1-associated cellular proteins. The removal of these cellular factors was monitored by blotting the proteins recovered from each GST-5ORF1 protein pulldown reaction with a GST-5ORF1 (data not shown) or a GST-9ORF1 probe (Fig. 5A). We found that the amount of recoverable 5ORF1-associated cellular protein in the extract was significantly reduced after three sequential GST-5ORF1 protein pulldown reactions. This depleted cell extract was then subjected to a GST-9ORF1 protein pulldown reaction, and recovered cellular proteins were also protein blotted with a GST-9ORF1 probe. The results showed that removal of 5ORF1-associated cellular proteins from the extract produced a concomitant loss of all 9ORF1-associated cellular proteins, except the 9ORF1-specific factor(s) p160 (Fig. 5A). This depletion of 9ORF1-associated cellular proteins by the GST-5ORF1 protein was specific, because 9ORF1-associated cellular proteins were not similarly eliminated from a cell extract after three sequential GST protein pulldown reactions were performed (Fig. 5B). These findings suggested that several different adenovirus E4 ORF1 transforming proteins share the ability to complex with the cellular factors p220, p155, and p140/p130.

In vivo association between the wild-type 9ORF1 protein and cellular polypeptides also requires functional 9ORF1 C-terminal region III sequences. It was next important to determine whether the 9ORF1 protein also associates in vivo with the observed cellular proteins. This possibility was tested by performing coimmunoprecipitation analyses, in which polypeptides recovered by immunoprecipitating 9ORF1 protein from 9ORF1-expressing TE85 cells (47) were protein blot-

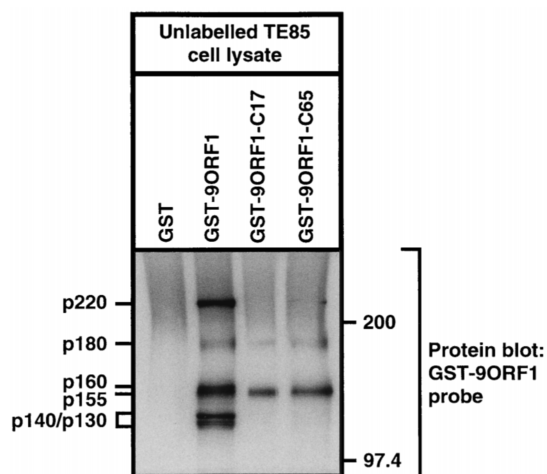

FIG. 3. 9ORF1 C-terminal protein fragments alone bind some 9ORF1-associated cellular proteins. TE85 cell proteins were recovered in the indicated GST pulldown reactions, and proteins were blotted with a GST-9ORF1 probe as described in the legend for Fig. 2A.

ted with a GST-9ORF1 probe. From such an experiment, we detected multiple cellular proteins that coprecipitated with the 9ORF1 protein and that reacted with a GST-9ORF1 probe in protein-blotting assays (Fig. 6). This result was specific, because these cellular factors were not detected in equivalent experiments performed either with 9ORF1 antiserum and extracts from control TE85 cells which do not express 9ORF1 protein (data not shown) or with preimmune serum and extracts from 9ORF1-expressing TE85 cells (Fig. 6). Significantly, the gel mobilities of the coprecipitated cellular proteins matched those of the cellular polypeptides recovered in GST-9ORF1 protein pulldown reactions (Fig. 6). In addition, the coprecipitated cellular proteins did not react with a GST-mutIII-A probe in protein-blotting assays (data not shown). These results suggested that the same cellular proteins detected in vitro also complexed with the wild-type 9ORF1 protein in vivo.

The results of coimmunoprecipitation analyses with TE85 cells were confirmed and extended in similar analyses using CREF cells expressing either wild-type or mutant 9ORF1 proteins (46). Consistent with results for TE85 cells, protein blotting of immunoprecipitates with a GST-9ORF1 probe showed that 9ORF1 antiserum, but not preimmune serum, coprecipitated most 9ORF1-associated proteins from wild-type 9ORF1-expressing CREF cells but not from control (vector) CREF cells which do not express 9ORF1 protein (Fig. 7). The reason that p160 and p155 coprecipitated inefficiently with 9ORF1 protein expressed in CREF cells is not clear. More important, compared to the wild-type 9ORF1 protein, transformation-defective 9ORF1 C-terminal region III mutant proteins showed significant defects in binding the cellular proteins in vivo. Specifically, none of the 9ORF1-associated cellular proteins coprecipitated with either mutIII-A or mutIII-B protein, and only p140/130 coprecipitated detectably with the mutIII-C protein (Fig. 7). In other experiments, however, some p220 was observed to coprecipitate with the mutIII-D protein (data not shown). These findings were concordant with in vitro results for these 9ORF1 mutant proteins (Fig. 1B and 2A and B), although most weak protein interactions of mutIII-C and mutIII-D with the 9ORF1-associated cellular proteins (Fig. 2A and B) were not detected in these coimmunoprecipitation analyses. Aside from mutIII-B, which is poorly expressed in CREF cells (46), comparable amounts of wild-type and each mutant 9ORF1 protein were immunoprecipitated in these experiments (Fig. 7). Therefore, C-terminal region III was also required for the 9ORF1 protein to associate with the cellular polypeptides in vivo.

DISCUSSION

The transforming potentials of DNA tumor virus oncoproteins most often derive from an ability to heterocomplex with cellular factors (33). This fundamental principle led us to predict that the 9ORF1 oncoprotein would also interact with cellular proteins. In support of this hypothesis, we demonstrated that the 9ORF1 protein binds to multiple cellular polypeptides both in vitro and in vivo (Fig. 1, 2, 6, and 7). These cellular factors may be important for transformation by the 9ORF1 protein because four different transformation-defective 9ORF1 mutants (mutIII-A, mutIII-B, mutIII-C, mutIII-D) exhibited greatly reduced capacities to associate with these polypeptides. Moreover, mutIII-A and mutIII-B failed to interact detectably with any of the cellular proteins, whereas mutIII-C and mutIII-D showed some reduced protein binding activity (Fig. 2A and B). The latter findings further strengthen the correlation between 9ORF1 transforming potential and binding to the cellular proteins because mutIII-A and mutIII-B lack transforming activity while mutIII-C and mutIII-D retain weak transforming activity in cells (Fig. 1B) (46). Therefore, the 9ORF1-associated polypeptides reported here represent strong candidates for cellular mediators of 9ORF1 oncogenic potential.

Proteins related in sequence and function might be expected to have common activities. In this regard, the E4 ORF1 transforming proteins from Ad5 (5ORF1) and Ad12 (12ORF1) were found to associate with a subset of the 9ORF1-associated cellular proteins (Fig. 4 and 5). As subgroup D Ad9 is unique among adenoviruses in generating mammary neoplasms in rats, the inability of 5ORF1 and 12ORF1 to bind certain 9ORF1-associated cellular proteins, together with the protein expression deficiencies observed for non-subgroup-D E4 ORF1 proteins in several different rodent cell lines (47), may contribute to the failure of the corresponding viruses to induce

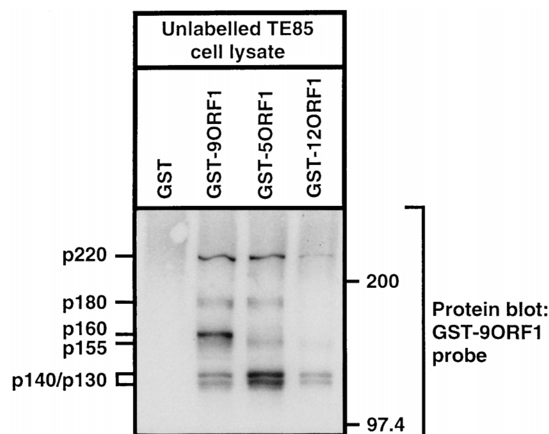

FIG. 4. Several different adenovirus E4 ORF1 transforming proteins from subgroup A Ad12 (12ORF1), subgroup C Ad5 (5ORF1), and subgroup D Ad9 (9ORF1) bind the same or similar cellular proteins. GST pulldown reactions were performed with the indicated wild-type adenovirus E4 ORF1 GST fusion protein and 0.8 mg of protein from extracts of unlabelled TE85 cells. Recovered proteins were detected by protein blotting with a radiolabelled GST-9ORF1 probe.

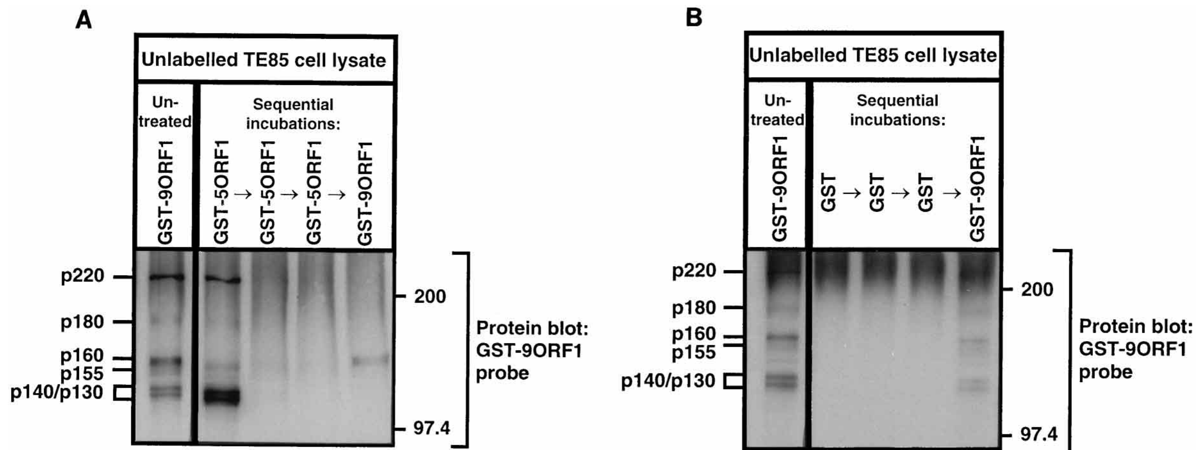

FIG. 5. Specific depletion of 9ORF1-associated cellular proteins from a cell extract by sequential incubations with GST-5ORF1 protein. An unlabelled TE85 cell extract containing 1 mg of total cell protein was subjected to three sequential GST pulldown reactions with either GST-5ORF1 protein (A) or GST protein (B). Each treated extract was then subjected to a GST-9ORF1 protein pulldown reaction. GST-9ORF1 protein pulldown reactions performed with the same amount of untreated TE85 cell extract were included as controls. Recovered cellular proteins were detected by protein blotting with a radiolabelled GST-9ORF1 probe.

rat mammary tumors. With respect to the replication of adenoviruses in permissive cells, the role of the E4 ORF1 protein is unknown, as mutant viruses unable to express this gene product show replication kinetics and yields similar to those of wild-type viruses in cultured human cell lines (3, 14, 17, 22). Nevertheless, the early gene products of DNA viruses generally act to establish conditions optimal for viral replication, often by targeting cellular proteins which control cell cycle progression (27, 33). Aside from the E4 ORF1 protein, the E4 transcription unit codes for five additional polypeptides (4, 8, 9, 26, 39, 40) and, interestingly, cellular targets for three of these early gene products, E4 ORF4, E4 ORF6, and E4 ORF6/7, have been identified as protein phosphatase 2A, p53, and E2F, respectively (10, 18, 26, 29, 32). Because adenovirus gene products with related functions frequently cluster within a single viral transcription unit (42), the cellular factors found to complex with the adenovirus E4 ORF1 proteins are similarly expected to represent important regulators of cellular proliferation. Thus, it follows that perturbation of the activity of such cellular factors, resulting from interactions with the 9ORF1 protein, may lead to neoplastic transformation of cells.

We previously identified three separate 9ORF1 protein regions (region I, region II, region III) required for transformation (Fig. 1B) (46). In this study, we demonstrated that 9ORF1 mutant proteins having alterations within C-terminal region III (mutIII-A, mutIII-B, mutIII-C, mutIII-D) were impaired for binding to cellular proteins. Moreover, 9ORF1 C-terminal fragments alone, which included region III sequences, retained the capacity to bind some of the cellular factors (Fig. 3). These findings suggest that 9ORF1 region III constitutes a principal component of a functional domain which mediates protein-protein interactions. The fact that 9ORF1 C-terminal fragments did not display wild-type binding to all 9ORF1-associated cellular proteins further indicates that other regions of the 9ORF1 protein also contribute to the binding activity of this essential domain. These other 9ORF1 regions needed to preserve full protein binding activity remain to be precisely localized.

A common feature of most viral oncoproteins is possession of several different functional domains (11, 30, 37). In this regard, transformation-defective 9ORF1 mutants having alterations in either region I or region II (mutI-A, mutII-A, mutII-B) displayed wild-type binding to 9ORF1-associated cellular proteins (Fig. 1A). Thus, the ability of 9ORF1 C-terminal region III to mediate interactions with cellular proteins may be necessary but not sufficient for transformation. This conclusion may further imply that 9ORF1 region I and region II perform additional unknown functions which, like that of region III,

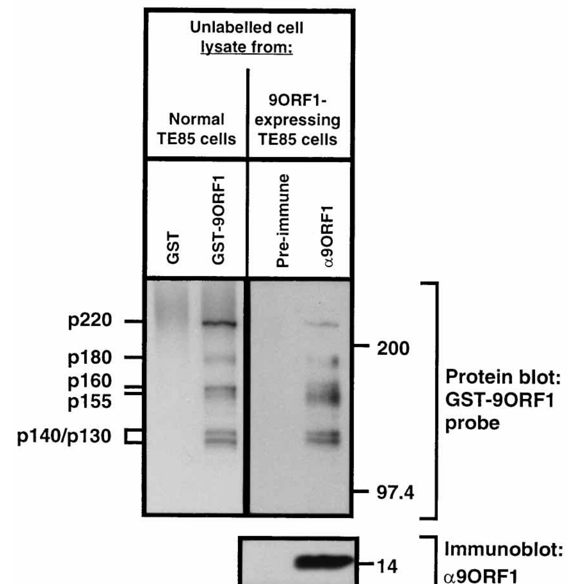

FIG. 6. 9ORF1-associated cellular proteins coimmunoprecipitate with the wild-type 9ORF1 protein expressed in TE85 cells. Immunoprecipitations were performed with either 9ORF1 antiserum (α9ORF1) or the matched preimmune serum, using 6.0 mg of protein from extracts of 9ORF1-expressing TE85 cells (47). The GST pulldown reactions were performed with the indicated GST fusion protein and 0.5 mg of protein from extracts of TE85 cells. (Upper panel) Proteins recovered from immunoprecipitations or GST pulldown reactions were detected by protein blotting with a radiolabelled GST-9ORF1 probe. The membrane with proteins recovered from GST pulldown reactions was exposed for 65 h without an intensifying screen, whereas the membrane with proteins recovered from immunoprecipitations was exposed for 74 h with an intensifying screen. (Lower panel) Equal amounts of each immunoprecipitate were subjected to immunoblot analysis with 9ORF1 antiserum.

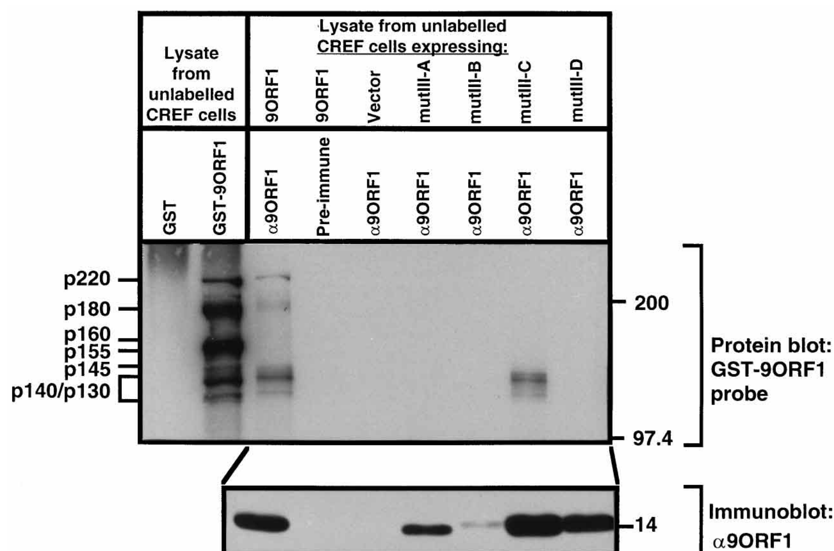

FIG. 7. 9ORF1-associated cellular proteins coimmunoprecipitate with wild-type but not with C-terminal region III mutant 9ORF1 proteins expressed in CREF cells. Immunoprecipitations were performed with either 9ORF1 antiserum (α9ORF1) or the matched preimmune serum, using 5.0 mg of protein from extracts of control (vector) CREF cells or wild-type or mutant 9ORF1-expressing CREF cells (46). GST pulldown reactions were performed with the indicated GST fusion protein and 4.5 mg of protein from extracts of CREF cells. (Upper panel) Proteins recovered from immunoprecipitations or GST pulldown reactions were detected by protein blotting with a radiolabelled GST-9ORF1 probe. (Lower panel) Equal amounts of each immunoprecipitate were subjected to immunoblot analysis with 9ORF1 antiserum.

are essential for transformation. Interestingly, 9ORF1 and dUTPase proteins exhibit sequence and predicted structural similarity, and comparisons with the crystal structure of *E. coli* dUTPase (5, 28) suggest that 9ORF1 region I and region II lie in close proximity to each other and distant from C-terminal region III in the native 9ORF1 molecule (47). This observation hints at the possibility that 9ORF1 region I and region II sequences participate in forming a single functional domain. Therefore, even though we failed to detect binding of human and avian adenovirus dUTPases to 9ORF1-associated cellular proteins (unpublished results), the observation that region II sequences are conserved within many eukaryotic dUTPase proteins (47) may indicate that 9ORF1 and dUTPase polypeptides have a common activity.

While the 9ORF1 protein was found to bind multiple cellular polypeptides, it was not possible to distinguish whether these 9ORF1-associated proteins are related or unrelated. Nevertheless, considering that a single 9ORF1 functional domain, defined by C-terminal region III, mediated binding to all of the detected cellular factors, we hypothesize that the 9ORF1-associated polypeptides will be found to be related proteins or proteins that have a common protein domain. Such a scenario may be analogous to the ability of nuclear viral oncoproteins adenovirus E1A, simian virus 40 large T antigen, and human papillomavirus E7 to interact with several different members (pRb, p107, p130) of the retinoblastoma tumor suppressor protein family. In these examples, the viral oncoproteins have a common LXCXE motif that binds directly to the related pocket domains of each pRb family member (33). With respect to the 9ORF1 protein, however, its location outside of the cell nucleus may suggest that 9ORF1-associated proteins are involved in signal transduction, similar to the cellular targets of polyomavirus middle T antigen (25), bovine papillomavirus E5 (36), and Epstein-Barr virus latent membrane protein 1 (31). In this regard, it may be pertinent that most 9ORF1-associated cellular proteins are phosphorylated (Fig. 1A). More important, a well-known feature of cellular proteins that function in signal transduction is that they contain various protein modules that mediate protein-protein interactions. Examples of such modules include SRC homology 2 (SH2), SH3, armadillo, WWP/WW, pleckstrin homology, PDZ, and phosphotyrosine-binding domains (1, 34, 35, 38). Therefore, one intriguing possibility is that 9ORF1 region III sequences bind selectively to one of these common protein domains found in cellular signaling molecules.

The observation that multiple cellular polypeptides associated with the 9ORF1 protein also makes it unclear whether association with one or more of these cellular factors may be required for transformation. Because the 9ORF1 mutant proteins mutIII-C and mutIII-D bound to an overlapping yet distinct subset of 9ORF1-associated cellular proteins (Fig. 2A and B), but because neither possesses wild-type transforming activity (Fig. 1B), we currently favor the idea that the full transforming activity of the 9ORF1 protein is achieved through an ability to complex with several cellular polypeptides. Construction and analysis of additional 9ORF1 C-terminal region III mutants should aid in resolving which interactions are important for transformation. Furthermore, considering that cellular factors which associate with viral oncoproteins often prove to play crucial roles in regulating normal cell proliferation and in oncogenesis (33), the eventual identification of such 9ORF1-associated proteins, in addition to revealing molecular mechanisms for 9ORF1-induced transformation, may also contribute to our understanding of cellular growth control and the development of human cancers.

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ADDENDUM IN PROOF

After submission of this manuscript for publication, we determined that 9ORF1 C-terminal region III contains a consensus PDZ domain-binding motif and identified 9ORF1-associated p140/p130 to be the PDZ domain-containing cellular factor DLG (S. S. Lee. R. S. Weiss, and R. T. Javier, Proc. Natl. Acad. Sci. USA **94:**6670–6675, 1997).

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