

# Productive Interaction between Transmembrane Mutants of the Bovine Papillomavirus E5 Protein and the Platelet-Derived Growth Factor $\beta$ Receptor

Char-Chang Lai,<sup>†</sup> Anne P. B. Edwards, and Daniel DiMaio\*

*Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06510*

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**The bovine papillomavirus E5 protein is a 44-amino-acid transmembrane protein that transforms cells by binding to the transmembrane region of the cellular platelet-derived growth factor (PDGF)  $\beta$  receptor, resulting in sustained receptor signaling. However, there are published reports that certain mutants with amino acid substitutions in the membrane-spanning segment of the E5 protein transform cells without activating the PDGF  $\beta$  receptor. We re-examined several of these transmembrane mutants, and here we present five lines of evidence that these mutants do in fact activate the PDGF  $\beta$  receptor, resulting in cellular signaling and transformation.**

The bovine papillomavirus E5 protein is a dimeric 44-amino-acid type II transmembrane protein that transforms cells by binding and activating the platelet-derived growth factor (PDGF)  $\beta$  receptor, a transmembrane tyrosine kinase (2, 16). Formation of a complex between the E5 protein and the PDGF  $\beta$  receptor results in ligand-independent receptor dimerization and *trans*-phosphorylation and leads to the assembly of a large signaling complex containing cellular signal transduction molecules (2, 3, 6, 10, 11, 14–16, 23). The transmembrane and juxtamembrane region of the PDGF  $\beta$  receptor contains all the specific contacts needed for complex formation with the E5 protein (12, 17, 20). The E5 protein also binds to a number of other cellular proteins, including a transmembrane subunit of the vacuolar ATPase, and these interactions have been proposed to mediate transformation in some settings or to account for certain aspects of the transformed phenotype (1, 5, 19, 21, 22).

The wild-type E5 protein is able to induce cellular signaling, cell proliferation, and transformation by interacting with the PDGF  $\beta$  receptor but not with other growth factor receptors, including the related PDGF  $\alpha$  receptor (2, 3, 6, 14, 18, 20). Furthermore, we observed a nearly perfect correlation between the ability of numerous E5 mutants to transform C127 cells, form stable complexes with the PDGF  $\beta$  receptor, and induce tyrosine phosphorylation of the receptor (7, 8, 13). Small transformation competent transmembrane proteins in which the central hydrophobic portion of the E5 protein was replaced with random hydrophobic amino acids also activated the PDGF  $\beta$  receptor and initiated PDGF receptor-dependent signaling (4). Taken together, these experiments provide compelling evidence that the PDGF  $\beta$  receptor is the major, if not the sole, target of the E5 protein in mediating cell transforma-

tion. In contrast, Schlegel and colleagues reported that certain E5 transmembrane substitution mutants transformed fibroblasts without binding or activating the PDGF  $\beta$  receptor, and on the basis of these results presented models for E5 structure and function (1, 19, 21, 22). One such mutant (E5-Q17S) contained serine at position 17 in place of the wild-type glutamine (19); however, previous studies in our laboratory showed that E5-Q17S was able to bind and activate the PDGF  $\beta$  receptor in C127 and Ba/F3 cells, resulting in PDGF  $\beta$  receptor-dependent cell proliferation and transformation (8). Furthermore, although E5-Q17S did not induce tyrosine phosphorylation of the immature, precursor form of the PDGF  $\beta$  receptor in NIH 3T3 cells (19), re-examination of the original published data clearly shows elevated tyrosine phosphorylation of the mature form of the PDGF  $\beta$  receptor in cells infected with this mutant (for an example, see Fig. 7 in reference 19). We re-examined several transformation-competent E5 mutants reported to be unable to undergo productive interaction with the PDGF  $\beta$  receptor, and here we show that these mutants do in fact bind and activate the receptor, resulting in mitogenic signaling and cell transformation.

**Analysis of the original NIH 3T3 cell lines transformed with epitope-tagged E5 missense mutants.** Most of our previous studies were carried out with the authentic E5 protein in C127 cells, whereas the studies from the Schlegel laboratory employed epitope-tagged E5 proteins and were carried out largely in NIH 3T3 cells (see, e.g., references 1 and 8). In addition, there were numerous technical differences in the details of sample preparation and analysis. To re-examine the original cell lines transformed by these E5 mutants, we obtained parental NIH 3T3 cells and NIH 3T3 cells transformed by the wild-type E5 protein (wt-E5) and a representative set of E5 mutants, E5-Q17S, E5-L24A (leucine 24 to alanine), and E5-L26A (leucine 26 to alanine), from R. Schlegel (Georgetown University). Each of these E5 proteins contained an AU1 epitope tag at its amino terminus. E5-Q17S and E5-L24A were reported to transform cells without binding to or activating the PDGF  $\beta$  receptor, and E5-L26A was reported to bind to the

\* Corresponding author: Mailing address: Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510. Phone: (203) 785-2684. Fax: (203) 785-6765. E-mail: daniel.dimaio@yale.edu.

<sup>†</sup> Present address: Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115.

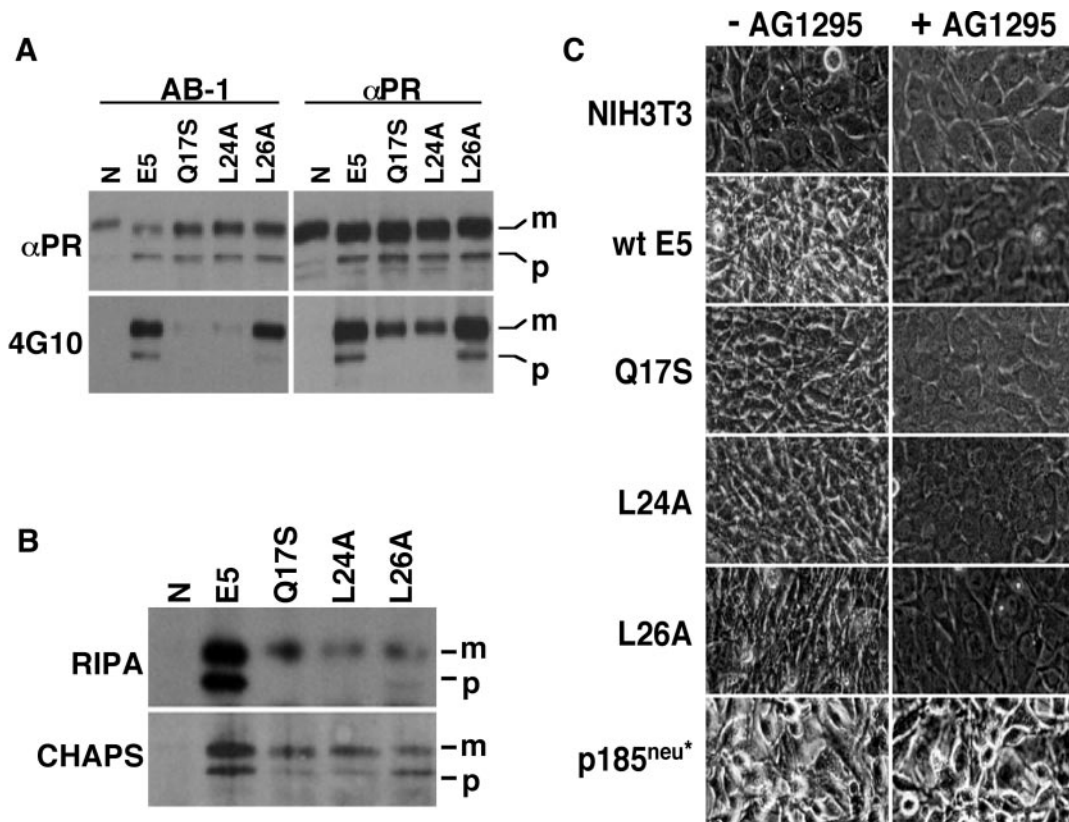


FIG. 1. Activity of E5 mutants in NIH 3T3 cells. (A) PDGF  $\beta$ -receptor tyrosine phosphorylation. After serum starvation, RIPA-MOPS extracts were prepared from untransformed NIH 3T3 cells (N) or NIH 3T3 cells expressing the indicated epitope-tagged E5 proteins. Equal amounts of extracted protein were subjected to immunoprecipitation with  $\alpha$ -PR or with commercial anti-PDGF  $\beta$ -receptor antibody AB-1, as indicated at the top, and then immunoblotted with  $\alpha$ -PR or 4G10, a monoclonal antiphosphotyrosine antibody. m and p indicate the mature and precursor forms of the PDGF  $\beta$  receptor, respectively. The top two panels are shown at the same exposure, as are the bottom two panels. (B) Complex formation between E5 proteins and the PDGF  $\beta$  receptor. Extracts of untransformed NIH 3T3 cells (N) and cells transformed with the indicated E5 proteins were prepared in RIPA-MOPS buffer or in CHAPS buffer. The extracts were then subjected to immunoprecipitation with the anti-E5 antiserum, gel electrophoresis, and immunoblotting, using  $\alpha$ -PR to detect PDGF  $\beta$  receptor in complex with the E5 protein. (C) Effect of PDGF receptor inhibition on transformed morphology. Photomicrographs of parental NIH 3T3 and NIH 3T3 cells stably expressing the wild-type E5 protein, the indicated E5 mutant, or the activated p185<sup>neu\*</sup> oncogene are shown in the absence of AG1295 (left) or after 4 days in the presence of 50  $\mu$ M AG1295 (right).

receptor without causing significant tyrosine phosphorylation (1, 19).

To determine whether the PDGF  $\beta$  receptor in transformed cells contained elevated tyrosine phosphorylation compared to that in parental cells, cells were starved for 24 h in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum, and cell extracts were prepared in modified RIPA-MOPS buffer (20 mM morpholinepropanesulfonic acid [pH 7.0], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10  $\mu$ g of leupeptin and aprotinin/ml). The extracts were divided into two equal portions that were immunoprecipitated with saturating amounts of two different PDGF receptor antibodies,  $\alpha$ -PR, a rabbit antiserum generated in our laboratory that recognizes an 11-amino-acid C-terminal PDGF  $\beta$  receptor peptide, or a commercial antibody (AB-1) used by Supryniewicz et al. (22). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the filters were immunoblotted with  $\alpha$ -PR or monoclonal antibody 4G10, which recognizes phosphotyrosine.

As shown in the top panels of Fig. 1A, all cells expressed similar levels of PDGF  $\beta$  receptor. However, substantially more PDGF  $\beta$  receptor was detected following immunoprecipitation with  $\alpha$ -PR than with AB-1. Moreover, following immunoprecipitation with  $\alpha$ -PR, both the mature and the precursor forms of the PDGF  $\beta$  receptor were highly tyrosine phosphorylated in cells transformed by wt-E5 or E5-L26A, in comparison to that in untransformed cells. E5-Q17S and E5-L24A also induced elevated tyrosine phosphorylation of the mature form of the PDGF  $\beta$  receptor, albeit to a lesser extent than wt-E5, but did not induce tyrosine phosphorylation of the precursor form. Similar results were obtained with multiple independent preparations of extracts. When AB-1 was used for immunoprecipitation, little receptor phosphotyrosine was detectable in cells transformed by E5-Q17S and E5-L24A, similar to the previous reports from the Schlegel laboratory (1, 19), although we detected abundant receptor phosphorylation in response to E5-L26A. However, prolonged exposure of the filters containing AB-1 immunoprecipitates revealed that E5-Q17S and E5-L24A induced elevated tyrosine phosphorylation

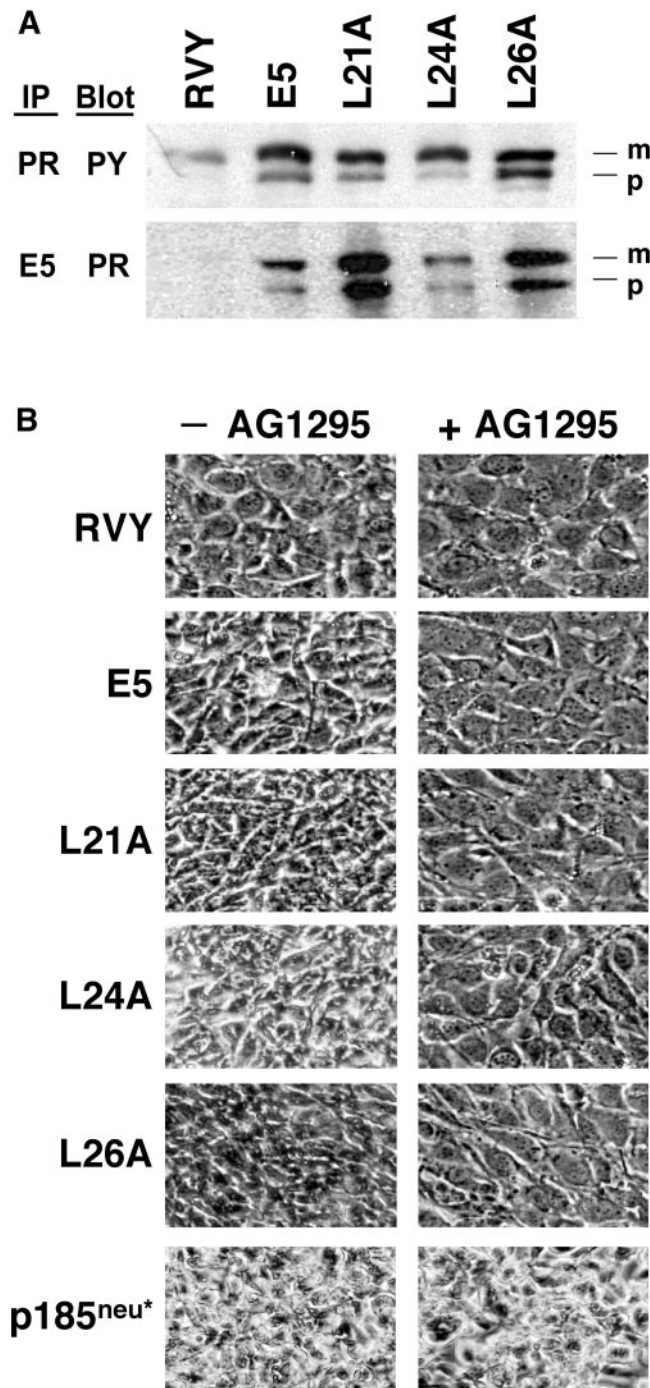


FIG. 2. Activity of E5 mutants in C127 cells. (A) PDGF  $\beta$ -receptor tyrosine phosphorylation and binding. After serum starvation, RIPA-MOPS extracts were prepared from control untransformed C127 cells (RVY) and C127 cells transformed by the wild-type E5 gene (E5) or the indicated transmembrane mutants. PDGF  $\beta$ -receptor tyrosine phosphorylation (top panel) and complex formation (bottom panel) were determined following immunoprecipitation with  $\alpha$ PR and  $\alpha$ E5, respectively. (B) Reversion of transformed morphology by PDGF receptor inhibitor. Photomicrographs of control and transformed C127 cells treated with AG1295 as described for Fig. 1C.

of the mature form of the PDGF  $\beta$  receptor compared to that in untransformed cells (data not shown). Similar results were obtained when three different antiphosphotyrosine antibodies were used for immunoblotting (4G10, PY99 [Transduction Laboratories; catalogue no. P38820], and PY54 [Santa Cruz; catalogue no. SC-70205]) (data not shown). These results show that these E5 missense mutants induce constitutive tyrosine phosphorylation of the PDGF  $\beta$  receptor in transformed NIH 3T3 cells. Evidently, the differences between our results and those reported by the Schlegel laboratory are due to differences in the preparation and analysis of extracts.

We used coimmunoprecipitation to assess the ability of the wild-type and mutant E5 proteins to form stable complexes with the PDGF  $\beta$  receptor in these transformed cells (8, 15). Extracts were prepared in either modified RIPA-MOPS or CHAPS {15 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate} buffer (CHAPS, 30 mM NaCl, 1 mM EDTA, and 50 mM Tris-HCl [pH 7.4]). These extracts were immunoprecipitated with an antiserum that recognized a carboxy-terminal peptide from the E5 protein and then subjected to immunoblotting with  $\alpha$ -PR to detect PDGF  $\beta$  receptor in complex with the E5 protein (Fig. 1B). As expected, the E5 antibody did not coimmunoprecipitate the PDGF  $\beta$  receptor from extracts of untransformed cells, but abundant mature and precursor PDGF  $\beta$  receptor was coimmunoprecipitated from both types of extracts from cells transformed by the wild-type E5 protein. The PDGF  $\beta$  receptor was also coimmunoprecipitated from extracts of cells transformed by all three E5 mutants, although less PDGF  $\beta$  receptor was immunoprecipitated than from cells transformed by the wild-type E5 protein. These results indicate that transmembrane E5 missense mutants form a stable complex with the PDGF  $\beta$  receptor in transformed NIH 3T3 cells.

To test whether PDGF  $\beta$  receptor signaling was required for transformation by these mutants, we used AG1295 (Calbiochem), a specific inhibitor of the PDGF receptor tyrosine kinase (9). We analyzed control NIH 3T3 cells and NIH 3T3 cells transformed by various epitope-tagged E5 proteins or by p185<sup>neu\*</sup>, an activated tyrosine kinase receptor that is unrelated to the PDGF  $\beta$  receptor. Western blotting showed that AG1295 caused a marked reduction in tyrosine phosphorylation of the PDGF  $\beta$  receptor in the cells transformed by wild-type or mutant E5 proteins but did not affect tyrosine phosphorylation of p185<sup>neu\*</sup> (data not shown). As shown in Fig. 1C, AG1295 caused reversion of the transformed morphology of cells expressing the wild-type or mutant E5 proteins but had no effect on the morphology of cells transformed by p185<sup>neu\*</sup>. Similarly, in the absence of AG1295, the wild-type and mutant E5 proteins caused marked acidification of the culture medium, a standard phenotypic marker of transformation, and AG1295 prevented acidification by cells transformed by the E5 proteins but not by cells transformed by p185<sup>neu\*</sup> (data not shown). These experiments indicate that sustained signaling by the PDGF  $\beta$  receptor is required to maintain the transformed state of NIH 3T3 cells expressing the wild-type or mutant E5 proteins.

**Analysis of transmembrane mutants in C127 cells.** To test the activity of the transmembrane mutants containing alanine substitutions in C127 cells, we reconstructed them without an epitope tag in the hygromycin-resistant retroviral vector RVY

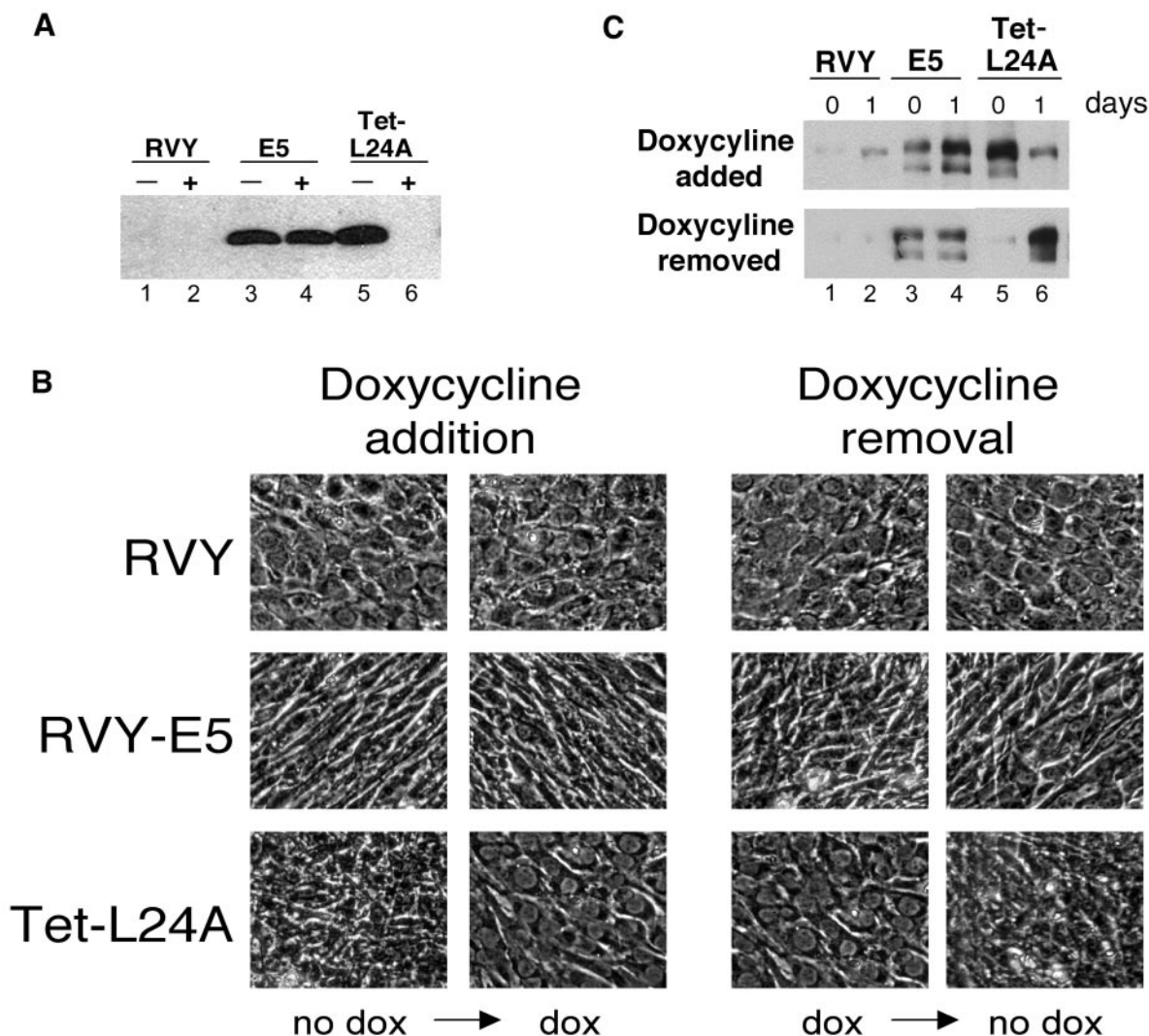


FIG. 3. Analysis of a regulated E5-L24A mutant. (A) Regulated expression of E5-L24A. Cells harboring the empty vector (RVY), a constitutively-expressed wild-type E5 protein (E5), or doxycycline-regulated E5-L24A (Tet-L24A) were grown in the presence (+) or absence (-) of doxycycline, representing basal or inducing conditions, respectively. The E5 protein was detected by immunoprecipitation and immunoblotting as previously described (8). (B) Cellular transformation in response to E5-L24A induction and repression. Control untransformed (RVY) and transformed (RVY-E5) C127 cells and cells expressing a doxycycline-regulated E5-L24A gene (Tet-L24A) were grown in the absence (inducing conditions, left pairs of panels) or presence (basal conditions, right pairs of panels) of doxycycline. Doxycycline was then added or removed as indicated, and the cells were incubated for an additional 3 days. (C) PDGF  $\beta$ -receptor tyrosine phosphorylation in response to E5-L24A induction and repression. The cell lines described in panel A were grown in the absence (top panel) or presence (bottom panel) of doxycycline for 7 days. Incubation was continued for 1 day in the same medium (0), or doxycycline was then added (top panel) or removed (bottom panel) for 1 day (1), as indicated, and PDGF  $\beta$ -receptor tyrosine phosphorylation was determined following immunoprecipitation with  $\alpha$ PR as described in the legend to Fig. 1A.

(18). We included in this set E5-L21A, which transforms cells but was reported to be defective in PDGF  $\beta$ -receptor tyrosine phosphorylation and binding (1). All three mutants efficiently induced focus formation in C127 cells. We analyzed transformed cell lines established from pooled hygromycin-resistant colonies. All of the mutants induced elevated tyrosine phosphorylation of the mature PDGF  $\beta$  receptor in comparison to that of untransformed cells and all formed a stable complex with the receptor (Fig. 2A). Furthermore, the PDGF receptor kinase inhibitor, AG1295, caused reversion of the transformed morphology and prevented acidification of the culture medium of C127 cells expressing the wild-type or mutant E5 proteins

but had no effect on cells transformed by p185<sup>neu\*</sup> (Fig. 2B and data not shown). These results indicate that the E5 transmembrane mutants interact productively with the PDGF  $\beta$  receptor in C127 cells as well as in NIH 3T3 cells.

To test whether PDGF  $\beta$ -receptor activation was a direct effect of the expression of a mutant E5 protein in transformed C127 cells, we cloned the E5-L24A mutant into RVY-tet, a self-contained doxycycline-repressible retrovirus vector (unpublished results). C127 cells were transduced with the RVY-tet-E5-L24A retrovirus in the absence of doxycycline (inducing conditions), and transformed foci were picked and expanded into cell lines for further analysis. Figure 3A shows

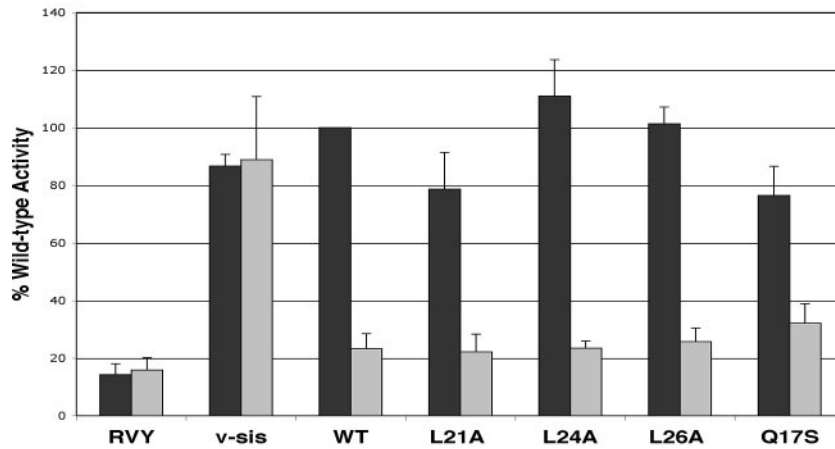


FIG. 4. Transient SIE reporter gene assay for PDGF  $\beta$ -receptor-dependent signaling in CV1 cells. The graph shows the relative firefly luciferase activity induced from the SIE element by v-sis, the wild-type E5 protein (wt), the indicated E5 mutants, or the empty vector (RVY) coexpressed with the wild-type PDGF  $\beta$  receptor (dark grey) or a receptor mutant with a heterologous transmembrane domain (light grey). The graphs show the normalized average (plus or minus the standard deviation) of the results of three independent transfection experiments, each performed in triplicate, expressed as a percentage of luciferase activity induced by coexpression of the wild-type E5 protein and the wild-type PDGF  $\beta$  receptor.

that E5-L24A was abundantly expressed in cells incubated in media lacking doxycycline (lane 5) but was undetectable under noninducing conditions (lane 6). Cells grown continuously in the absence of doxycycline appeared morphologically transformed, but addition of 0.4  $\mu$ M doxycycline caused morphological reversion of cells harboring the regulated E5-L24A mutant (Fig. 3B, left two sets of panels). Importantly, repression of E5-L24A expression by addition of doxycycline also caused a substantial decrease in tyrosine phosphorylation of the PDGF  $\beta$  receptor within 1 day (Fig. 3C, compare lane 6 to lane 5 in top panel). Conversely, removal of doxycycline from RVY-tet-E5-L24A cells previously grown in its presence led to rapid acquisition of PDGF  $\beta$ -receptor tyrosine phosphorylation (Fig. 3C, lane 6, bottom panel) and morphological transformation (Fig. 3B, right two sets of panels). In contrast, doxycycline treatment had no significant effect on the morphology of untransformed cells or cells constitutively expressing the wild-type E5 protein or on their levels of PDGF  $\beta$ -receptor tyrosine phosphorylation. Thus, tyrosine phosphorylation of the PDGF  $\beta$  receptor and morphological transformation are rapid and reversible responses to E5-L24A expression, suggesting that this E5 mutant directly activates the receptor.

**Transient signaling by E5 transmembrane mutants in CV1 cells.** We also used a transient reporter gene assay to assess the ability of the E5 transmembrane mutants to induce receptor signaling (4). CV1 monkey cells, which do not express endogenous PDGF  $\beta$  receptor, were cotransfected with plasmids encoding the wild-type or mutant E5 protein and the PDGF  $\beta$  receptor together with a reporter plasmid containing three tandem copies of either a serum inducible element (SIE) or a STAT-responsive gamma interferon activated site (GAS) element, both of which respond to PDGF  $\beta$ -receptor signaling by driving expression of a linked firefly luciferase gene. We used either the wild-type PDGF  $\beta$  receptor gene or a gene encoding a chimeric PDGF  $\beta$  receptor containing a transmembrane domain derived from p185<sup>neu</sup>, which does not interact with the wild-type E5 protein (17). The cells were also cotransfected

with a plasmid encoding renilla luciferase to normalize for transfection efficiency. As shown in Fig. 4 for the SIE reporter plasmid, the mutant E5 proteins, like the wild-type E5 protein, induced signaling by the wild-type PDGF  $\beta$  receptor but not by the chimeric receptor. As expected, both receptors were activated by the PDGF homologue, v-sis. Similar results were obtained with the GAS reporter plasmid (data not shown). Therefore, acute signaling by these mutants requires expression of the PDGF  $\beta$  receptor and a specific interaction with the transmembrane domain of the receptor.

**Discussion.** We present five lines of evidence that the mutant E5 proteins studied here underwent a productive interaction with the PDGF  $\beta$  receptor. (i) The mutants formed a stable complex with the PDGF  $\beta$  receptor and (ii) induced PDGF  $\beta$ -receptor tyrosine autophosphorylation. (iii) Maintenance of the transformed state was inhibited by treatment with a specific PDGF receptor kinase inhibitor, (iv) PDGF  $\beta$ -receptor tyrosine phosphorylation and morphological transformation were acutely induced by expression of a mutant E5 protein and were rapidly reversed by its repression, and (v) acute activation of two different transcriptional response elements by these mutants required coexpression of the wild-type PDGF  $\beta$  receptor. We conclude that the PDGF  $\beta$  receptor is a crucial target mediating transformation by the mutant E5 proteins tested, as it is for the wild-type E5 protein. This conclusion is consistent with our earlier finding that E5-Q17S transformed C127 and Ba/F3 cells in a PDGF  $\beta$ -receptor-dependent fashion (8).

The original assessment that these mutants were defective for PDGF  $\beta$ -receptor activation was based primarily on the examination of tyrosine phosphorylation of the precursor form of the PDGF  $\beta$  receptor (1, 19). However, as we have shown here and previously, some E5 mutants caused tyrosine phosphorylation of the mature form only (7, 8). In addition, substantially more PDGF  $\beta$  receptor was immunoprecipitated by our antipeptide antibody than by the commercial antibody used by Suprynowicz et al. (22). We conclude that the inability

of Schlegel and colleagues to detect receptor tyrosine phosphorylation in response to these E5 mutants was largely a consequence of their exclusive focus on the precursor form of the receptor and of inefficient immunoprecipitation of the PDGF  $\beta$  receptor from cell extracts or other technical differences. Although the transmembrane mutants studied here clearly interact productively with the PDGF  $\beta$  receptor, this interaction does not appear equivalent to the interaction involving the wild-type E5 protein. For example, in NIH 3T3 cells, E5-Q17S and E5-L24A proteins were less effective at inducing tyrosine phosphorylation of the PDGF  $\beta$  receptor than was the wild-type E5 protein, and several mutants displayed reduced complex formation with the receptor.

In summary, the E5 mutants analyzed here activated a PDGF  $\beta$ -receptor-dependent mechanism to transform C127 and NIH 3T3 cells and to initiate signaling in CV1 cells. Although it remains possible that certain activities of the E5 protein are not mediated by the PDGF  $\beta$  receptor, our results demonstrate that the mutants analyzed here interact productively with the endogenous PDGF  $\beta$  receptor in mouse fibroblasts and with an exogenous PDGF  $\beta$  receptor in monkey epithelial cells and that this interaction is required to initiate mitogenic signaling and induce and maintain morphological transformation. These findings should be incorporated into models for E5 structure and function.

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