E5 Oncoprotein Mutants Activate Phosphoinositide 3-Kinase Independently of Platelet-derived Growth Factor Receptor Activation

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The E5 oncoprotein of bovine papillomavirus type 1 is a Golgi-resident, 44-amino acid polypeptide that can transform fibroblast cell lines by activating endogenous platelet-derived growth factor receptor β (PDGF-R). However, the recent discovery of E5 mutants that exhibit strong transforming activity but minimal PDGF-R tyrosine phosphorylation indicates that E5 can potentially use additional signal transduction pathway(s) to transform cells. We now show that two classes of E5 mutants, despite poorly activating the PDGF-R, induce tyrosine phosphorylation and activation of phosphoinositide 3-kinase (PI 3-K) and that this activation is resistant to a selective inhibitor of PDGF-R kinase activity, tyrphostin AG1296. Consistent with this independence from PDGF-R signaling, the E5 mutants fail to induce significant cell proliferation in the absence of PDGF, unlike wild-type E5 or the sis oncoprotein. Despite differences in growth factor requirements, however, both wild-type E5 and mutant E5 cell lines form colonies in agarose. Interestingly, activation of PI 3-K occurs without concomitant activation of the ras-dependent mitogen-activated protein kinase pathway. The known ability of constitutively activated PI 3-K to induce anchorage-independent cell proliferation suggests a mechanism by which the mutant E5 proteins transform cells.

Phosphoinositide 3-kinases (PI 3-Ks) are a family of enzymes that phosphorylate inositol phospholipids specifically at the D-3 position of the inositol ring. The phosphorylated lipid products of PI 3-Ks serve as second messengers that are involved in the regulation of cell growth and differentiation, apoptosis, cytoskeletal organization, cell motility, membrane trafficking, and glucose metabolism (18–20). Heterodimeric (p85/p110) PI 3-K plays a key role in growth factor-induced mitogenic signaling and is important for cellular transformation by v-src, v-tos, and the middle-T antigen of polyoma virus (19, 21–24). The expression of constitutively active heterodimeric PI 3-K is sufficient to induce characteristics of cellular transformation, including anchorage-independent growth (25).

Although it has been reported that wt BPV-1 E5 elevates basal PI 3-K activity in immunoprecipitates from NIH 3T3 cells, PI 3-K activation was presumed to result solely from the constitutive activation of receptor tyrosine kinases (26). In the present study, we investigated the possibility that PDGF-R-independent activation of PI 3-K constitutes an alternative pathway by which several new BPV-1 E5 mutants transform cells. We show that these new E5 mutants induce tyrosine phosphorylation and activation of PI 3-K without significantly activating the PDGF-R or the ras-dependent mitogen-activated protein kinase (MAPK) signal transduction pathway. Thus, it appears that the E5 oncoprotein utilizes an additional signaling pathway for activating PI 3-K and mediating cell transformation that is independent of PDGF-R activity.

MATERIALS AND METHODS

Generation and Maintenance of Cell Lines—NIH 3T3 cells and cell lines were maintained at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies, Inc.). To analyze signal transduction pathways in the absence of growth factors, 50% confluent cultures were washed twice with Dulbecco’s phosphate-buffered saline (D-PBS) and were in-
cultivated in DMEM containing 0.1% FBS for 20–24 h before experiments.

Stable NIH 3T3 cell lines expressing wt BPV-1 E5 and the E5 point mutants Q17G, Q17S, L24A, and L26A were generated as described previously (14, 27) by geneticin G418 selection of cells co-transfected with E5 DNA and the neomycin resistance-conferring plasmid, LNCX, at a ratio of 9:1 (E5 DNA:LNCX) using Ca,(PO4)2-DNA coprecipitation (28). All E5 constructs were tagged at their N terminus with the 6-amino acid AU1 epitope, which is recognized by the AU1 monoclonal antibody (29). The presence of this epitope does not affect the biological activity of E5 (27). Stable NIH 3T3 cell lines expressing the sis oncogene were similarly transfected and selected using a v-sis construct from J. Pierce (National Institutes of Health).

Proliferation Assays—To assess the proliferation of attached cells in defined media, 2 × 105 cells were plated in 60-mm tissue culture dishes in 4 ml of DMEM containing 2.5% FBS. After 24 h, 1 dish of each cell line was harvested using trypsin/EDTA treatment and counted (Coulter Electronics) to determine the initial cell density. The remaining cultures were washed with D-PBS, and the medium was replaced with 4 ml of DMEM containing 0.5% FBS (basal medium). To assay PDGF-dependent growth, basal medium was supplemented with recombinant human PDGF BB (Life Technologies, Inc.) at a final concentration of 20 ng/ml. Insulin-dependent growth was assayed in basal medium supplemented with bovine insulin (Life Technologies, Inc.) at a final concentration of 5 μg/ml. After 3 days in defined media, the cells were harvested and counted to determine the final cell density. Results are expressed as the percent increase in the number of cells, which is 100 × (final density − initial density)/initial density. To analyze the contribution of PDGF-R signaling to proliferation, the selective PDGF-R kinase inhibitor, tyrphostin AG1296 (30), was added to cultures along with MeSO (final 0.5%) as the cells were shifted into basal medium ≥ growth factors. A 5 μm stock solution of AG1296 (Calbiochem or Alexis Biochemicals) was prepared in MeSO and stored as aliquots at −70 °C.

To assay anchorage-independent growth, 1 ml of 0.3% agarose containing 1.7 × 106 cells was layered over 1 ml of 0.6% agarose in 35-mm dishes. A sterile 3% agarose stock solution was prepared in D-PBS and diluted to the above concentrations by mixing with DMEM (minus phenol red containing 10% FBS and antibiotics (Life Technologies, Inc.). Cultures initially were overlaid with 0.5 ml of medium and were given further additions as necessary to prevent desiccation for a period of 3–4 weeks.

Immunoprecipitation and Electrophoresis—E5 expression was analyzed by immunoprecipitating epitope-tagged E5 protein from 100-mm tissue culture dishes of 80–90% confluent [35S]methionine-labeled cells using the AU1 monoclonal antibody (Berkeley Antibody Co.) as described previously (9).

For anti-PDGF-R and anti-phosphotyrosine immunoprecipitations, 150–200-mm tissue culture dishes of 80–90% confluent cells were plated on ice and washed with 25 ml of D-PBS containing 1 mM Na2VO4. Cells were scraped into 0.9 ml of SDS lysis buffer (0.4% SDS, 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 10 mM HEPES-NaOH, pH 7.0) and 100 μl of lysis buffer (buffer A containing 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin) at 4 °C. After ultrasonic disruption and clarification, tyrosine-phosphorylated polypeptides were detected by means of chemiluminescence.

PI 3-Kinase Assay—PI 3-K activity was measured essentially as described by Salfitt et al. (31). Briefly, cells from 150-mm tissue culture dishes at 80–90% confluence were washed twice with 25 ml of buffer A (137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 20 mM HEPES-NaOH, pH 7.5) and scraped into 1 ml of lysis buffer (buffer A containing 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin) at 4 °C. After ultrasonic disruption and clarification, tyrosine-phosphorylated proteins were immunoprecipitated from aliquots of lysates containing 0.5 mg of protein as described above. Immunoprecipitates were washed 3 times with lysis buffer and 3 times with kinase assay buffer (100 mM NaCl, 1 mM EDTA, 0.2 mM Na3VO4, 10 mM HEPES-NaOH, pH 7.0). PI 3-K activity of the immunoprecipitates was assayed by adding 5 μl of kinase buffer (10 μg/ml MgCl2, 250 mM NaCl, 2 mM ATP, 100 μM Na3VO4, 2 mM MgCl2, 20 μM β-glycerophosphate) at 4 °C.

RESULTS

Transforming E5 Mutants Are Defective for PDGF-R Activation and Induction of Growth Factor Independence

To study how BPV-1 E5 oncoprotein mutants transform fibroblast cell lines without activating the PDGF-R, we generated NIH 3T3 cell lines that stably express wt BPV-1 E5 and the E5 point mutants Q17S, L24A, L26A, and Q17G. Q17S E5, in which serine is substituted for glutamine at position 17, transforms NIH 3T3 cells with 39% the efficiency of wt E5 in a focus formation assay (27) but does not induce PDGF-R tyrosine phosphorylation, indicative of receptor activation (17). The L24A and L26A mutants, in which leucine residues at positions
Epitope-tagged E5 protein was immunoprecipitated from exponentially growing cells labeled with $^{35}$S-methionine. Equivalent amounts of cell protein were immunoprecipitated for each lane using the AU1 monoclonal antibody and were resolved on 14% SDS gels. Molecular mass markers (in kDa) are indicated on the left.

24 and 26 of E5 are replaced with alanine, transform NIH 3T3 cells with 305% and 225% the efficiency of wt E5 but do not induce tyrosine phosphorylation of the PDGF-R (14). As a negative control, we used Q17G E5, in which glycine is substituted for glutamine at position 17. This mutant does not transform NIH 3T3 cells (0% focus formation) (27) and does not activate the PDGF-R (17). As shown in Fig. 1, wt and mutant E5 proteins were expressed at similar levels in the cell lines. The expression of L24A E5 was somewhat higher, and E5 was not detected in normal NIH 3T3 cells. In some experiments, we employed an NIH 3T3 cell line that stably expresses the sis oncogene as a control. Constitutive expression of the PDGF B-like sis gene product in these cells causes transformation due to persistent activation of endogenous PDGF-Rs (32, 33). E5 was not detected in the sis-expressing cells (Fig. 1).

**PDGF-R Tyrosine Phosphorylation**—The ability of E5 mutants to activate the PDGF-R was evaluated in the cell lines used in this study by analyzing levels of tyrosine-phosphorylated PDGF-R in the absence of exogenous growth factors. Initially, tyrosine-phosphorylated proteins were immunoprecipitated from serum-starved cells and then were immunoblotted to detect tyrosine-phosphorylated PDGF-Rs. For this analysis, cells were lysed in the presence of SDS and immediately boiled to avoid potential post-lysis phosphorylation/dephosphorylation or proteolysis. Boiling in SDS also served to disrupt protein-protein interactions, so that the anti-PDGF-R immunoblots reflected tyrosine-phosphorylated PDGF-Rs rather than unphosphorylated PDGF-Rs associated with tyrosine-phosphorylated protein(s). In addition, cells were incubated with Na$_3$VO$_4$ for 10 min before lysis to inhibit tyrosine phosphatases and optimize the detection of tyrosine-phosphorylated PDGF-R on the immunoblots. As shown in Fig. 2A, serum-starved, control 3T3 cells exhibited basal levels of tyrosine-phosphorylated mature PDGF-R (mPDGF-R), whereas cells stimulated with 10% FBS showed enhanced tyrosine phosphorylation. In contrast, all other cells lines expressing either transformation-competent E5 constructs (wt, L26A, Q17S, or L24A) or the transformation-defective Q17G E5 mutant showed levels of mPDGF-R tyrosine phosphorylation that were comparable with control 3T3 cells. As anticipated, although wt E5 did not increase tyrosine phosphorylation of the mPDGF-R, it did induce significant tyrosine phosphorylation of the immature PDGF-R (iPDGF-R) (Fig. 2A), consistent with its primary localization in the Golgi apparatus (8). No other E5 mutant induced significant phosphorylation of the iPDGF-R, although L24A E5 did promote a very low level of phosphorylation (Fig. 2A).

These results were further evaluated by performing the reverse experiment under the same experimental conditions, i.e. immunoprecipitation with anti-PDGF-R antibodies and labeling the immunoblot with anti-phosphotyrosine antibodies. As shown in Fig. 2B, a high level of mPDGF-R tyrosine phosphorylation was observed in normal 3T3 cells after the addition of 10% FBS, and an equally elevated level of iPDGF-R tyrosine phosphorylation was seen in the wt E5 cell line. Unstimulated 3T3 cells and mutant E5 cell lines exhibited very low levels of PDGF-R tyrosine phosphorylation. Increased PDGF-R tyrosine phosphorylation in the wt E5 cell line was not due to enhanced expression of the PDGF-R; in fact, these cells contained somewhat lower levels of the receptor (Fig. 2C). Therefore, as judged by tyrosine phosphorylation, activation of the mPDGF-R was induced only by serum, which is compatible with exogenous PDGF binding to cell-surface PDGF-Rs. Only wt E5 was able to induce significant phosphorylation of the iPDGF-R. In agreement with recent studies (14, 17), a number of transforming E5 mutants (L26A, Q17S, and L24A) induced only minimal phosphorylation of either the mPDGF-R or iPDGF-R.

**Proliferation Assays**—To employ criteria other than phosphorylation to demonstrate that the L26A, Q17S, and L24A transforming E5 mutants do not significantly activate the PDGF-R, cell lines expressing wt and mutant E5 proteins were tested for their ability to proliferate in 20-fold reduced levels of growth factors. As shown in Fig. 3A, normal NIH 3T3 cells increased in number by only 60% over a period of 3 days in basal medium (DMEM + 0.5% FBS), and cells that expressed the nontransforming Q17G E5 mutant increased by only 160%. When basal medium was supplemented with recombinant PDGF, the number of 3T3 cells and Q17G E5-expressing cells increased by 340% and 440%, respectively. Moreover, the stimulation of cell proliferation by PDGF was sensitive to tyrphostin AG1296, a selective inhibitor of PDGF-R tyrosine kinase activity (30). 20 μM tyrphostin AG1296 reduced the proliferation of 3T3 cells and Q17G E5-expressing cells to 120% and 140%, respectively, which were similar to levels of proliferation.
and sis-expressing cells to levels characteristic of control cells in basal medium (i.e. 3T3 and Q17G E5 cells) confirmed that wt E5 and sis enable proliferation in basal medium due to their activation of the PDGF-R.

The proliferation in basal medium of cell lines expressing the transforming L26A, Q17S, and L24A E5 mutants was strikingly similar to that of control cells that do not activate the PDGF-R (Fig. 3A). Cells expressing L26A E5 increased in number by 40%, which was less than the proliferation of normal 3T3 cells. Q17S E5- and L24A E5-expressing cells increased by 140%, which was less than the proliferation of cells that expressed the nontransforming Q17G E5 mutant. The slowed proliferation of these cells in basal medium, however, was not the consequence of a general defect in cell growth, since the L26A E5 and L24A E5 cell lines multiplied at the same rate as normal 3T3 cells, Q17G E5 expressers, and sis-expressing cells in medium containing 10% FBS (Fig. 3B). Cells expressing Q17S E5 multiplied at even a slightly higher rate (Fig. 3B). The results of these proliferation assays are therefore in agreement with the analysis of PDGF-R tyrosine phosphorylation and indicate that among the E5 constructs employed in this study only wt E5 significantly activates the PDGF-R.

The identity of mitogenic signal(s) responsible for the partially enhanced proliferation of Q17G E5-expressing cells relative to normal NIH 3T3 cells in basal medium (additional 100% increase in cell number) is not known. Regardless, this signaling does not induce PDGF-R tyrosine phosphorylation (Fig. 2, A and B) and does not lead to transformation, since the Q17G E5 mutant is nontransforming in focus formation assays.

Transferring E5 Mutants Confer Anchorage Independence

Although the L26A, Q17S, and L24A E5 mutants have previously been shown to induce foci in NIH 3T3 cells (14, 27), they do not significantly activate the PDGF-R or support growth factor-independent proliferation. To determine whether these mutants can induce additional characteristics of the transformed phenotype, we evaluated their ability to promote anchorage-independent proliferation.

The proliferation in basal medium of cell lines expressing the nontransforming Q17G E5 mutant generated relatively few small colonies, whereas cells expressing wt E5 formed many colonies, both small and large in size. The L26A and L24A E5 cell lines also formed many small and large colonies. The transforming Q17S E5 mutant triggered an increase predominantly in the number of small colonies compared with the Q17G E5 control (Fig. 4). In summary, transferring E5 mutants that are defective for PDGF-R activation are unable to induce growth factor-independent proliferation but can induce anchorage-independent proliferation.

E5 Does Not Constitutively Activate the Erk/MAPK Signal Transduction Pathway

Numerous growth and differentiation signals initiate a protein phosphorylation cascade in which activated Ras triggers the sequential activation of Raf, MAPK kinase (MEK), and the 44 kDa and 42 kDa MAPKs, Erk1, and Erk2 (34–36). Since constitutively active forms of Ras, Raf, and MEK are oncogenic (34, 35, 37–39), we asked whether transferring E5 mutants might directly or indirectly activate component(s) of the Erk/MAPK signal transduction pathway, leading to the constitutive activation of Erk1 and Erk2.

The activation of Erk1 and Erk2 involves their phosphorylation (by MEK) on proximal N-terminal threonine and tyrosine residues (40). Levels of active Erk1/Erk2 can be quantified on immunoblots using antibodies raised against a doubly phosphorylated peptide corresponding to the Erk phosphorylation
site (41). As shown in Fig. 5A, active Erk1/Erk2 were not detectable on immunoblots of serum-starved NIH 3T3 cells but were readily detected 10 min after 10% FBS was added to the cells (compare the first and second lanes). There was no evidence of Erk activation in serum-starved cells expressing wt E5 (third lane), demonstrating that wt E5 does not constitutively activate these kinases to levels associated with acute serum-induced signaling. Active Erk1/Erk2 also were not detected in exponentially growing 3T3 cells in the continuous presence of 10% FBS (fourth lane). Immunoblots labeled with an antibody that recognizes Erk2 irrespective of its phosphorylation state showed that these same samples contained equal amounts of Erk2 protein (lower panel of Fig. 5A).

To detect lower levels of Erk activation in exponentially growing and serum-starved cells, it was necessary to increase the amount of phosphorylated Erk1 and Erk2 on the immunoblots. Treatment of serum-starved 3T3 cells with Na3VO4 (to inhibit serine/threonine dephosphorylation) and okadaic acid (to inhibit tyrosine dephosphorylation) for 10 min before lysis made possible the detection of active Erk1/Erk2 (Fig. 5A; compare the second and sixth lanes). As expected, cells grown in the continuous presence of 10% FBS and treated with these same inhibitors for 10 min showed even higher levels of Erk activation (fifth lane). The ability to detect active Erk1/Erk2 in serum-starved 3T3 cells now permitted an analysis of the effects of the various E5 mutants on Erk phosphorylation (upper panel of Fig. 5B). Treatment of serum-starved cells expressing wt or mutant E5 constructs with Na3VO4 and okadaic acid demonstrated that Erk activation was identical in all cell lines tested. Thus, none of the transforming E5 constructs (including wt E5) constitutively activate the Erk/MAPK signaling pathway as determined by the phosphorylation state of Erk1 and Erk2.

**Transforming E5 Mutants Activate PI 3-K**

Heterodimeric PI 3-K is a critical component of signal transduction pathways that are involved in the mitogenic response to various growth factors and in oncogenic transformation (18, 19). In rodent fibroblasts, expression of constitutively active PI 3-K in the presence of serum is sufficient to induce characteristics of cellular transformation, including anchorage-independent growth (25). Given our finding that transforming E5 mutants do not activate Erk/MAPK signaling, studies were undertaken to ascertain whether or not these oncoproteins activate PI 3-K signaling.

**PI 3-K Tyrosine Phosphorylation**—The activation of heterodimeric PI 3-K often involves phosphorylation of the 85-kDa regulatory subunit (p85) on tyrosine (23, 24, 42, 43). Therefore, we measured levels of tyrosine-phosphorylated p85 in serum-starved 3T3 cell lines expressing wt and mutant E5 proteins to screen for possible effects of E5 on PI 3-K activity. Tyrosine-phosphorylated proteins were immunoprecipitated from cell lysates, and the immunoprecipitates were probed for p85 on immunoblots. As with our analysis of PDGF-R phosphorylation, lysates were boiled in SDS to disrupt protein-protein interactions before immunoprecipitation so that anti-p85 immunoblots would not contain unphosphorylated p85 that was associated with another tyrosine-phosphorylated protein(s). This technique proved to be quantitative within a defined range of protein concentrations, since the amount of tyrosine-phosphorylated p85 that was detected increased linearly with increasing quantities of lysate (Fig. 6A).

Tyrosine-phosphorylated p85 was readily detected in serum-starved NIH 3T3 cells 10 min after adding FBS (Fig. 6B, first lane) but was present at a 10-fold lower level if FBS was not added (second lane). Tyrosine phosphorylation of p85 also was detected in cells expressing the sis oncogene (fifth lane). These results are consistent with the well documented activation of PI 3-K by the PDGF-R tyrosine kinase (23, 24, 43). The non-
transforming E5 mutants that were defective for PDGF-R activation nevertheless induced tyrosine phosphorylation of p85 in the absence of growth factors (sixth to eighth lanes). Serum-starved cell lines expressing L26A E5, Q17S E5, or L24A E5 contained tyrosine-phosphorylated p85 at 51, 22, or 130% of the FBS-stimulated level, respectively. These values represent a 2–13-fold increase in p85 tyrosine phosphorylation relative to control 3T3 cells and Q17G E5-expressing cells. The total amount of p85 present in each of the cell lines varied by at most 16% (Fig. 6C) and therefore could not account for the much greater variation in levels of tyrosine phosphorylated p85.

To further clarify the role of PDGF-R activation in E5-induced PI 3-K activation, serum-starved cell lines expressing wt E5 or L26A E5 were treated with 0.3–3 μM tyrphostin AG1296 for 60 min before lysis and analysis of tyrosine phosphorylated p85. Tyrophostin AG1296 previously has been shown to completely inhibit PDGF-R kinase activity in intact Swiss 3T3 cells and in isolated cell membranes at these concentrations (30). As indicated in Fig. 6D, 3 μM tyrphostin AG1296 decreased p85 tyrosine phosphorylation by 70% in wt E5-expressing cells, suggesting that a component of PI 3-K activation in these cells is the consequence of PDGF-R activation. However, 3 μM tyrphostin AG1296 had no effect on the tyrosine phosphorylation of p85 in cells expressing L26A E5. Thus, E5 mutants that cannot significantly activate the PDGF-R, such as L26A E5, apparently activate PI 3-K by a separate, tyrphostin-insensitive mechanism. It is also likely that the residual (30%) tyrphostin-resistant activity of wt E5 indicates utilization of the same alternative pathway to PI 3-K activation.

**PI 3-K Activity**—*In vitro* lipid kinase assays were performed to determine whether E5-induced tyrosine phosphorylation of the PI 3-K p85 regulatory subunit indeed reflected increased PI 3-K activity. Tyrosine-phosphorylated proteins were immunoprecipitated from serum-starved cells and tested for their ability to phosphorylate exogenous PI in the presence of [γ-32P]ATP. As expected, essentially no PI kinase activity was detected in control NIH 3T3 cells or in cells expressing the nontransforming Q17G E5 mutant (Fig. 7A, second and third lanes). When PDGF-R-signaling pathways were activated by adding FBS to the medium (first lane) or by constitutive expression of wt E5 (fourth lane) or the sis oncogene (fifth lane), 32P-labeled PI phosphate was increased. However, since Ras activates PI 3-K independently of p85 phosphorylation (22), it is not surprising that the addition of serum, which rapidly activates Ras, elevated PI 3-K activity to a greater extent than p85 tyrosine phosphorylation. An elevated level of PI 3-K activity also was present in cells expressing the L26A, Q17S, and L24A E5 mutants that do not activate the PDGF-R (sixth through eighth lanes). These results show that a number of transforming E5 mutants induce phosphorylation and activation of heterodimeric PI 3-K by means of a pathway that does not require activation of the PDGF-R.

The independence of PI 3-K activation from PDGF-R signaling in L26A E5-expressing cells was further evidenced by its insensitivity to tyrphostin AG1296. PI 3-K activity in these cells was not affected by treatment with 0.3–3 μM tyrphostin AG1296 for 60 min before lysis, whereas identical tyrphostin AG1296 treatment of wt E5-expressing cells partially lowered levels of PI 3-K activity (Fig. 7B). These results are in agreement with the effects of tyrphostin AG1296 on p85 tyrosine phosphorylation induced by wt and L26A E5 (Fig. 6D) and support the view that wt E5 utilizes both PDGF-R-dependent and PDGF-R-independent mechanisms to activate PI 3-K.
E5 Oncoprotein Activates PI 3-Kinase

Two new classes of E5 oncoprotein mutants have been identified that are hypertransforming but show only minimal PDGF-R activation (14, 17). To investigate the mechanism of cell transformation by these mutants, we sought to identify other mitogenic signal transduction pathways that are constitutively activated in cell lines.

Transforming E5 Mutants Defective for PDGF-R Activation—wt BPV-1 E5 induces trans-phosphorylation and activation of the PDGF-R by effectively cross-linking endogenous PDGF-R monomers via transmembrane interactions (10, 14–16). Nevertheless, E5 mutants that induce little or no receptor phosphorylation because they are defective for PDGF-R binding (Q17S, L24A, and L24A) or homodimerization (L7A, L25A, and L26A) can efficiently transform fibroblast cell lines (14, 17). In the current study, we show that members of both classes of transforming E5 mutants (Q17S, L24A, and L26A) not only fail to efficiently stimulate PDGF-R phosphorylation in NIH 3T3 cells (Fig. 2) but also fail to functionally activate the PDGF-R (17). It remains to be determined whether these opposite activities derive from different levels of E5 expression, from epitope-tagging of the protein, or from the use of different cell lines. Regardless of the results with Q17S E5, however, we have employed two additional E5 mutants that only minimally activate the PDGF-R yet exhibit a 2–3-fold increase in transforming activity relative to wt E5.

E5 Activates Heterodimeric PI 3-K Independently of PDGF-R Activation—We have shown that PI kinase activity is present in anti-phosphotyrosine immunoprecipitates of serum-starved cells that express wt E5 or the Q17S-, L24A-, or L26A-transforming E5 mutants. In contrast, PI kinase activity is barely detectable in similar immunoprecipitates of serum-starved normal NIH 3T3 cells and control cells expressing the non-transforming Q17G E5 mutant. Therefore, three different transforming E5 mutants that are defective for PDGF-R activation are still able to activate a PI-kinase(s). PI-kinase activation appears to be constitutive since it occurs in serum-starved cells.

There is compelling evidence that the activated PI-kinase detected in our study is heterodimeric (p85/p110) PI 3-K, a key component of mitogenic signaling pathways (18, 19, 22, 24). Heterodimeric PI 3-K can phosphorylate PI (Fig. 7) as well as PI 4-phosphate and PI 4,5-bisphosphate and is present in anti-phosphotyrosine immunoprecipitates (Fig. 6), unlike PI 4-kinases and other classes of PI 3-Ks (18–20, 24, 45). Moreover, the activation of heterodimeric PI 3-K by the PDGF-R involves tyrosine phosphorylation of the p85 regulatory subunit (23, 24, 42, 43). We show that constitutive activation of the PDGF-R in cell lines that express wt E5 or the sis oncogene leads to increased PI kinase activity in anti-phosphotyrosine immunoprecipitates and to increased tyrosine phosphorylation of the PI 3-K p85 subunit on immunoblots. Activation of receptor tyrosine kinases (including the PDGF-R) by 10% FBS also elicits a concomitant increase in immunoprecipitable PI kinase activity and p85 tyrosine phosphorylation. Finally, increased tyrosine phosphorylation of the PI 3-K p85 subunit is correlated with the increased PI kinase activity induced by Q17S E5, L24A E5, and L26A E5, which do not significantly activate the PDGF-R.

Because heterodimeric PI 3-K can be directly activated by the PDGF-R, it appears that wt E5 induces PI 3-K activation via two signaling pathways: one in which PDGF-R activation is an intermediate step and another, which does not require PDGF-R activation. Transforming E5 mutants that are defective for PDGF-R activation have to activate PI 3-K via the latter pathway. This model is supported by our observation that the highly selective PDGF-R kinase inhibitor, tyrphostin AG1296, partially inhibits PI 3-K activation and tyrosine phosphorylation of the PI 3-K p85 subunit induced by wt E5 but does not inhibit PI 3-K activation and p85 tyrosine phosphorylation induced by L26A E5, which elicits little or no PDGF-R activation.

Mechanism of PI 3-K Activation by E5 Mutants—It has been reported that expression of the BPV-1 E5 oncoprotein in NIH 3T3 cells constitutively activates Ras and elevates levels of PI 3-K activity present in anti-epidermal growth factor receptor (EGF-R) immunoprecipitates (26). Although Ras can activate heterodimeric PI 3-K (22), it seems unlikely that Ras is involved in the activation of PI 3-K by the transforming E5 mutants that we have studied. Typically, Ras activates the Erk/MAPK signaling pathway, which includes sequential activation of the Raf, MEK, and Erk protein kinases (34–36). We have not observed activation of Erk1 and Erk 2 in serum-starved NIH 3T3 cells that express mutant or wt E5 proteins. Since E5 is predominantly localized in membranes of the Golgi

FIG. 7. PI kinase assays. A, immunoprecipitation of PI 3-K activity from serum-starved NIH 3T3 cells and cell lines expressing E5 constructs or the sis oncogene. Thin-layer chromatography was used to detect the phosphorylation of PI by anti-phosphotyrosine immunoprecipitates in the presence of [γ-32P]ATP. The cells were treated with 1 mM Na3VO4 (or 1 mM Na3VO4 + 10% FBS where indicated) for 10 min at 37 °C before lysis, and equivalent amounts of cell protein were immunoprecipitated for each lipid kinase assay. B, PI 3-K activity in anti-phosphotyrosine immunoprecipitates of serum-starved wt E5 and L26A E5 cell lines after treatment with 0.3–3 μM tyrphostin AG1296 for 60 min at 37 °C. The basal level of PI 3-K activity associated with an anti-phosphotyrosine immunoprecipitate from serum-starved normal 3T3 cells is shown for comparison.

DISCUSSION

There is compelling evidence that the activated PI-kinase detected in our study is heterodimeric (p85/p110) PI 3-K, a key component of mitogenic signaling pathways (18, 19, 22, 24). Heterodimeric PI 3-K can phosphorylate PI (Fig. 7) as well as PI 4-phosphate and PI 4,5-bisphosphate and is present in anti-phosphotyrosine immunoprecipitates (Fig. 6), unlike PI 4-kinases and other classes of PI 3-Ks (18–20, 24, 45). Moreover, the activation of heterodimeric PI 3-K by the PDGF-R involves tyrosine phosphorylation of the p85 regulatory subunit (23, 24, 42, 43). We show that constitutive activation of the PDGF-R in cell lines that express wt E5 or the sis oncogene leads to increased PI kinase activity in anti-phosphotyrosine immunoprecipitates and to increased tyrosine phosphorylation of the PI 3-K p85 subunit on immunoblots. Activation of receptor tyrosine kinases (including the PDGF-R) by 10% FBS also elicits a concomitant increase in immunoprecipitable PI kinase activity and p85 tyrosine phosphorylation. Finally, increased tyrosine phosphorylation of the PI 3-K p85 subunit is correlated with the increased PI kinase activity induced by Q17S E5, L24A E5, and L26A E5, which do not significantly activate the PDGF-R.

Because heterodimeric PI 3-K can be directly activated by the PDGF-R, it appears that wt E5 induces PI 3-K activation via two signaling pathways: one in which PDGF-R activation is an intermediate step and another, which does not require PDGF-R activation. Transforming E5 mutants that are defective for PDGF-R activation have to activate PI 3-K via the latter pathway. This model is supported by our observation that the highly selective PDGF-R kinase inhibitor, tyrphostin AG1296, partially inhibits PI 3-K activation and tyrosine phosphorylation of the PI 3-K p85 subunit induced by wt E5 but does not inhibit PI 3-K activation and p85 tyrosine phosphorylation induced by L26A E5, which elicits little or no PDGF-R activation.

Mechanism of PI 3-K Activation by E5 Mutants—It has been reported that expression of the BPV-1 E5 oncoprotein in NIH 3T3 cells constitutively activates Ras and elevates levels of PI 3-K activity present in anti-epidermal growth factor receptor (EGF-R) immunoprecipitates (26). Although Ras can activate heterodimeric PI 3-K (22), it seems unlikely that Ras is involved in the activation of PI 3-K by the transforming E5 mutants that we have studied. Typically, Ras activates the Erk/MAPK signaling pathway, which includes sequential activation of the Raf, MEK, and Erk protein kinases (34–36). We have not observed activation of Erk1 and Erk 2 in serum-starved NIH 3T3 cells that express mutant or wt E5 proteins. Since E5 is predominantly localized in membranes of the Golgi

In contrast to our results with NIH 3T3 cells, Klein et al. (44) found that Q17S E5 increases PDGF-R phosphorylation 7-fold in a different murine cell line, C127. This discrepancy may derive from the use of different cell lines, since Q17S E5 has a 7.5-fold higher transformation efficiency in C127 cells than in NIH 3T3 cells (27). A similar biological disparity exists in murine hematopoietic cells. Although another Q17S E5 construct appears to activate the PDGF-R and induce interleukin-3 (IL-3)-independent proliferation when co-expressed with exogenous PDGF-Rs in Ba/F3 murine hematopoietic cells (44), our Q17S E5 mutant fails to induce IL-3-independent proliferation in 32D murine hematopoietic cells when co-expressed with the PDGF-R (17).
apparatus (8) and Ras-mediated activation of Raf occurs at the plasma membrane (37, 46), cellular compartmentalization may prevent the downstream activation of MEK and Erk. More importantly, Ras activation of PI 3-K involves the GTP-dependent interaction of Ras with the PI 3-K p110 catalytic subunit and does not result in tyrosine phosphorylation of the PI 3-K p85 regulatory subunit (22). Our finding that PI 3-K activation induced by wt E5 and the Q17S, L24A, and L26A E5 mutants is associated with increased tyrosine phosphorylation of the PI 3-K p85 subunit argues against Ras-mediated activation. Since E5 has no intrinsic protein kinase activity, this finding implies that the Q17S, L24A, and L26A E5 mutants activate a protein-tyrosine kinase other than the PDGF-R, which in turn activates PI 3-K by phosphorylating p85.

Receptor tyrosine kinases other than the PDGF-R are known to activate PI 3-K, including the colony-stimulating factor-1 receptor, EGF-R, and insulin receptor (19, 24), and could potentially explain the ability of the PDGF-R-independent E5 mutants to transform cells. However, a number of results argue against a role for these kinases in PI 3-K activation by E5. 1. Goldstein et al. (15) show that the co-expression of wt BPV-1 E5 and the PDGF-R causes IL-3-independent proliferation in 32D murine hematopoietic cells that normally require IL-3 for growth. In contrast, IL-3-independent proliferation was not observed when the colony-stimulating factor-1 receptor or EGF-R were co-expressed with wt E5, indicating that E5 does not activate these receptors. NIH 3T3 cells have approximately 10% as many EGF-Rs as PDGF-Rs (47, 48), and the addition of EGF to serum-starved NIH 3T3 cells increases PI 3-K activity in anti-EGF-R immunoprecipitates by only 45% (26). In our study, E5 mutants that are defective for PDGF-R activation increase PI 3-K tyrosine phosphorylation up to 13-fold. 3. PI 3-K activation by the insulin receptor does not involve tyrosine phosphorylation of the PI 3-K p85 subunit but rather is a direct result of p85 binding to distinct, tyrosine-phosphorylated insulin-receptor substrate proteins (19, 49). E5 mutants that are deficient in PDGF-R activation do not support growth factor-independent proliferation in basal medium. Growth factor-independent proliferation does occur in cells where the PDGF-R is constitutively activated due to the expression of wt E5 or the ssrc oncogene. If alternative growth factor receptors were activated by E5 mutants, one might expect reduced growth factor requirements in these cells. However, despite these reservations, additional experiments will be required to definitively rule out the participation of alternative receptor tyrosine kinases. PI 3-K has also been detected in complexes with numerous nonreceptor tyrosine kinases, including focal adhesion kinase, Src, Fyn, Lyn, Abl, and Lck (50–55). These interactions normally explain the ability of the PDGF-R-independent E5 mutant to transform NIH 3T3 cells, but do not eliminate the growth factor requirement for proliferation (25). PI 3-K activation is sufficient to cause serum-starved cells to enter $S$ phase of the cell cycle, but progression through the entire cell cycle additionally requires growth factor receptor signaling (25). It may be that PI 3-K activation abrogates anchorage requirements for proliferation because anchorage normally is required for the activation of PI 3-K in response to growth factors (57).

Constitutive activation of the focal adhesion kinase and Abl tyrosine kinases, and Rho and Cdc42 small G-proteins, leads to cell proliferation that is anchorage-independent but growth factor-dependent (58). It is likely that some of these signaling proteins may confer anchorage independence by activating PI 3-K, whereas others may be downstream targets of PI 3-K that are important for enabling anchorage-independent growth (18, 19, 50, 54). Since anchorage-independent growth is closely correlated with tumorigenicity in animal models (59) and transient expression of constitutively active PI 3-K promotes carcinoma invasion (56), dissecting the effect of the E5 oncoprotein on PI 3-K-dependent signaling pathways may provide valuable insights into normal cell proliferation and neoplasia.

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