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Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein

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The bovine papillomavirus E5 gene encodes a 44 amino acid membrane-associated protein that can induce tumorigenic transformation of rodent fibroblast cell lines. Genetic studies suggest that the E5 protein may transform cells by influencing the activity of cellular proteins involved in growth regulation. We report here that the endogenous cellular β type receptor for the platelet-derived growth factor (PDGF) is constitutively activated in C127 and FR3T3 cells stably transformed by the E5 protein, but not in these cell types transformed by a variety of other oncogenes. In C127 cells, a metabolic precursor as well as the mature form of the receptor is activated by E5 transformation. Activation of the receptor also occurs upon acute E5-mediated transformation of these cells and precedes mitogenic stimulation in this system. Moreover, activation of the receptor by addition of PDGF or the v-*sis* gene to untransformed cells is sufficient to induce DNA synthesis and stable growth transformation. We propose that the PDGF receptor is an important cellular intermediate in the transforming activity of the bovine papillomavirus E5 protein. There is a short region of sequence similarity between the fibropapillomavirus E5 proteins and PDGF, suggesting that the E5 proteins may activate the PDGF receptor by binding directly to it.

Key words: signal transduction / tyrosine kinase / viral oncogene

Introduction

DNA viruses frequently encode proteins capable of stimulating host cell proliferation and inducing tumors in animals. In the best understood cases, these viral proteins appear to act by influencing the activity of cellular proteins that have been implicated in tumorigenesis. In no case, however, has a DNA tumor virus transforming protein been shown to transform cells by influencing the activity of a cellular protein with a clearly defined role in controlling proliferation of normal cells.

One of the more intriguing viral transforming proteins is the 44 amino acid E5 protein of bovine papillomavirus type 1 (BPV). This protein can induce tumorigenic transformation of established lines of mouse and rat fibroblasts and is thus the shortest protein with documented transforming activity (Bergman *et al.*, 1988; DiMaio *et al.*, 1986; Schiller *et al.*, 1986). Its amino-terminal two thirds is extremely hydrophobic whereas its hydrophilic carboxyl-terminal third contains several charged and polar amino acids. There is no extensive sequence similarity between the papillomavirus E5 proteins and other known viral or cellular proteins. The E5 protein has been identified in BPV transformed cells as a membrane-associated homodimer with a subunit size of 7 kilodaltons (kd) (Schlegel *et al.*, 1986). The protein is localized largely in the Golgi apparatus, with a subfraction in the plasma membrane (Burkhardt *et al.*, 1989), and it is associated with a 16 kd cellular protein (Goldstein and Schlegel, 1990).

The biological activities of constructed viral mutants and peptide fragments indicate that a single functional domain at the carboxyl-terminus of the E5 protein is largely responsible for its ability to induce focus formation, acute morphologic transformation, and cellular DNA synthesis (Green and Lowenstein, 1987: Horwitz *et al.*, 1988, 1989; Rawls *et al.*, 1989; Settleman *et al.*, 1989). The seven amino acids in this domain that are essential for efficient transformation are the same residues that are the best conserved among other fibropapillomaviruses (which, like BPV, induce fibroblast proliferation during natural infection). Two of these amino acids are cysteines which mediate dimer formation, suggesting that dimerization is necessary for E5 function.

The functional analysis of the E5 protein and its unusual composition and size indicate that it is unlikely to express enzymatic activity. On the basis of our studies, we proposed that the E5 protein may transform cells by altering the activity of a cellular membrane protein involved in cell growth and differentiation (Horwitz *et al.*, 1988). One such class of proteins is comprised of trans-membrane growth factor receptor tyrosine kinases. The interaction between a growth factor and such a receptor increases the intrinsic protein tyrosine kinase activity of the receptor, resulting in specific phosphorylation of tyrosine residues on the receptor itself as well as on other substrates. This tyrosine phosphorylation appears to initiate a series of reactions which culminate in cell proliferation.

Recent experiments by Martin *et al.* (1989) provided evidence that E5 expression can affect the activity and metabolism of growth factor receptors. These workers found that co-transfected human genes encoding the receptors for epidermal growth factor (EGF) or colony stimulating factor-1 (CSF-1) cooperated with the BPV E5 gene in the induction of stable transformation of mouse NIH3T3 cells. Although these stably transformed cells expressed elevated levels of exogenous, tyrosine-phosphorylated EGF receptors, the most obvious difference compared with normal cells was the increased half life of the receptor and its retarded internalization in response to ligand. It was speculated that E5 enhances the effective activity of these receptors by increasing their half life and thus the number of receptors accessible to ligand. However, these experiments did not examine the effect of the E5 protein on endogenous growth factor receptors. To identify potential endogenous cellular targets of the E5 protein, we compared tyrosine-

phosphorylated proteins in untransformed cells and in cells transformed by the E5 protein. We found that the β receptor for the platelet-derived growth factor (PDGF) is constitutively activated in E5-transformed cells, and we propose that the PDGF receptor is an important mediator of the transforming activity of the BPV E5 protein.

Results

A novel phosphotyrosine-containing protein in E5-transformed FR3T3 cells is the PDGF receptor

Because several potential targets of the E5 protein are protein tyrosine kinases, phosphotyrosine-containing proteins in BPV-transformed cells were examined. In initial experiments, a cloned cell line of FR3T3 cells stably transformed by the BPV E5 gene alone (see Materials and methods) was compared with a cloned line of untransformed cells established in parallel. Phosphotyrosine proteins were immunoprecipitated from cell extracts using an anti-phosphotyrosine monoclonal antibody, separated by polyacrylamide gel electrophoresis, and identified by immunoblot analysis using an affinity purified anti-phosphotyrosine antiserum. An abundant phosphotyrosine protein of ~190 kd was readily detected in the transformed cell line, but not in the untransformed cells (Figure 1A, lanes O). Immunoprecipitation of this 190 kd phosphotyrosine protein was eliminated by incubation of the antibody with phosphotyrosine but not with phosphoserine or phosphothreonine, and similar results were obtained using a second anti-phosphotyrosine monoclonal antibody for blotting (data not shown).

Because tyrosine phosphorylation is a hallmark of activated growth factor receptors, we attempted to induce the phosphorylation of the 190 kd protein in untransformed cells by treatment with serum or purified growth factors (Figure 1A). Treatment of both untransformed and transformed FR3T3 cells with epidermal growth factor (EGF) (lanes E) caused tyrosine phosphorylation of a 175 kd protein with a mobility clearly distinct from that of the 190 kd protein constitutively phosphorylated in the transformed cells. An antiserum specific for the EGF receptor immunoprecipitated the 175 kd phosphotyrosine protein from extracts of EGF-stimulated cells (data not shown), thereby identifying this protein as the EGF receptor. However, the anti-EGF receptor antibody did not precipitate a 190 kd phosphotyrosine protein from unstimulated or EGF-stimulated cells (data not shown). Therefore, the 190 kd constitutively tyrosine-phosphorylated protein was not the EGF receptor.

In contrast to the results obtained with EGF, treatment with serum immediately prior to extraction (lanes S) induced the tyrosine phosphorylation of a 190 kd protein in untransformed FR3T3 cells with a mobility identical to that of the protein constitutively phosphorylated in E5-transformed cells. These results suggested that the novel protein in E5-transformed cells was the platelet-derived growth factor (PDGF) receptor, a 180–185 kd tyrosine kinase (Williams, 1989). In support of this hypothesis, treatment of untransformed cells with purified PDGF resulted in the tyrosine phosphorylation of a protein with the same mobility as the protein constitutively phosphorylated in E5-transformed cells (Figure 1B, right four lanes). To establish that the novel protein was the PDGF receptor, a specific anti-peptide antibody that recognizes the β form of the PDGF receptor was used to immunoprecipitate the receptor from the cell extracts, and the immunoprecipitates were immunoblotted using an anti-phosphotyrosine monoclonal antibody (Figure 1B, left four lanes). As expected, PDGF receptor was tyrosine phosphorylated in untransformed cells only when they were treated with PDGF. In contrast, tyrosine phosphorylated PDGF receptor was present in the E5-transformed cells whether or not they were treated with PDGF. Similar results were obtained using two

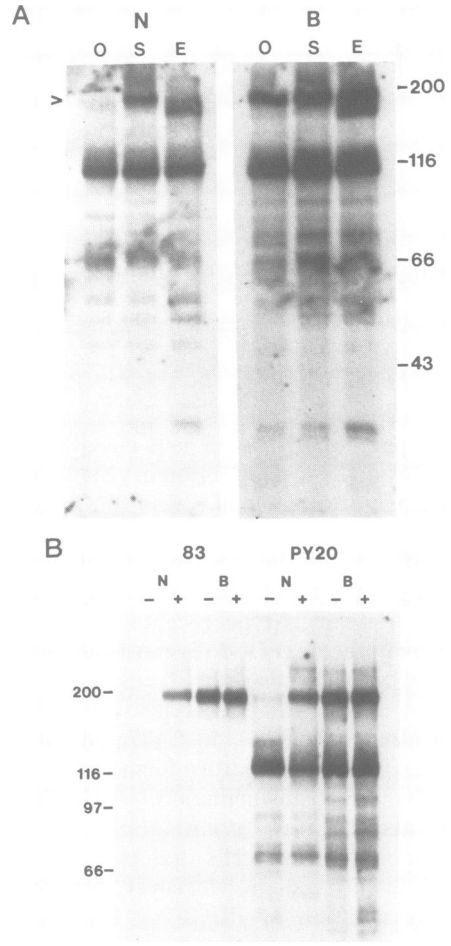

Fig. 1. Analysis of tyrosine-phosphorylated proteins in FR3T3 cells. **A.** Constitutive tyrosine phosphorylation of a novel 190 kd protein in E5-transformed FR3T3 cells. Normal untransformed FRE2 (N) or BPV E5-transformed FRC5 (B) FR3T3 cells were starved overnight in DME-0.1 and then incubated for 20 min at 37 C in DME-0.1 (lane 0), DME containing 10% fetal calf serum (lane S), or DME-0.1 containing 100 ng/ml EGF (lane E). Phosphotyrosine proteins were immunoprecipitated from cell extracts with anti-phosphotyrosine monoclonal antibody (PY20), electrophoresed and immunoblotted using the affinity-purified anti-phosphotyrosine antiserum as described in Materials and methods. Arrow points to a novel 190 kd phosphotyrosine protein in transformed cells. **B.** Identification of the novel 190 kd phosphotyrosine protein in E5-transformed cells as the PDGF receptor. Normal untransformed FRE2 (N) or BPV E5-transformed FRC5 (B) FR3T3 cells were starved overnight and then incubated for 15 min in the presence (+) or absence (−) of 10 ng/ml of PDGF (Collaborative Research). PDGF receptor or total phosphotyrosine proteins were immunoprecipitated from cell lysates with anti-PDGF receptor (83) antiserum or anti-phosphotyrosine monoclonal antibody (PY20), respectively. Immunoprecipitates from ~400 μg of protein were loaded in each lane, electrophoresed and immunoblotted with anti-phosphotyrosine antiserum.

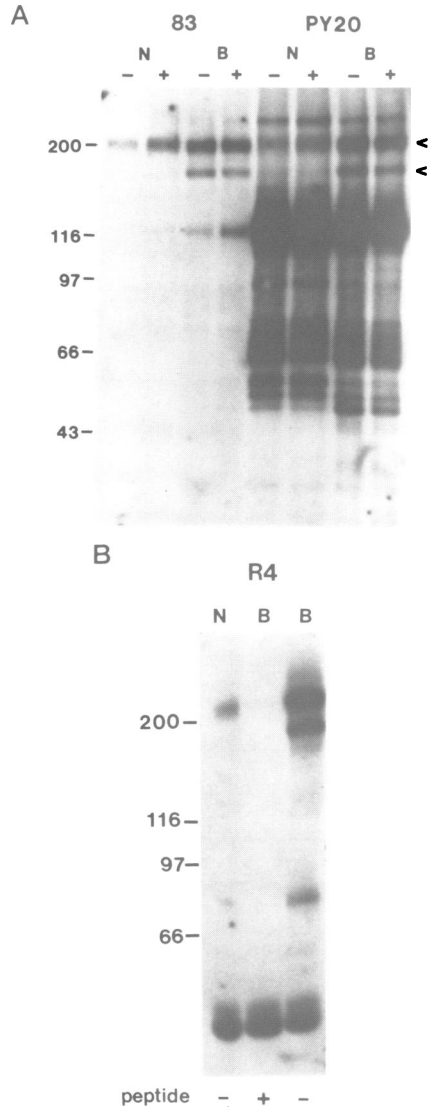

Fig. 2. Analysis of tyrosine-phosphorylated PDGF receptors in C127 cells. **A.** Normal untransformed R7-3 (N) or BPV E5-transformed L1D-1 (B) C127 cells were starved overnight and then incubated in the absence (−) or presence (+) of 10 ng/ml of PDGF (Collaborative Research). PDGF receptor or total phosphotyrosine proteins were immunoprecipitated from cell lysates with anti-PDGF receptor antiserum 83 or anti-phosphotyrosine monoclonal antibody PY20, respectively. Immunoprecipitates from equal amounts of extracted protein (~390 μg) were loaded in each lane, electrophoresed and immunoblotted using anti-phosphotyrosine antiserum. Arrows on the right point to the 200 kd PDGF receptor and the 165 kd protein specifically tyrosine phosphorylated in E5-transformed cells. **B.** Extracts were prepared as above from serum-starved R7-3 (N) or L1D-1 (B) C127 cells. PDGF receptor was immunoprecipitated with anti-PDGF receptor antiserum PR4 in the presence (+) or absence (−) of competing peptide and immunoprecipitates were analyzed for tyrosine phosphorylation as described in the legend to panel A.

additional antibodies specific for different PDGF receptor peptides, and immunoprecipitation of the tyrosine-phosphorylated PDGF receptor was blocked by addition of immunizing peptide (data not shown, see Figure 2 for similar experiments with extracts of E5-transformed C127 cells). Thus, the 190 kd protein constitutively tyrosine phosphorylated in BPV E5-transformed FR3T3 cells is the β type PDGF receptor. Immunoblot analysis of whole cell extracts demonstrated that E5-transformed FR3T3 cells do not contain elevated levels of the PDGF receptor (data not shown), indicating that there is an increase in receptor phosphorylation, not just in receptor number in the transformed cells.

Tyrosine phosphorylation of the PDGF receptor in E5-transformed C127 cells

Because mouse C127 fibroblasts are the standard cells used for most studies of E5-mediated transformation, we determined whether the PDGF receptor was also activated by the E5 protein in this cell line. Untransformed or E5-transformed C127 cells were either untreated or treated with PDGF, and extracts were prepared. Specific immunoprecipitation of the PDGF receptor from these extracts followed by immunoblotting using anti-phosphotyrosine antibody was used to detect tyrosine phosphorylated forms of the PDGF receptor (Figure 2A, left four lanes). A low level of an ~200 kd tyrosine phosphorylated PDGF receptor was present in extracts of untransformed cells, and the amount of tyrosine phosphorylation was increased significantly upon PDGF treatment. However, the basal level of receptor phosphorylation was more variable in untransformed C127 cells compared to untransformed FR3T3 cells. The level of tyrosine phosphorylation of this 200 kd form of the receptor in extracts of E5-transformed cells was high prior to PDGF addition, suggesting that the receptor is constitutively activated in E5-transformed C127 as well as in FR3T3 cells. Strikingly, the PDGF receptor antiserum also precipitated a faster migrating, 165 kd protein that was specifically tyrosine phosphorylated in E5-transformed but not in untransformed C127 cells. Similar results were obtained using two other antisera recognizing different PDGF receptor peptides (one of which is shown in Figure 2B), and immunoprecipitation of both the 200 kd and 165 kd forms was blocked by addition of immunizing peptide (Figure 2B). In addition, the 200 and 165 kd tyrosine-phosphorylated proteins are not detectable if immunoprecipitation is carried out with non-immune serum (Figure 3A, lanes a and e). The tyrosine phosphorylation of this 165 kd protein was a more specific and reproducible response to E5 transformation than was increased tyrosine phosphorylation of the 200 kd receptor species. Tyrosine phosphorylation of the 165 kd protein was also apparent in immunoblots of total phosphotyrosine immunoprecipitates of transformed cells but not of untransformed cells (Figure 2A, right four lanes).

The 165 kd phosphoprotein immunoprecipitated by the PDGF receptor antiserum might be either a form of the PDGF receptor itself or a protein in a complex with the PDGF receptor. To distinguish between these possibilities, cell extracts were denatured by boiling in 0.5% SDS prior to precipitation with receptor antibody and blotting with anti-phosphotyrosine antibody. As shown in Figure 3A, lanes g and h, the 165 kd phosphotyrosine protein is detectable in extracts of transformed cells even if the extracts are denatured prior to immunoprecipitation. Similar results were obtained if 5 mM DTT was included in the denaturation step (data not shown). We conclude that the 165 kd protein containing phosphotyrosine in E5-transformed cells is a form of the PDGF receptor itself. We note that several proteins immunoprecipitated from the native extracts of transformed cells are absent from immunoprecipitates of denatured extracts (compare lane f with lanes g and h). This suggests

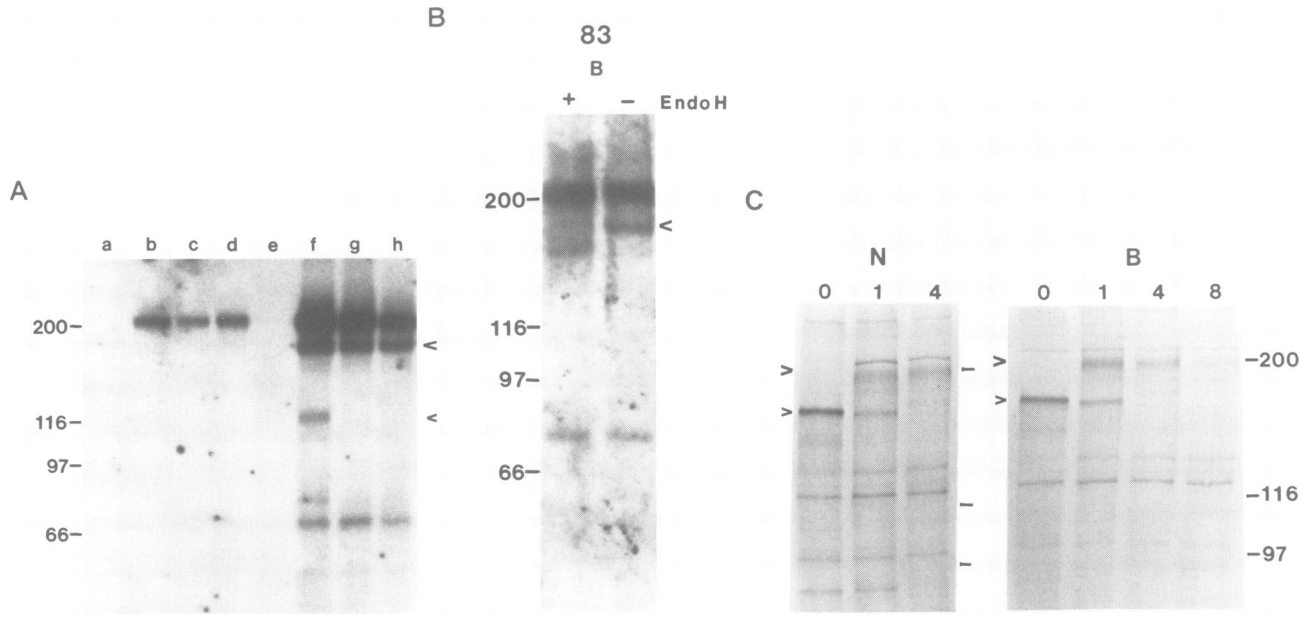

Fig. 3. Analysis of the 165 kd protein specifically tyrosine phosphorylated in E5-transformed C127 cells. **A.** Immunoprecipitation of denatured protein extracts. Extracts of normal untransformed R7-3 (lanes a–d) or BPV E5-transformed L1D-1 (lanes e–h) C127 cells were divided into aliquots. One was immunoprecipitated directly with preimmune serum (lanes a and e) and another with anti-PDGF receptor antibody 83 (lanes b and f). Proteins in the other aliquots were denatured by boiling in SDS and then immunoprecipitated with antiserum 83 either directly (lanes c and h) or after dilution of the SDS (lanes d and g). Immunoprecipitates from ~97 or 285 μg of protein extracted from untransformed or E5-transformed cells, respectively, were electrophoresed and immunoblotted using anti-phosphotyrosine antiserum. Large arrowhead points to the 165 kd PDGF receptor form and the small arrowhead to a protein of ~120 kd that co-precipitates from native but not from denatured extracts. **B.** Endo H sensitivity. PDGF receptor was immunoprecipitated from an extract of BPV E5-transformed L1D-1 C127 cells using anti-PDGF receptor antibody 83. The immune complex was eluted from the protein A-Sepharose beads and divided into two equal aliquots that were then incubated in either the presence (+) or absence (-) of endo H as described in Materials and methods. Each aliquot was electrophoresed and immunoblotted using anti-phosphotyrosine antiserum. Arrow points to the 165 kd protein in the untreated lane. **C.** Pulse–chase experiment. Normal untransformed R7-3 (N) or BPV E5-transformed L1D-1 (B) C127 cells were metabolically labeled with Trans^{35}S-Label for 15 min and then either lysed immediately (0) or incubated with an excess of cold methionine and cysteine for 1, 4 or 8 h, as indicated, prior to lysis. PDGF receptor was immunoprecipitated from these extracts using antibody 77 and detected by SDS–polyacrylamide gel electrophoresis and autoradiography. Arrows indicate positions of the 165 and 200 kd PDGF-receptor forms.

that the proteins eliminated by this treatment were in a complex with the PDGF receptor and therefore that the denaturation procedure was effective.

The mobility of the 165 kd form of the PDGF receptor phosphorylated in E5-transformed C127 cells is similar to that of the major intracellular precursor of the receptor in mouse NIH 3T3 cells (Keating and Williams, 1987). If the 165 kd form was a precursor, it should be sensitive to digestion with endoglycosidase H (endo H), an enzyme which digests high mannose oligosaccharides on glycoprotein precursors. PDGF receptor immunoprecipitated from E5-transformed cells was either untreated or treated with endo H and then electrophoresed and immunoblotted with anti-phosphotyrosine antibody (Figure 3B). Endo H treatment converted the 165 kd activated PDGF receptor form into an even faster migrating form, whereas the 200 kd form was unaffected. Thus, the 200 kd form is most likely the mature PDGF receptor and the 165 kd form is a precursor with immature carbohydrate side chains.

A pulse–chase experiment provided further evidence that the 165 kd form of the receptor is a precursor. Untransformed and E5-transformed C127 cells were metabolically labeled with [^{35}S]methionine and cysteine for 15 min and then incubated with excess unlabeled amino acids. PDGF receptor was immunoprecipitated from cell extracts prepared after different lengths of chase, electrophoresed, and identified by autoradiography (Figure 3C). Only a 165 kd form of the receptor was present immediately after the cells were pulse labeled. After a one hour chase, the amount of the 165 kd form decreased while a new 200 kd form appeared, implying that the smaller form was converted to the larger form through post-translational processing. Specific immunoprecipitation of the pulse-labeled 165 kd receptor form with anti-phosphotyrosine antibodies documented that it is tyrosine-phosphorylated within one hour after synthesis in transformed cells (data not shown). After longer chase periods, the 165 kd form was entirely replaced with the 200 kd form, and eventually degradation of the 200 kd form became evident. These results provide strong evidence that the 165 kd form of the receptor is a metabolic precursor to the mature 200 kd form. As determined by scanning densitometry of a similar experiment performed with a second anti-receptor antibody, the half lives of the precursor form and the mature form were similar for both untransformed and E5-transformed cells (data not shown), indicating that the rate of synthesis and of processing of the PDGF receptor is similar in normal and transformed C127 cells. The PDGF receptor half life was also similar in untransformed and E5-transformed FR3T3 cells (data not shown).

Increased protein tyrosine kinase activity in PDGF receptor immune complexes

To examine protein kinase activity associated with the PDGF receptor, PDGF receptor was immunoprecipitated from C127 cells, and a portion of the immune complex was

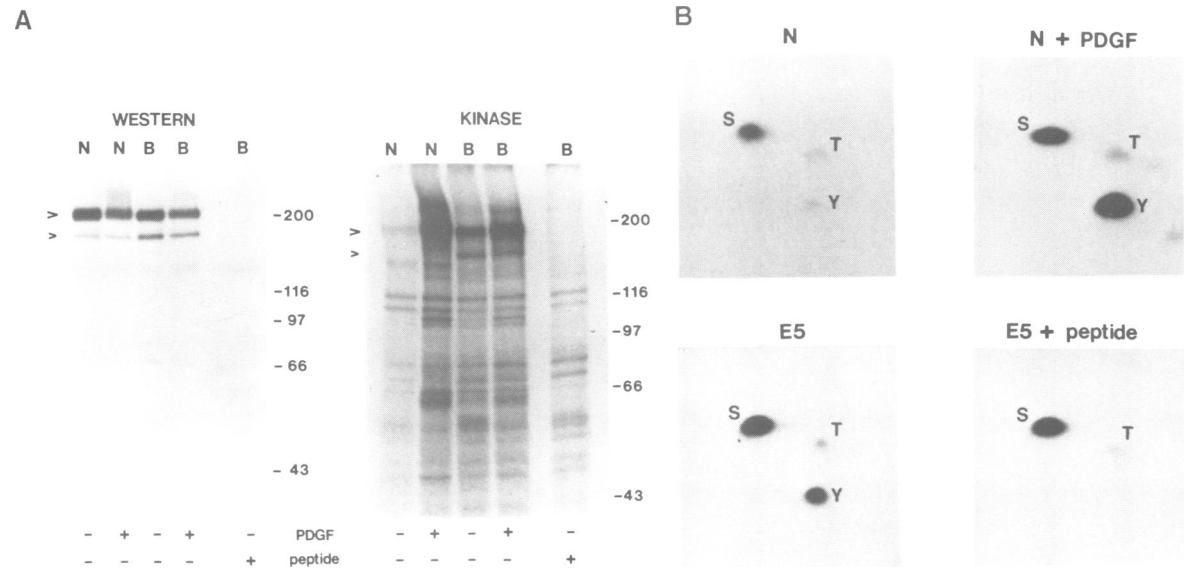

Fig. 4. Presence of the PDGF receptor in a complex with increased tyrosine kinase activity in E5-transformed cells. **A.** Using antibody PR4, PDGF receptor was immunoprecipitated from lysates of normal untransformed R7-3 (N) or BPV E5-transformed L1D-1 (B) C127 cells incubated in the presence (+) or absence (−) of PDGF BB homodimer (PDGF, Inc.). Competing peptide was included in the immunoprecipitations as indicated. Left panel. 10% of each immunoprecipitate was electrophoresed, transferred to nitrocellulose and its PDGF receptor content was determined by blotting with anti-PDGF receptor antiserum PR4. Right panel. 90% of each immunoprecipitate was incubated with 10 μCi of [γ-^{32}P]ATP, washed and then 10% of the reaction was resuspended in protein sample buffer as described in Materials and methods. Reaction products were electrophoresed and ^{32}P-labeled proteins were detected by autoradiography of the dried gel. Large arrow points to the major protein phosphorylated *in vitro*, which displays mobility characteristic of the mature PDGF receptor and the small arrow points to a product with a mobility characteristic of the precursor form of the PDGF receptor. **B.** Phosphoamino acid analysis. 10% of the *in vitro* kinase reactions carried out with PDGF receptor immune complexes as described in the legend to panel A were subjected to phosphoamino acid analysis as described in the Materials and methods. The hydrolysis products were separated by 2-dimensional electrophoresis on a thin layer plate and detected by autoradiography (anode was on the left for the first dimension and at the top for the second dimension). All four samples were run on the same plate and the film was exposed for the same amount of time. The spots are identified as phosphoserine (S), phosphthreonine (T) and phosphotyrosine (Y). Source of immune complex: N, normal R7-3 cells; E5, E5-transformed L1D-1 cells.

assayed for receptor content by immunoblotting. As shown in Figure 4A (left panel), there was no significant difference in the amount of mature receptor in normal and transformed cells and only a slight increase of precursor (~2-fold as determined by scanning densitometry of multiple experiments) in the transformed cells. This is consistent with the similar half life of the receptor in normal and transformed cells.

The rest of the immune complex was incubated with [γ-^{32}P]ATP under kinase reaction conditions, and phosphorylated proteins were detected by gel electrophoresis and autoradiography (Figure 4A, right panel). Immune complexes from untransformed cells catalyzed a low level of phosphorylation that appeared to be largely non-specific because it was similar to the phosphorylation seen when immunoprecipitation was blocked with PDGF receptor peptide. In contrast, treatment with PDGF or transformation by the E5 protein caused phosphorylation of novel bands, the major ones of which comigrated with the PDGF receptor and (in E5-transformed cells) the PDGF receptor precursor. A portion of the reaction products was also subjected to acid hydrolysis and phosphoamino acid analysis. As shown in Figure 4B, the low level of phosphorylation catalyzed by immunoprecipitates from untransformed cells occurred primarily at serine residues. However, PDGF receptor immune complexes prepared from transformed cells or cells treated with PDGF clearly catalyzed increased phosphorylation of tyrosine. This increase in tyrosine phosphorylation (but not phosphorylation of serine or threonine) is prevented by inhibition of PDGF receptor immunoprecipitation with a competing peptide. These results indicate that there is a more active protein tyrosine kinase within the PDGF receptor complex in E5-transformed cells and is consistent with the suggestion that the kinase activity of the receptor itself is increased in these cells. However, we have not ruled out the possibility that the E5 protein activates another tyrosine kinase in the PDGF receptor complex, such as pp60$^{c\text{-}src}$ (Kypta *et al.*, 1990).

Specificity of PDGF receptor activation

To determine whether the presence of the tyrosine-phosphorylated PDGF receptor was a specific marker of cells transformed by E5, stably transformed cells were established in parallel following infection with the E5-containing retrovirus or transfection with a variety of oncogenes, and their content of phosphotyrosine proteins was determined by immunopreciptation and immunoblotting. As shown in Figure 5A the 190 kd PDGF receptor was readily detectable in several FR3T3 cell lines transformed by the E5 retrovirus (C8, C11, C5; lanes b−d), by the full length BPV1 genome (lanes e and f) or by the BPV1 genome containing a frameshift mutation in the E6 transforming gene (lanes g and h). In contrast, this protein was barely detectable in untransformed cells (lane a), cells transformed by the polyoma middle T antigen (lanes i and j), v-Ha-*ras* (lanes k and l), or SV40 large T antigen gene (lane m).

Similar experiments were performed in C127 cells (Figure 5B). In this set of cell lines, the mature receptor form in untransformed cells displayed a high level of tyrosine phosphorylation that was not increased in any of the

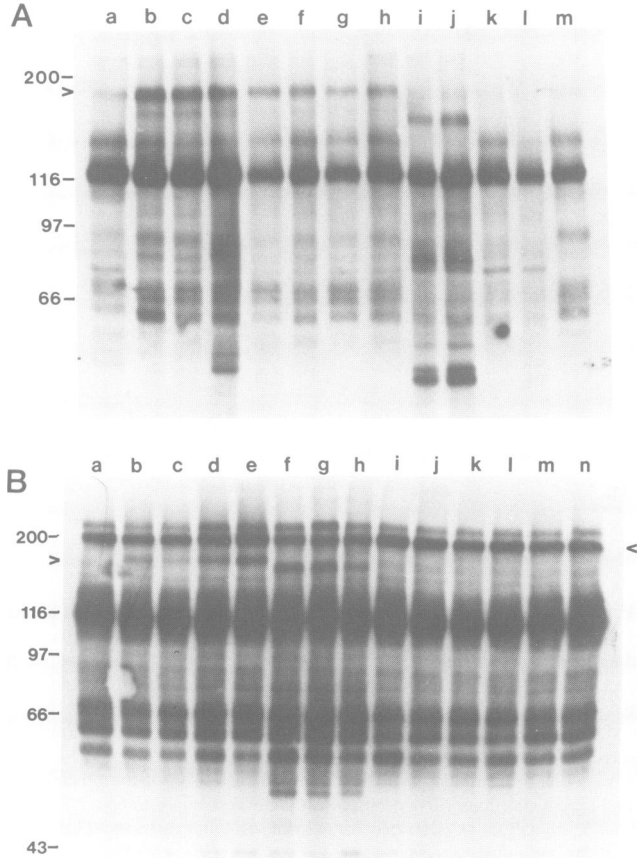

Fig. 5. Phosphotyrosine proteins present in FR3T3 and C127 cells transformed by various oncogenes. **A**. Independent cell lines were derived from FR3T3 cells transformed by the E5 retrovirus (C8, C11, C5; lanes b–d, respectively) or by transfection with full length BPV DNA (lanes e and f), BPV DNA containing a frameshift in ORF E6 [mutant E6fs2 (Neary and DiMaio, 1989)] (lanes g and h), or with plasmids encoding polyomavirus middle T antigen (lanes i and j), Harvey-*ras* (lanes k and l), or SV40 large T antigen (lane m). Untransformed FRE2 cells (lane a) were included as a negative control. Phosphotyrosine proteins were detected in extracts (240 μg protein) of starved cells by immunoprecipitation and Western blotting with anti-phosphotyrosine antibody as described in legend to Figure 1. Arrowhead points to the novel 190 kd tyrosine phosphorylated protein detected in BPV-transformed FR3T3 cells. **B**. C127 cells were transformed by the E5 retrovirus (L1D-1, lane b) or by transfection with full length BPV DNA (lane c), BPV ORF E6 mutant E6fs2 (lanes d and e), or with plasmids encoding the polyoma middle T antigen (lanes f–h), Harvey-*ras* (lanes i and j), the v-*mos* (lanes k and l), or the SV40 large T antigen (lanes m and n). Phosphotyrosine proteins in cell extracts (760 μg protein) were detected as in panel A. Untransformed C127 cells transfected with calf thymus DNA (lane a) were included as negative control. Arrowhead on the left of points to the novel 165 kd tyrosine phosphorylated protein detected in BPV-transformed C127 cells. Arrowhead on the right indicates position of mature PDGF receptor in C127 cells.

transformed cells. In contrast, although tyrosine phosphorylated 165 kd PDGF receptor precursor was not detected in extracts of C127 cells transfected with calf thymus DNA (lane a) or transformed by the polyoma virus middle T antigen (lanes f–h), v-Ha *ras* (lanes i and j), v-*mos* (lanes k and l), or SV40 large T antigen gene (lanes m and n), it was readily detected in all BPV transformants tested (lanes b-e), Thus, increased tyrosine phosphorylation of the PDGF receptor is a specific characteristic of transformation by the BPV E5 protein.

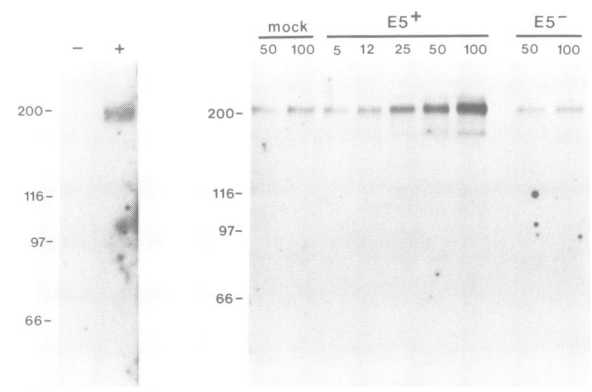

Fig. 6. PDGF receptor activation upon acute transformation by E5. **Left** panel. FR3T3 cells were infected in DME-10 with wild type Pava1 virus (+) or a mutant virus containing a frameshift mutation in the E5 gene (–) as described in Materials and methods. After 48 h, cells were lysed and anti-PDGF receptor antiserum 83 was used to immunoprecipitate the receptor from extracts containing ~560 μg of protein. Activated PDGF receptor was detected by electrophoresis and immunoblotting with anti-phosphotyrosine monoclonal antibody. **Right** panel. C127 cells were mock infected (mock) or infected with the indicated amounts (in μl) of wild type ($E5^+$) or E5 mutant ($E5^-$) virus stocks. After 31 h in DME-2, PDGF receptor was immunoprecipitated from extracts containing 195 μg of protein and detected by immunoblotting as described for the left panel.

Activation of the PDGF receptor during acute transformation by the E5 protein

We have developed an SV40/BPV recombinant virus that induces acute, E5-mediated transformation of C127 cells (Settleman and DiMaio, 1988; Settleman *et al.*, 1989). Most cells in an infected culture are rapidly induced by the E5 protein to undergo DNA synthesis and morphologic transformation. The acute morphologic and mitogenic response to the E5 protein is proportional to the amount of virus added to the cells (Settleman *et al.*, 1989). Importantly, the acute cellular response to the E5 protein can be analyzed in the absence of nonspecific secondary events which might occur during stable transformation.

To examine the effect of acute E5 expression on the PDGF receptor, cells were infected at high multiplicity with the wild type recombinant virus or a mutant virus containing a frameshift mutation in the E5 gene. Within 48 h of infection, the cells were lysed and their PDGF receptor was immunoprecipitated with receptor antibody and immunoblotted using anti-phosphotyrosine antibody. Acute expression of the wild type E5 protein but not the mutant protein induced tyrosine phosphorylation of the PDGF receptor in FR3T3 cells (Figure 6, left panel). In C127 cells, acute expression of the E5 protein induced increased tyrosine phosphorylation of both the precursor and mature forms of the PDGF receptor (Figure 6, right panel). As shown in the figure, the extent of tyrosine phosphorylation of both receptor forms was proportional to the multiplicity of infection at doses of virus where the morphological response was proportional to multiplicity. In contrast, even high multiplicity infection with the E5 mutant virus did not increase tyrosine phosphorylation of the PDGF receptor compared to uninfected cells. Thus, acute transformation by E5 expression induces tyrosine phosphorylation of the PDGF receptor in both FR3T3 and C127 cells, the specific metabolic forms of the receptor phosphorylated recapitulated

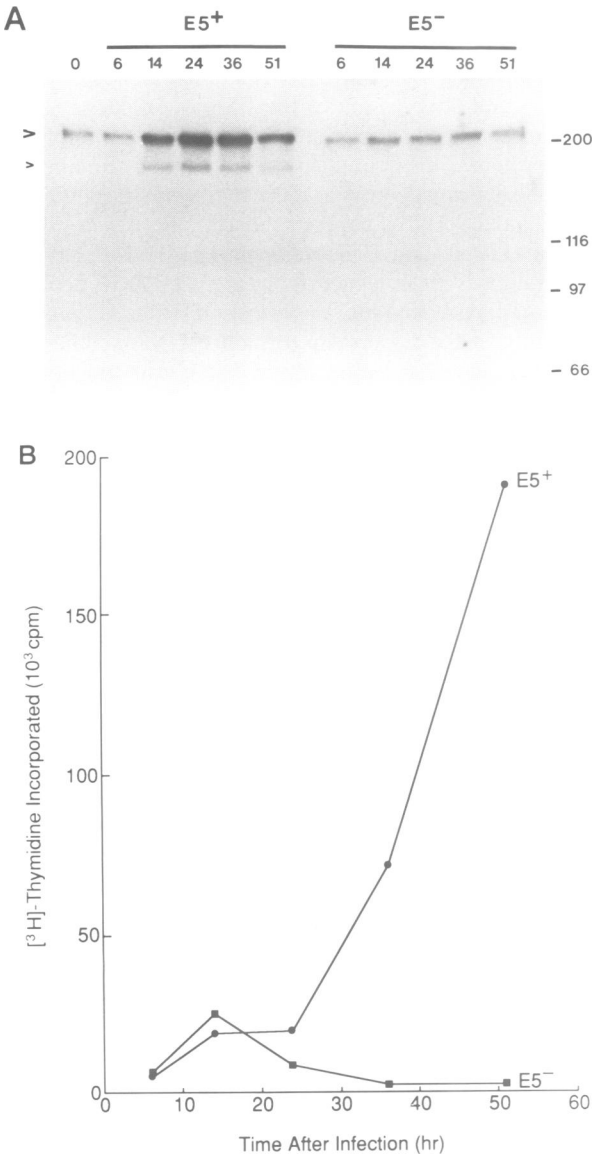

Fig. 7. Kinetics of PDGF receptor activation and DNA synthesis during acute transformation by E5. Serum-starved C127 cells were infected in parallel in DME-0 in 60 mm dishes and 24-well plates with either Pava1 ($E5^+$) or the E5 mutant virus ($E5^-$). At the indicated h after infection, cells in 60 mm dishes were assayed for PDGF receptor activation (panel A) and cells in a 24-well plate were assayed in duplicate for DNA synthesis (panel B). **A**. PDGF receptor activation was assayed by immunoprecipitating PDGF receptor from 105 μg of extracted protein with antibody 83, electrophoresis and immunoblotting using anti-phosphotyrosine monoclonal antibody 4G10. Mature PDGF receptor and receptor precursor are indicated by the large and small arrowheads, respectively. **B**. DNA synthesis induced by the wild type ($E5^+$) or mutant ($E5^-$) virus during a 2 h pulse was assayed by measuring [^{3}H]thymidine incorporation as described in Materials and methods. Average values of duplicate determinations are shown.

what was seen in stably transformed cell lines, and E5-stimulated PDGF receptor activation showed a dose dependence similar to E5-induced transformation.

We also determined the relative time course of receptor activation and DNA synthesis induction following introduction of the E5 gene. Quiescent C127 cells were infected with the wild type or the E5 mutant virus and then assayed at various times for PDGF receptor activation and for DNA synthesis. As shown in Figure 7A, there was little tyrosine phosphorylation of the mature form and none of the 165 kd form of the receptor during the first 6 h of infection with the E5 virus or at any time after infection with the E5 mutant virus. In contrast, by 14 h after infection with the wild type virus, a significant increase in tyrosine phosphorylation of both mature and precursor PDGF receptor was detectable and persisted for many hours. In cells infected in parallel at the same multiplicity, significant E5-specific stimulation of cellular DNA synthesis was not evident until 36 h after infection (Figure 7B). Thus, activation of the PDGF receptor by the E5 protein clearly preceded the mitogenic response of the cells to this protein. Similar results were obtained in a separate experiment performed with independently prepared virus stocks. The lag period prior to receptor activation presumably reflects at least in part the time necessary for the viral DNA to reach the nucleus and for sufficient levels of the E5 protein to accumulate. 14 h after infection with either the wild type or mutant virus, there was a modest burst of DNA synthesis that appears analogous to the transient mitogenic effect of polyomavirus capsid proteins in murine fibroblasts (Zullo *et al.*, 1987).

Effect of PDGF on FR3T3 and C127 cells

To determine the response of untransformed FR3T3 and C127 cells to PDGF, quiescent cells were treated with recombinant human PDGF BB homodimer and assayed for cellular DNA synthesis. In both cell types, PDGF treatment induced cellular DNA synthesis (Table I), indicating that activation of the PDGF receptor by its ligand is sufficient to elicit this response. Significant stimulation of C127 cell DNA synthesis occurred within 15–20 h after ligand addition, considerably earlier than it occurred following infection with the virus expressing the E5 protein. Moreover, a transfected v-*sis* gene can induce focus formation in C127 cells (L.Nilson and D.DiMaio, unpublished results). v-*sis*, the homologue of the structural gene for the PDGF B chain (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983), is a dominant oncogene thought to induce transformation via stimulation of PDGF receptors (Huang *et al.*, 1984; Leal *et al.*, 1985; Johnsson *et al.*, 1985).

Discussion

We have shown that the β type PDGF receptor is constitutively activated in FR3T3 and C127 cells stably and acutely transformed by the BPV E5 gene. Activation was demonstrated by two criteria, increased tyrosine phosphorylation of the receptor *in vivo* and the presence of the receptor in a complex with increased *in vitro* tyrosine kinase activity. The rapid, dose-dependent phosphorylation of the receptor observed upon acute transformation implies that activation is a direct effect of the E5 protein. PDGF treatment causes association of the receptor with downstream substrates implicated in cellular growth control, including the $p21^{ras}$-associated GTPase activator protein (GAP) (Molloy, *et al.*, 1989; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990). Recently, we have found that the GAP protein is constitutively in a complex with the PDGF receptor in E5-transformed C127 cells but not in normal cells, providing further evidence that the E5 protein causes biologically significant activation of the receptor (L.Nilson, S.Bulfone and D.DiMaio, unpublished results).

The level of tyrosine phosphorylation of the mature

```
PDGF A  ...GLU - HIS - LEU - GLU -  CYS - THR - CYS - THR...
                |    ||    |     ||    ||    |    ||    ||
BPV E5  ...ASP -  HIS - PHE - GLU - CYS - SER -  CYS - THR...
                ||    ||    |           ||          ||
PDGF B  ...ASP -  HIS - LEU - ALA -  CYS - LYS -  CYS - GLU...
                ||    |     |     |    ||    ||    ||    ||
DPV E5  ...ASP - GLN -PHE - GLY  - CYS - LYS -  CYS - GLU...
```

Fig. 8. Sequence similarity between fibropapillomavirus E5 proteins and PDGF. The amino acid sequence of amino acids 33-40 of the carboxyl-terminal regions of the BPV E5 protein and the deer papillomavirus E5 protein are shown, as is a related sequence of the A chain of *Xenopus laevis* and the B chain of human platelet-derived growth factor (amino acids 174-181 of the primary translation product) (Mercola *et al.*, 1988). Amino acids identical to PDGF are indicated by double lines; conserved amino acids by single lines. Amino acids essential for C127 cell transformation by the BPV E5 protein are shown in bold face.

receptor in the absence of the E5 protein or PDGF is variable and occasionally high in C127 cells (see Figure 5B). However, this basal level of phosphorylation does not appear to represent true activation because it is not accompanied by increased tyrosine kinase activity, cellular DNA synthesis, morphological transformation, or association with GAP. In contrast, the E5-mediated increase in tyrosine phosphorylation of the receptor precursor in C127 cells is observed reproducibly and correlates well with physiological measures of activation. Thus, we have focused on tyrosine phosphorylation of the receptor precursor in C127 cells because it is a less ambiguous measure of activation than is phosphorylation of the mature receptor. Nevertheless, several results suggest that E5 expression activates the mature as well as the immature form of the receptor in C127 cells, but that basal phosphorylation of the mature form may impair detection of further phosphorylation in response to the E5 protein. First, when basal phosphorylation of the mature receptor is low increased tyrosine phosphorylation in response to E5 expression is detectable (eg. Figures 2, 6, and 7). Second, the E5 protein causes increased apparent auto-phosphorylation of both receptor forms in an *in vitro* kinase assay (Figure 4A). Finally, the E5 protein causes association of both receptor forms with the GAP protein (L.Nilson, S.Bulfone and D.DiMaio, unpublished results). The lack of phosphorylation of the precursor form in FR3T3 cells may reflect differences in the kinetics of receptor maturation or E5 protein localization in these cells.

The effect of the E5 protein on the PDGF receptor is specific because the EGF receptor, which appears to be abundant in transformed FR3T3 cells, is not detectably tyrosine-phosphorylated in E5-transformed cells without the addition of ligand (Figure 1A). Our findings differ from those of Martin *et al.* (1989), who identified the EGF receptor as a potential target of the BPV E5 protein and showed that down regulation of the human EGF receptor was inhibited in E5-transformed mouse NIH3T3 cells. In our experiments, the E5 protein did not alter PDGF receptor half life or induce increased levels of PDGF receptors. In the experiments of Martin *et al.* (1989), the state of activation of the PDGF receptor was not determined, nor was receptor status assessed using an acute transformation system less prone to secondary events that may occur during establishment of stable transformants. However, we feel that the most important difference is that Martin *et al.* (1989) examined transfected, heterologous receptors whereas we studied endogenous receptors. It is possible that the E5 protein can influence the behavior of multiple different receptors, depending on the host cell, the abundance of the various receptors and E5 protein, and the species from which the receptor was derived. Activation of endogenous PDGF receptors in two rodent species as a consequence of either acute or stable expression of the E5 protein suggests that PDGF receptor activation is a normal response of fibroblasts to this protein. This notion is supported by our finding that infection of primary bovine dermal fibroblasts with BPV virions induces tyrosine phosphorylation of the PDGF receptor (L.Petti and D.DiMaio, unpublished results).

Several lines of evidence indicate that PDGF receptor activation plays a role in transformation by the E5 protein. The ability of the E5 protein to induce DNA synthesis in serum-starved cells demonstrates that it can functionally replace growth factors in this assay (Green and Lowenstein, 1987; Jaskulski *et al.*, 1987; Settleman *et al.*, 1989). Moreover, acute morphological transformation and receptor activation in C127 cells require a similar dose of the E5 gene. In cells susceptible to E5-mediated transformation, acute activation of the PDGF receptor by PDGF BB homodimer is sufficient to induce DNA synthesis; and sustained activation of the receptor by the v-sis protein is sufficient to induce stable growth transformation. Both PDGF BB homodimer and the v-*sis* gene product are thought to exert their mitogenic effects in fibroblasts primarily via the β type receptor (Heldin *et al.*, 1988; Hart *et al.*, 1988). This demonstrates that activation of the β receptor by its 'normal' ligands is sufficient to induce effects similar to those induced by the E5 protein.

On the basis of these results, we propose that in fibroblasts the E5 protein activates the PDGF receptor which in turn initiates a series of events that culminate in DNA synthesis and morphologic transformation. This model is strongly supported by the findings that PDGF receptor activation precedes DNA synthesis in the acute assay and that PDGF receptor activation is not a property common to FR3T3 and C127 cells stably transformed by other oncogenes. To test this model that the PDGF receptor plays a central role in transformation of fibroblasts by the E5 protein, we are determining the transforming activity of the E5 gene in cells lacking PDGF receptors and in derivative cells containing a transfected PDGF receptor gene.

The BPV E5 protein appears to activate both the mature and precursor forms of the PDGF receptor. In cells transformed by v-*sis*, an activated intracellular precursor of the PDGF receptor has been proposed to deliver the transforming signal (Huang *et al.*, 1984; Keating and Williams, 1988; Bejcek *et al.*, 1989; Fleming *et al.*, 1989), although cell surface localization may also be required (Johnsson *et al.*, 1985; Fleming *et al.*, 1989; Hannick and Donoghue, 1988). Further experiments are required to assess the contribution of specific PDGF receptor forms to transformation by the E5 protein.

Inspection of the amino acid sequences of the BPV E5 protein and PDGF itself revealed that these proteins share a short stretch of similar amino acid sequences (Figure 8). This similarity is also evident in the comparison between PDGF B and the deer papillomavirus E5 protein, which we have recently shown is capable of transforming C127 cells and activating both the precursor and mature forms of the PDGF receptor (Groff and Lancaster, 1985; R.Kulke and D.DiMaio, in preparation). Thus, the fibropapillomavirus E5 proteins and PDGF share structural as well as functional similarity, implying that constitutive activation of the receptor

Table I. Stimulation of cellular DNA synthesis by platelet-derived growth factor

Treatment	C127 cells	FR3T3 cells
None	6483	2942
10% serum	285 900	79 846
10 ng/ml PDGF	76 608	101 464

Incorporation of [^{3}H]thymidine into cellular DNA during a 2 h pulse after mock treatment or addition of growth factor. Average values of duplicate wells are shown.

may result from a direct physical interaction between the E5 protein and the PDGF receptor. This hypothesis is supported by genetic evidence showing that many of the specific amino acids required for efficient BPV E5-mediated transformation are located in the region of similarity with PDGF (Figure 8) (Horwitz *et al.*, 1988), and that v-*sis* transformation is inhibited by point mutations and carboxyl-terminal deletions that impinge upon this region (Giese *et al.*, 1987; Sauer *et al.*, 1988). Experiments are currently underway to determine whether the E5 protein and the PDGF receptor bind to one another and whether the region of sequence similarity between the E5 proteins and PDGF is important for this interaction and for receptor activation. Another membrane-associated transforming protein, polyomavirus middle T antigen, appears to act by binding to the product of the *src* proto-oncogene (Courtneidge and Smith, 1983) and activating its protein tyrosine kinase activity (Bolen *et al.*, 1984). However, unlike the *src* family of proteins, the PDGF receptor has a well-defined role in normal cell growth, namely the transmission of mitogenic signals from extracellular growth factors.

The ability of the fibropapillomaviruses to induce fibroblast proliferation in animals appears to reside in the E5 gene (reviewed by DiMaio, 1990). Our findings in cultured cells suggests that the abundance of PDGF receptors on fibroblasts may account for this tumorigenic potential *in vivo*. Many of the human papillomaviruses, which do not induce fibroblast proliferation, are predicted to encode short, hydrophobic E5 proteins with no apparent similarity to PDGF. It will be of interest to determine whether these proteins also interfere with growth regulatory pathways and whether such activities play a role in tumor induction by these viruses.

Materials and methods

Cell culture

Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (DME-10). BPV E5-transformed FR3T3 (FRC- series) and C127 (L1D- series) cells were established by infection with a recombinant retrovirus containing E5 and no other BPV genes (C.Leptak, B.Horwitz and D.DiMaio, manuscript in preparation). These cloned cell lines were morphologically transformed and expressed the E5 gene. Untransformed cells used as controls were FR3T3 (FRE2) and C127 (R7-3) cells which contained the retrovirus vector alone. Untransformed cells were passaged regularly at subconfluence.

Calcium phosphate-mediated DNA transfections were performed as described previously (DiMaio *et al.*, 1986) with 300 ng of *Bam*HI-digested pBPV-142.6 (wild type BPV DNA) and pE6fs2 [BPV DNA containing a frameshift mutation in ORF E6 (Neary and DiMaio, 1989)] or with 2 μg of intact plasmids containing the Harvey-*ras* (pHaSV), SV40 large T antigen (pCCW), polyoma virus middle T antigen (pPyMT), and v-*mos* (pmos) genes. Stable cell lines were established from individual or pooled foci 12–21 days after DNA transfer. No foci appeared on plates that were transfected with calf thymus DNA alone.

For most experiments, cells were grown to confluence in 10 cm plates and then incubated overnight in either serum-free medium (DME-0) or medium containing 0.1% fetal calf serum (DME-0.1) prior to preparation of extracts. For some experiments, starved cells were incubated for 15 to 20 min at 37°C in DME-0 or DME-0.1 containing either 10 ng/ml human PDGF (Collaborative Research, Inc.) or recombinant human PDGF B chain homodimer (PDGF Inc.) and 5–10 mg/ml bovine serum albumin (BSA), 100 ng/ml EGF (Sigma), or 10% fetal calf serum; untreated control cells were incubated with DME-0.1 alone or DME-0 containing 10 mg/ml BSA. The recombinant PDGF caused substantially more tyrosine phosphorylation of the PDGF receptor than did an equivalent amount of the other preparation.

Virus infections

Cells were infected with BPV/SV40 recombinant viruses as described (Settleman and DiMaio, 1988). Briefly, FR3T3 or C127 cells at 90% confluence in 6 cm dishes were infected at multiplicity of infection of ~1500 with either wild type Pava1 or Pava-E5d29 which contains a frameshift mutation in the E5 open reading frame (Settleman and DiMaio, 1988). After infection, the FR3T3 cells were incubated in DME-10 for another 48 h and then lysed for analysis. For the dose dependence experiment shown in Figure 6 C127 cells were mock infected or infected with the indicated amounts (in μl) of Pava1 or Pava-E5d29 virus (with each of these virus stocks, infection of a 60 mm plate with 100 μl corresponds to a multiplicity of about 1500). After 31 h in DME-2, extracts were prepared for analysis. For the time course shown in Figure 7, C127 cells were incubated in DME-0 for 15 h and then infected in parallel at a multiplicity of infection of about 1500 of wild type or E5 mutant virus in 24-well plates (for DNA synthesis assays) and in 60 mm dishes (for receptor activation assays). After infection, cells were maintained in DME-0 and harvested at various times after infection.

Antibodies

Rabbit anti-PDGF β receptor peptide sera, 77, 88 and 83 (Keating and Williams, 1987), were gifts from M.Keating and L.T.Williams, UCSF. Antisera 77 and 88 recognize extracellular and cytoplasmic epitopes, respectively. Antiserum 83, which specifically recognises the PDGF β receptor is directed against a cytoplasmic epitope that is preferentially recognized after the receptor binds PDGF (Keating *et al.*, 1988). However, in extracts of FR3T3 and C127 cells lysed in EBC buffer (see below), antiserum 83 appears to immunoprecipitate free and PDGF-bound forms of the receptor with comparable efficiency (data not shown). Rabbit anti-PDGF β receptor peptide antiserum PR4, which recognizes the carboxyl-terminal 13 amino acids of the receptor (Kypta *et al.*, 1990), was the gift of S.Courtneidge. An anti-phosphotyrosine monoclonal antibody, PY20 (ICN Biochemicals, Inc.), was used for immunoprecipitations. Rabbit anti-phosphotyrosine antibodies used for immunoblotting were raised against poly(Glu-P-Tyr-Ala) coupled to keyhole limpet hemagglutinin, affinity purified on a phosphotyramine column, and eluted with nitro-phenyl phosphate (Decker, 1984; Kamps and Sefton, 1988) and were a gift from D.Stern (Yale). Mouse monoclonal anti-phosphotyrosine antibody 4G10 was the gift of T.Roberts (Dana-Farber) and was also used for immunoblotting.

Immunoprecipitations

Immunoprecipitations were performed according to the method of DeCaprio *et al.* (1988) with some modifications. Cell monolayers were washed twice with cold phosphate buffered saline (PBS) containing 1 mM phenylmethylsulfonylfluoride (PMSF). The cells were then lysed with 1 ml (for 10 cm plates) or 0.5 ml (for 6 cm plates) of cold EBC buffer (50 mM Tris–HCl, pH 8.0; 120 mM NaCl; 0.5% Nonidet P-40; 1 mM PMSF; 0.1 mM sodium orthovanadate) for 20 min on ice. Lysate was cleared of nuclei and cell debris by centrifugation in a microcentrifuge for 5 min. Each supernatant was assayed for protein concentration using a Coomassie blue assay reagent (Pierce). Approximately 1 μl (1 μg) of PY20 (ICN), 0.75 μl of 83, 2 μl of 77 or 88, or 10-20 μl of PR4 antibody was added per ml of extract and the mixture was incubated on ice for 2–4 h. 75–100 μl of a 1:1 suspension of protein A–Sepharose (Pharmacia) in TBS–BSA (25 mM Tris–HCl, pH 8.0; 120 mM NaCl; 10% w/v BSA) was added, and the mixture was rotated for 30–60 min at 4°C. The protein A–Sepharose beads were collected by centrifugation and washed five times with 1 ml of cold NET-N buffer (20 mM Tris–HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5% Nonidet P-40; 1 mM PMSF). For blocking experiments, prior to addition of extract the PR4 antiserum was incubated with an excess of the peptide against which the antibody was raised and PY20 monoclonal antibody was incubated with 10 mM phosphotyrosine, phosphoserine, or phosphothreonine.

For denaturation prior to immunoprecipitation, extracts of L1D-1 cells and R7-3 cells in EBC buffer were each divided into four aliquots. One

was immunoprecipitated with non-immune rabbit serum, and a second was immunoprecipitated as above with antiserum 83. Sodium dodecyl sulfate (SDS) (final concentration 0.5%) was added to the other two aliquots which were boiled for 5 min and then chilled on ice. Cold EBC buffer was added to one of the denatured aliquots to adjust the final SDS concentration to 0.1%, and both denatured aliquots were immunoprecipitated with antiserum 83.

Endo H digestion and in vitro kinase assays

For endo H digestion, PDGF receptor was immunoprecipitated from L1D-1 cells using anti-PDGF receptor antibody 83. The immune complex was dissociated and eluted from the protein A−Sepharose beads by boiling in 30 μl of elution buffer (150 mM Tris−HCl, pH 6.8; 1% SDS) for 5 min. After the Sepharose beads were pelleted, the supernatant was divided into two 14 μl aliquots and 14 μl of 0.15 M sodium citrate, pH 5.5, was added to each. Endo-β-*N*-acetylglucosaminidase H (endo H, Boehringer Mannheim) was added to one of the aliquots at a final concentration of 10 mU/ml. Both samples were incubated at 37°C overnight and then resuspended in 30 μl of 2× protein sample buffer.

For the *in vitro* immune complex kinase assay, PR4 anti-PDGF receptor antibody was used to prepare immunoprecipitates from precleared extracts of serum-starved cells or cells incubated with 10 ng/ml PDGF BB homodimer (PDGF, Inc.). Extracts were prepared and kinase assays were performed as described in Kypta *et al.*, (1990). Briefly, immune complexes from ~300 μg of each extract were incubated in 20 μl of kinase buffer (20 mM HEPES, PH 7.5; 10 mM $MnCl_2$) containing 10 uCi [γ-^{32}P]ATP (Amersham) for 15 min at 4°C. The immunoprecipitates were washed three times with TBS (25 mM Tris−HCl, pH 8.0; 120mM NaCl) and eluted from *S. aureus* by boiling for 5 min in 50 μl kinase elution buffer [100 mM Tris−HCl, pH 7.6; 2% SDS; 1 mM dithiothriotol (DTT)]. An equal portion of each eluate was solubilized by boiling in protein sample buffer (150 mM Tris−HCl, pH 6.8; 2% SDS; 10% v/v glycerol; 0.02% bromophenol blue; 5% v/v 2-mercaptoethanol; 0.1 mM DTT) and analyzed by SDS−polyacrylamide gel electrophoresis and autoradiography. The remainder of the eluate was stored at −70C and used for phosphoamino acid analysis.

Phosphoamino acid analysis

0.5 ml of 50 mM ammonium bicarbonate, 0.1% SDS, 1 mM EDTA, 10 μg/ml BSA was added to 10% of the eluted *in vitro* kinase reaction products, which were then precipitated with trichloroacetic acid and subjected to partial acid hydrolysis as described by Stern *et al.* (1986). Phosphoamino acids were resolved by two-dimensional electrophoresis on a thin layer cellulose plate as described by Stern *et al.* (1988). Electrophoresis in the first dimension was at pH 1.9, 2.5 kV, for 15 min., and in the second dimension was at pH 3.5, 2.0 kV, for 10 min. Unlabeled phosphoserine, phosphothreonine, and phosphotyrosine standards were mixed with the samples and located by ninhydrin staining.

SDS−polyacrylamide gel electrophoresis and immunoblotting

Immunoprecipitates to be resolved directly by polyacrylamide gel electrophoresis were prepared from equivalent amounts of extracted protein (~100−760 μg in different experiments), resuspended in protein sample buffer, and solubilized by boiling. Samples were electrophoresed on discontinuous 7.5% acrylamide−0.17% bis acrylamide as described (Stern et al., 1986; Sefton *et al.*, 1978). Gels containing ^{35}S- or ^{32}P-labeled proteins were fixed in a staining solution of 0.03% Coomassie brilliant blue in 25% isopropanol, 10% glacial acetic acid, destained in 10% isopropanol, 10% glacial acetic acid and processed for autoradiography (for ^{32}P-labeled proteins) or fluorography using Amplify (Amersham) (for ^{35}S-labeled proteins). For Western blotting, gels were soaked for 5 min in transfer buffer (25 mM Tris; 192 mM glycine; 20% v/v methanol; 0.1% SDS) and transferred electrophoretically to nitrocellulose in the cold at 50 V, 400−600 mA overnight or at 100 V, 1 A for 2 h. Blots were stained with 10% Ponceau S (Sigma) to determine the migration of size markers. Phosphotyrosine immunoblots were processed using the method of Kamps and Sefton (1988). Briefly, blots were incubated in blocking buffer (5% BSA; 10 mM Tris−HCl, pH 7.4; 165 mM NaCl; 0.01% NaN_2) for 1−2 h and then reacted for 2 h at 22°C with 1−2 μg/ml of monoclonal antibody 4G10 or affinity-purified anti-phosphotyrosine antibody in filtered blocking buffer. Immunoblots were washed sequentially with rinsing buffer (10 mM Tris−HCl, pH 7.4; 165 mM NaCl) (twice), rinsing buffer containing 0.05% Triton X-100 (once), and rinsing buffer (twice). Blots were then incubated with filtered blocking buffer containing 1 uCi/ml [^{125}I]protein A (ICN, 50 μCi/μg), and washed as described above. The PDGF receptor immunoblot shown in Figure 4A was processed in a similar manner except that the blot was blocked in rinsing buffer containing 3% BSA, incubated with a 1:200 dilution of R4 antiserum in filtered blocking buffer, and washed throughout with rinsing buffer containing 0.2% NP40.

On the figures, the positions of co-electrophoresed markers are indicated by dashes, and the molecular weight of the proteins (in kd) is indicated.

Pulse−chase experiment

The pulse−chase experiment was performed essentially as described by Keating and Williams (1987). Confluent R7-3 and L1D-1 cell monolayers in 6 cm dishes were washed twice with PBS and preincubated for 40 min in serum- and methionine-free medium. Cells were then incubated in fresh serum- and methionine-free medium containing 0.3 mCi/ml [^{35}S]methionine-containing Tran^{35}S-label (ICN) for 15 min at 37°C. Cells were washed twice with PBS and either immediately lysed in EBC buffer or incubated in fresh DMEM containing 150 μg/ml of L-methionine and 120 μg/ml L-cysteine for various times before lysis. After immunoprecipitation with anti-PDGF receptor antiserum 77 and electrophoresis as described above, gels were subjected to fluorography.

DNA synthesis assays

Quiescent cells in 24 well plates were incubated for 2 h with 1.5 μCi/ml [^{3}H]thymidine at various times after virus infection. Duplicate wells were assayed for cold trichloroacetic acid-precipitable, hot perchloric acid soluble radioactivity as previously described (Settleman *et al.*, 1989). For the experiment shown in Table I, C127 cells were starved for 24 h prior to treatment, and then assayed 14.5 h after addition of serum or platelet-purified PDGF; FR3T3 cells were starved for 12 h and assayed 25 h after addition of serum or recombinant PDGF BB homodimer.

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