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The BPV-1 E5 protein, the 16 kDa membrane pore-forming protein and the PDGF receptor exist in a complex that is dependent on hydrophobic transmembrane interactions

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The E5 oncoprotein of bovine papillomavirus type 1 is a 44 amino acid, highly hydrophobic protein that induces the stable transformation of immortalized murine fibroblasts, presumably through its activation of growth factor receptors. Previous studies have shown that the E5 protein complexes with the 16 kDa (16k) pore-forming protein of vacuolar H^+-ATPases. This integral membrane protein is essential for the acidification and function of subcellular compartments that process growth factor receptors. Using an SV40 expression system in COS cells, we analyzed whether the E5-16k complexes bind additional cellular proteins, including growth factor receptors. These studies demonstrate that E5 binds to both the 16k protein and the PDGF receptor and that this tri-component complex can be isolated with antibodies specific for each protein. Importantly, the 16k protein bound to the PDGF receptor in the absence of E5, suggesting that E5 binds to the PDGF receptor via its interaction with the 16k protein. An E5 mutant lacking the hydrophilic carboxyl-terminal 14 amino acids retained binding to both 16k and the PDGF receptor, indicating that E5 binds to these proteins through its hydrophobic, membrane-associating domain. These studies reveal that hydrophobic, intramembrane interactions govern the association of E5, 16k and the PDGF receptor, suggesting a ligand-independent mechanism for receptor activation and a potential link between receptor signal transduction pathways and membrane pore activity.

Introduction

Papillomaviruses are small DNA viruses that induce the benign proliferation of infected epithelial cells. In some cases, such as in human cervical cancers, this viral-induced cellular proliferation can progress to malignancy (Orth, 1987; zur Hausen and Schneider, 1987). Similar to other DNA tumor virus-transforming proteins, papillomavirus oncoproteins alter the activities of cellular proteins that control cell growth and differentiation. For example, cellular immortalization induced by human papillomavirus types 16 or 18 is a consequence of the E6 and E7 oncoproteins binding to (and presumably inactivation of) the cellular tumor suppressor proteins p53 and Rb, respectively (Dyson *et al.*, 1989; Münger *et al.*, 1989; Werness *et al.*, 1990).

The primary transforming protein of bovine papillomavirus type 1 (BPV-1), when assayed on immortalized rodent cells, is the E5 protein (Dvoretzky *et al.*, 1980; Lowy *et al.*, 1980; DiMaio *et al.*, 1986; Groff and Lancaster, 1986; Schiller *et al.*, 1986; Schlegel *et al.*, 1986). Although consisting of only 44 amino acids, this protein induces the efficient transformation of murine fibroblast cell lines such as NIH3T3 and C127 in the absence of other viral genes. The E5 protein is highly hydrophobic and consists of two distinct domains: an amino-terminal region that is predicted to traverse the cell membrane and a 14 amino acid hydrophilic carboxyl-terminal domain, which contains two cysteine residues that mediate homodimer formation (Schlegel and Wade-Glass, 1987; Horwitz *et al.*, 1988). Several amino acids within this carboxyl-terminal region are conserved among the fibropapillomaviruses and appear to be essential for E5 transforming ability. In contrast, many conservative amino acid substitutions within the hydrophobic domain do not significantly alter E5 activity (Horwitz *et al.*, 1988, 1989). Nevertheless, the requirement for a glutamine residue at the center of this hydrophobic region (position 17) highlights the critical role of this domain in protein function (Goldstein *et al.*, 1992; Kulke *et al.*, 1992).

Biochemical studies indicate that E5 transforming activity of both BPV-1 and HPV-16 involves the activation of growth factor receptors (Martin *et al.*, 1989; Petti *et al.*, 1991; Leechanachai *et al.*, 1992; Pim *et al.*, 1992). Experiments by Martin *et al.* (1989) revealed that BPV-1 E5 expression led to the ligand-independent stimulation of the EGF and CSF-1 receptors. The increase in EGF receptor activity was accompanied by an increase in receptor half-life and a decrease in internalization of activated receptor. In addition, BPV-1 E5 transformation of mouse fibroblast cell lines was also shown to involve the specific stimulation of phosphorylation of the endogenous β-type receptor for the platelet derived growth factor (PDGF; Petti *et al.*, 1991). The specific activation of the PDGF receptor was further supported by recent data demonstrating stable complex formation between E5 and the receptor (Petti and DiMaio, 1992). A short region of sequence similarity between the carboxyl-terminal amino acids of E5 and a domain found in PDGF led to the speculation that E5 may be activating phosphorylation of the receptor by direct interaction with the receptor through this region of homology (Petti *et al.*, 1991). Supporting the suggestion that this region is important for interaction with cellular targets is the observation that several of the amino acids that are essential for E5 activity are conserved among the fibropapillomaviruses (Horwitz *et al.*, 1988).

The E5 oncoprotein has recently been shown to associate with the 16 kDa (16k), integral-membrane subunit of the proton-ATPase found in vacuoles (V-ATPases), clathrin-coated vesicles, lysosomes, endosomes and Golgi vesicles (Goldstein and Schlegel, 1990; Goldstein *et al.*, 1991). This enzyme is essential for the acidification of these intracellular compartments and may play an important role in protein sorting, processing and endocytosis at the plasma membrane

(Nelson and Taiz, 1989; Nelson and Nelson, 1990). Although the link between the activity of E5 and the function of the proton pump remains uncertain, mutagenesis studies revealed that complex formation between E5 and 16k appears necessary for efficient E5 cellular transformation. Several mutants lacking the glutamine residue in the center of the hydrophobic region (position 17) have both a reduced capacity to associate with the 16k protein and a decreased ability to transform murine fibroblast cell lines in focus formation assays (Goldstein *et al.*, 1992; unpublished data). Interestingly, the binding of E5 protein to 16k appears to be mediated entirely by intramembrane hydrophobic domains.

To determine whether E5 interacts with additional transmembrane hydrophobic domains (such as those present in growth factor receptors) or whether E5 was complexed simultaneously with several hydrophobic molecules, we performed co-precipitation studies in cells expressing E5, 16k and several membrane receptor molecules including PDGF receptor, EGF receptor, MHC and the α subunit of the interleukin-2 receptor. Our studies demonstrate that E5 is bound to both the 16k protein and the PDGF receptor and that this tri-component complex can be isolated with antibodies specific for each protein. We also show that the 16k protein binds to the PDGF receptor in the absence of E5, suggesting that E5 may be binding to the PDGF receptor via its interaction with the 16k protein. Perhaps even more important, the association of the 16k protein with PDGF receptor delineates the first physical association of a growth factor receptor with a component of the proton pump and may help to define the link between ligand-induced receptor activation and alterations in cellular ion fluxes. Finally, these studies have also used an E5 deletion mutant to demonstrate that the binding of E5 to 16k and the PDGF receptor is not dependent on its hydrophilic, carboxyl-terminal domain. Several potential mechanisms for E5-mediated activation of growth factor receptors are discussed.

Results

Co-immunoprecipitation of E5, 16k and the PDGF receptor

We have previously demonstrated in NIH3T3 and COS cells that epitope-tagged E5 proteins (HA1–E5) bound to the 16 kDa protein component of the vacuolar H^+-ATPase (Goldstein and Schlegel, 1990; Goldstein *et al.*, 1991). In addition, Petti and DiMaio (1992) have demonstrated complex formation between E5 and the β-type PDGF receptor. To determine the nature of E5 interaction with its cellular targets, the β-type PDGF receptor and the E5 genes were cloned into an SV40-based vector (pSVL) for expression in COS cells (Figure 1A). Following metabolic labelling with ^{35}S-methionine, overexpression of the 185 kDa PDGF receptor was demonstrated by immunoprecipitation with either an antibody that recognizes only the β-type receptor (lane 4) or one that recognizes both α- and β-type receptors (lane 5). No PDGF receptor was detected by either antibody in cells transfected with the parental plasmid vector (pSVL; lanes 1 and 2). In addition, no receptor was detected using monoclonal antibody, 12CA5, which was used for immunoprecipitating epitope-tagged E5 protein (lane 3; Goldstein and Schlegel, 1990).

To determine whether the PDGF receptor could associate with the E5 protein in this expression system, pSVL plasmids containing the PDGF receptor (pSVL-PR) and the HA1–E5 gene (pTA6) were used to co-transfect COS cells (Figure 1B). Following immunoprecipitation with antibodies to either HA1–E5 or the β-type receptor, samples of precipitated proteins were divided equally and electrophoretically separated

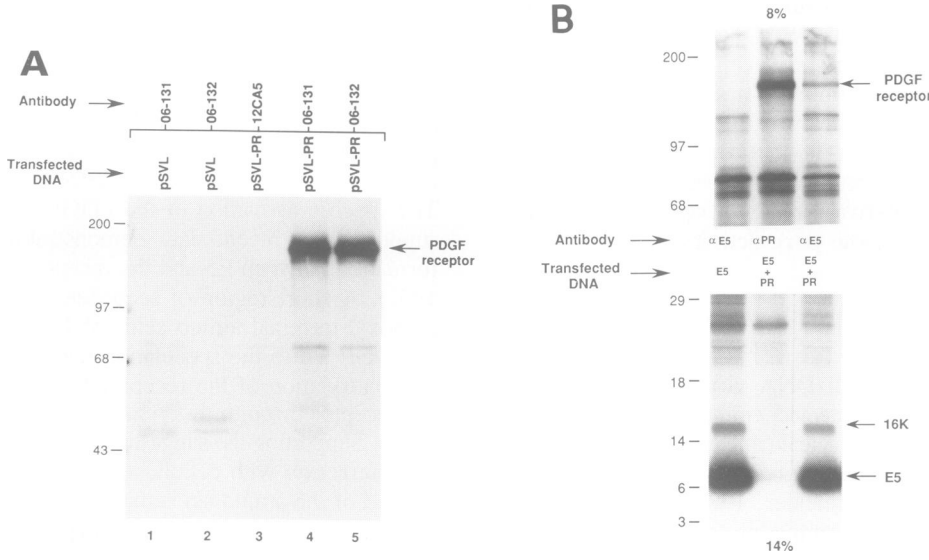

Fig. 1. The E5–16k complex binds to the PDGF receptor. (**A**) Overexpression of the β PDGF receptor in COS cells. Subconfluent monolayers of COS cells were transfected with the DNAs indicated above the lanes. 48 h post-transfection, cells were metabolically labelled with [^{35}S]methionine, extracted and immunoprecipitated with the antibodies shown. Precipitated proteins were electrophoretically separated on 8% SDS–polyacrylamide gels and visualized by fluorography. Position of the PDGF receptor is indicated on the right-hand side of the gel. The positions of molecular weight standards (in kDa) are indicated on the left-hand of the gel. (**B**) Co-precipitation of the PDGF receptor with the E5–16k complex. Extracts of COS cells transfected with DNAs indicated between the panels: E5, HA1–E5-expressing plasmid, pTA6 and PR, PDGF receptor-expressing plasmid, pSVL-PR, were precipitated with the antibodies shown (αE5, HA1-specific antibody, 12CA5; αPR, PDGF receptor polyclonal antiserum, 06-131). Samples of precipitated proteins were divided equally and electrophoretically separated on 8 (upper panel) and 14% (lower panel) SDS–polyacrylamide gels. The positions of PDGF receptor, 16k protein and the HA1–E5 protein are indicated to the right of the gel and the molecular weight standards to the left.

on both 8% and 14% SDS−polyacrylamide gels. Lane 1 of Figure 1B shows co-precipitation of HA1−E5 with endogenous 16k protein when cells were transfected with the E5-expressing plasmid. When cells were transfected with both E5 and PDGF receptor constructs, HA1−E5-specific antibodies (αE5) co-precipitated PDGF receptor as well as the endogenous 16k protein (lane 3). In addition, PDGF receptor antibodies (αPR) co-precipitated low levels of HA1−E5 protein (lane 2; seen only when overexposed). We consistently observed more co-precipitated PDGF receptor with E5-specific antibodies than E5 protein with PDGF receptor antibodies, which may indicate that the receptor antibodies preferentially recognize uncomplexed receptor or that fewer E5 molecules are in complex with the receptor. In addition, the mobility of the two PDGF receptor species that were immunoprecipitated directly with PDGF receptor antiserum or co-precipitated with epitope-tagged E5 protein is similar to previously published data indicating that the faster migrating species is the major intracellular precursor and the slower migrating species represents the mature, fully processed receptor (Keating and Williams, 1987).

Specificity of E5−transmembrane protein interactions

Since it was a possibility that overexpression might facilitate non-specific interactions between E5 and the PDGF receptor, the E5 protein was evaluated for binding to other highly expressed, membrane-associated proteins. COS cells transfected with the HA1−E5 plasmid were immunoprecipitated with either 12CA5 (αE5) or class I MHC antigen antibodies (Barnstable *et al.*, 1978; Figure 2). Although COS cells express high levels of the components of the MHC receptor (α-chain and β_2-microglobulin), neither component was co-precipitated with E5 antibody (Figure 2A). Similarly, anti-MHC antibodies did not co-precipitate E5 protein. In addition, an SV40 expression plasmid containing the Tac antigen (α chain of the interleukin-2 receptor; Cosson *et al.*, 1991) was co-transfected with the E5-expressing plasmid and analyzed by immunoprecipitation with E5 and Tac antibodies (Figure 2B). Whereas E5 and two forms of the Tac receptor were expressed at much higher levels (>20-fold) than the PDGF receptor, no co-precipitation of E5 with the Tac receptor was detected with either antibody, even when the fluorogram was dramatically overexposed (data not shown).

Martin *et al.* (1989) have previously shown that E5 stimulated the transforming activity of the overexpressed EGF receptor. Petti *et al.* (1991), however, failed to detect endogenous EGF receptor activation in E5 transformed cell lines. To determine whether the E5 protein binds to the endogenously expressed EGF receptor, COS cells were transfected with the HA1−E5-expressing plasmid or parent vector and immunoprecipitated with either HA1 epitope-E5 (αE5) or αEGF receptor antibodies (Figure 2C). We have observed that COS cells, although expressing undetectable levels of PDGF receptors, express EGF receptor as shown by immunoprecipitation (Figure 2, lane 4) and immunofluorescence using a monoclonal antibody directed against the EGF receptor (unpublished data). In contrast to the data for the PDGF receptor (Figure 1B and lane 1 of Figure 2C), no co-precipitation of the EGF receptor with the E5 protein was detected (lanes 3 and 4). The studies with MHC, Tac antigen and the EGF receptor therefore suggest that the HA1−E5 protein displays a specific binding to the PDGF receptor. It

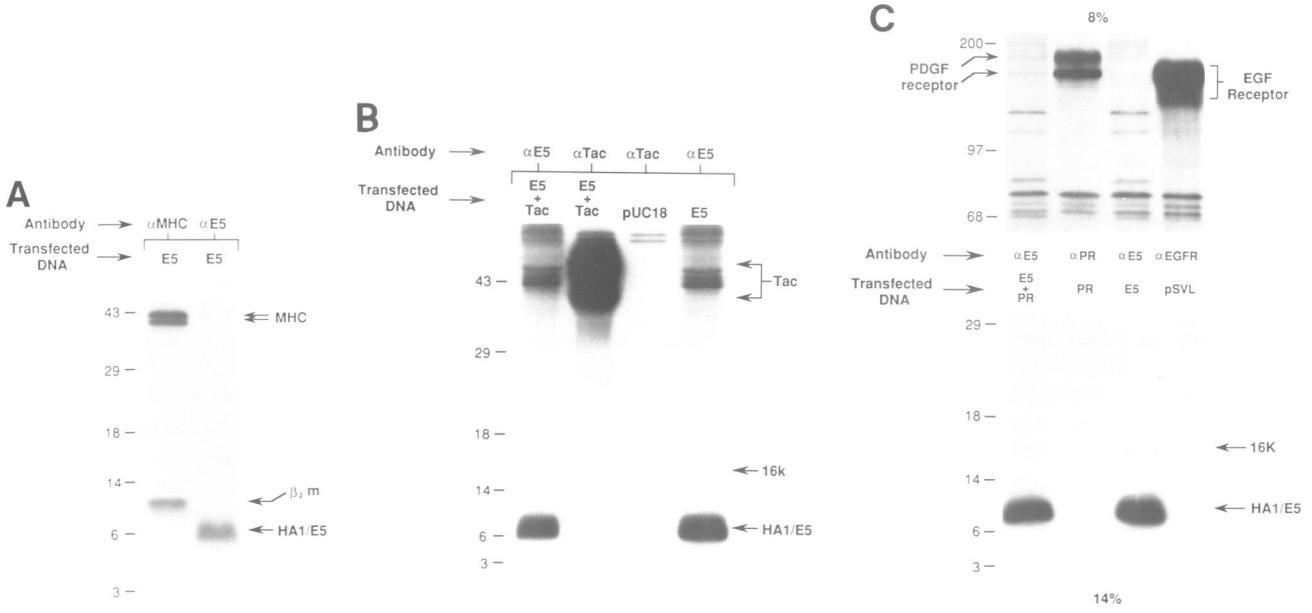

Fig. 2. The E5−16k complex fails to bind other membrane receptors. **(A)** E5 does not bind to the highly expressed MHC class I molecules. Immunoprecipitation of COS cells transfected with the HA1−E5-expressing plasmid (pTA6) and immunoprecipitation with either anti-MHC class I antibodies (W6/32; Barnstable *et al.*, 1978) or 12CA5 (αE5). Longer exposures revealed only co-precipitated 16k protein and no co-precipitated MHC proteins with 12CA5 antibody and also no co-precipitated E5 with anti-MHC antibodies. Proteins precipitated with 12CA5 that appear to have similar migration to MHC on 14% gels (lane 2), did not co-migrate with MHC on an 8% gel (data not shown). **(B)** E5 does not bind to the α chain of the IL-2 receptor. COS cells transfected with Tac antigen and HA1−E5-expressing plasmids were extracted and immunoprecipitated with anti-Tac antibodies (αTac; Cosson *et al.*, 1991) or HA1−E5 antibodies (αE5) and precipitated proteins were separated on a 14% SDS−polyacrylamide gel. The positions of HA1−E5 and the two forms of the Tac proteins are indicated on the right-hand side of the gel. Positions of molecular weight markers are indicated on the left-hand side. The autoradiogram was exposed for 4 h. **(C)** E5 does not bind to the endogenously expressed EGF receptor. COS cells transfected with the DNAs indicated between the panels were immunoprecipitated with the indicated antibodies. Samples of precipitated proteins were divided equally and electrophoretically separated on 8 (upper panel) and 14% (lower panel) SDS−polyacrylamide gels. The positions of EGF receptor, 16k protein and the HA1−E5 protein are indicated to the right of the gel and the PDGF receptor and molecular weight standards to the left.

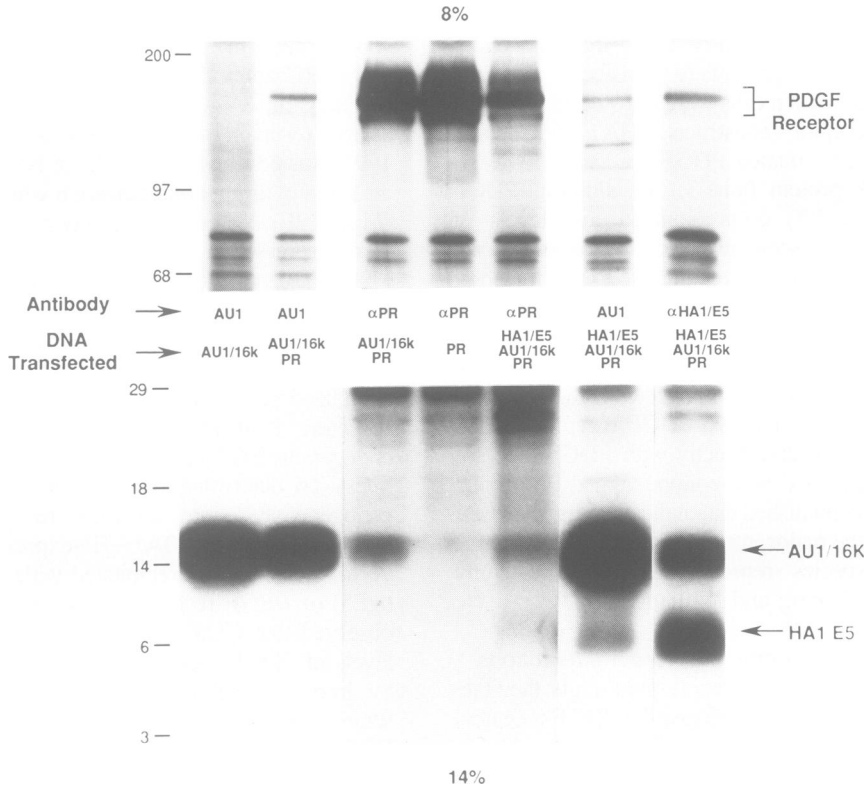

Fig. 3. Immunoprecipitation of the E5−16k−PDGF receptor complex with antibodies specific for each component. COS cells were transfected with DNAs expressing the HA1−E5, AU1−16k and PDGF receptor (PR) proteins as indicated. 48 h following transfection, cells were metabolically labelled and immunoprecipitated with the antibodies shown. Samples of precipitated proteins were divided equally and electrophoretically separated on either an 8 (upper panel) or 14% (lower panel) SDS−polyacrylamide gel. The positions of PDGF receptor, AU1−16k protein and the HA1−E5 protein are indicated on the right-hand side and the positions of molecular weight standards on the left-hand side.

is important to note, however, that unlike the PDGF receptor, the EGF receptor in these experiments was not overexpressed using the expression vector and that failure to detect E5 binding may in fact be due to lower levels of receptor on an individual cell basis.

Overexpression of the 16k protein and association with PDGF receptor

The co-immunoprecipitation experiments described above demonstrate complex formation between E5, 16k and the PDGF receptor. Antibodies to the epitope-tagged E5 protein co-precipitated both endogenously expressed 16k protein and overexpressed PDGF receptor. In addition, antibodies to the PDGF receptor co-precipitated overexpressed PDGF receptor and low levels of the overexpressed E5 protein. However, virtually no endogenously expressed 16k protein was detected in immune complexes when anti-PDGF receptor antibodies were used (Figure 1B). This may be due to low levels of labelled 16k present in the immunoprecipitates. To investigate further the nature of the complex formation between the PDGF receptor, E5 and the 16k protein, a series of immunoprecipitations were performed on transfected COS cells overexpressing all three proteins. For overexpression of the 16k protein, a previously described cDNA of the bovine 16k gene (Goldstein *et al.*, 1991) was cloned into pSVL. To circumvent the lack of high affinity antibodies to the 16k protein, we chose to epitope-tag the 16k protein using a six amino acid sequence from the BPV-1 L1 protein. This sequence is recognized by the monoclonal antibody AU1 (Lim *et al.*, 1990) and was shown recently to recognize fusion proteins containing this six amino acid sequence (unpublished data). Oligonucleotides corresponding to the AU1 epitope were added to the amino-terminal end of the 16k gene using PCR techniques. As detected by immunoprecipitation using AU1 antibodies, AU1-tagged 16k protein can be overexpressed in transiently transfected COS cells (Figure 3, lane 1).

COS cells were triple transfected with constructs expressing PDGF receptor, HA1−E5 and AU1−16k proteins. Figure 3 demonstrates that antibodies directed against any of the three components of the complex precipitated all three proteins. Thus, antibodies against either E5 (lane 7), PDGF receptor (lane 5) or 16k (lane 6) can precipitate the three expressed proteins. Unexpectedly, the AU1−16k protein was found to complex with PDGF receptor in the absence of E5, and the presence of both of these proteins in a complex was verified on 8 and 14% gels using antibodies to either protein (lanes 2 and 3). These findings further confirm that E5 forms a complex with both 16k and PDGF receptor and that the PDGF receptor can interact with the 16k protein in the absence of E5. No binding of the 16k protein to either overexpressed Tac antigen or MHC was detected indicating that 16k, like E5, shows specificity for binding to transmembrane proteins (data not shown). Very low levels of the 16k protein were also detected when only PDGF receptor was used in transfections, suggesting complex formation between the receptor and endogenous 16k protein (lane 4). However, definitive association between the 16k protein and the PDGF receptor can be demonstrated best when the 16k protein is overexpressed.

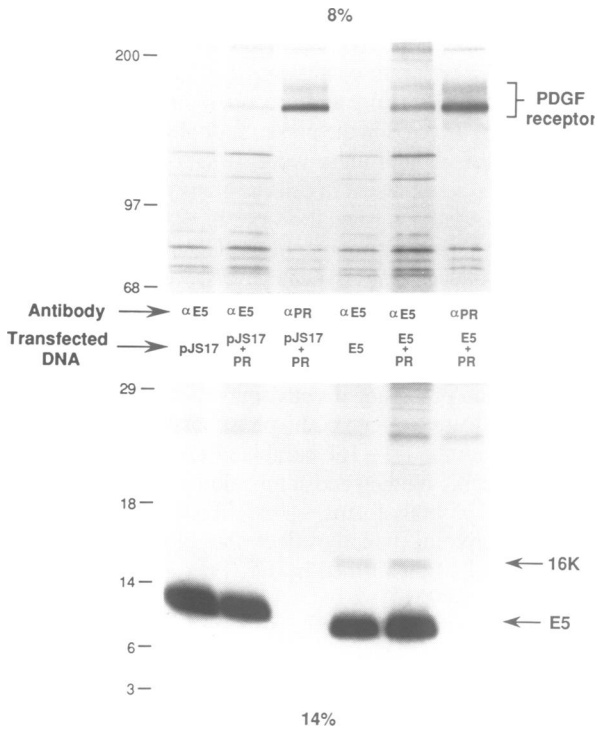

Fig. 4. The E5 mutant that is defective for 16k binding is also defective for PDGF receptor binding. COS cells were transfected with plasmid pJS17, which expresses an HA1−E5 protein containing a glycine substitution at position 17, in the presence (lanes 2 and 3) and absence (lane 1) of the PDGF receptor plasmid and are compared with the wild type HA1−E5 expression and association of 16k and PDGF receptor (right-hand three lanes). Extracts were immunoprecipitated with the indicated antibodies and analyzed for the presence of HA1−E5, 16k and the PDGF receptor. Precipitated proteins are indicated on the right-hand side of the gel.

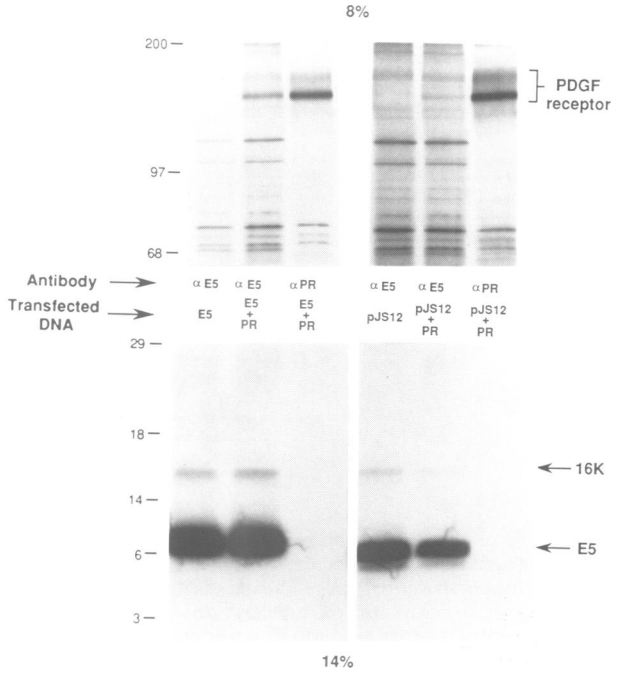

Fig. 5. The E5 hydrophobic domain is sufficient for 16k or PDGF receptor binding. COS cells were transfected with plasmid pJS12, which expresses the truncated HA1−E5 protein, in the presence (lanes 2 and 3) and absence (lane 1) of the PDGF receptor plasmid and are compared with the wild type HA1−E5 protein for the ability to associate with the 16k and PDGF receptor proteins. Extracts were immunoprecipitated with the antibodies indicated. Precipitated proteins are shown on the right-hand side of the gel.

An E5 mutant defective for association with 16k binds less PDGF receptor

Previous studies have demonstrated that substitution of the glutamine residue in the center of the E5 hydrophobic region (position 17) for a glycine residue significantly decreased binding to the 16k protein. To determine whether this mutation had a similar effect on the ability of the E5 protein to associate with the PDGF receptor, an HA1−E5 gene encoding a glycine at position 17 was introduced into the pSVL vector and used with the PDGF receptor plasmid to co-transfect COS cells (Figure 4). Immunoprecipitated proteins were separated by SDS−PAGE on 8 and 14% polyacrylamide gels and analyzed by fluorography. As demonstrated previously (Goldstein and Schlegel, 1990; Goldstein *et al.*, 1992), the mutated E5 protein co-precipitated significantly less 16k protein than wild type E5 (compare lanes 1 and 2 with lanes 4 and 5). The upper half of Figure 4 (8% gel) shows that the HA1−E5 antibody (12CA5) co-precipitated a decreased amount of the PDGF receptor when the receptor construct was co-transfected with the mutated E5 DNA (pJS17) as compared with the wild type HA1−E5 protein (compare lane 2 with 5) even though equivalent levels of mutated E5 protein were precipitated. As determined by densitometric scanning, the levels of both 16k and PDGF receptor proteins co-precipitated by the mutated E5 protein were comparably decreased to 20−25% of that co-precipitated by the wild type protein. This result indicates that the glutamine residue in the E5 hydrophobic segment, in addition to being necessary for 16k binding, also contributes (either directly or indirectly) to the co-precipitation of the PDGF receptor.

The hydrophobic domain of E5 is sufficient for 16k and PDGF receptor binding

On the basis of amino acid sequence similarity between the carboxyl-terminus of E5 and a region of PDGF growth factor, Petti *et al.* (1991) speculated that the E5 protein may be acting as a ligand for the PDGF receptor. To determine if the carboxyl-terminus of E5 was responsible for binding the PDGF receptor, an HA1−E5 truncation mutant was constructed that lacked the entire 14 amino acid segment containing the homology to PDGF. Thus, this truncated E5 molecule consisted only of a hydrophobic membrane-associating segment with an amino-terminal HA1 epitope. Extracts prepared from cells transfected with the mutant HA1−E5 gene (pJS12) and PDGF receptor DNAs were immunoprecipitated with epitope-E5 antibodies (Figure 5). As predicted, an HA1−E5 protein of slightly faster mobility was detected on a 14% polyacrylamide gel (lanes 4 and 5). However, despite the complete absence of the carboxyl-terminus, this E5 mutant bound 16k protein as efficiently as wild type E5 protein (lanes 4 and 5). Furthermore, detectable levels of PDGF receptor were observed when these same immunoprecipitated proteins were separated on 8% polyacrylamide gels (lane 5). When corrected for the lower levels of mutant E5 expressed by this plasmid, the levels of PDGF receptor co-precipitated with the mutant protein are comparable to that co-precipitated with the wild type E5. These results indicate that an E5 mutant lacking the carboxyl-terminal sequences retained the ability to associate with both 16k and the PDGF receptor.

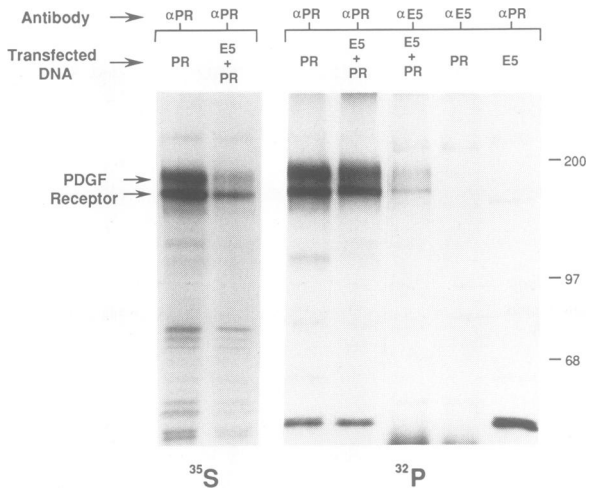

Fig. 6. The E5 protein stimulates the phosphorylation of the overexpressed precursor and mature PDGF receptor forms. COS cells were transfected with the DNAs indicated above (E5, HA1−E5-expressing plasmid, pTA6; PR, PDGF receptor-expressing plasmid, pSVL-PR) and either metabolically labelled with [^{35}S]methionine (left-most two lanes) for 4 h before immunoprecipitation or precipitated directly without metabolic labelling and used for *in vitro* kinase assays (right-hand five lanes). Metabolically labelled proteins were precipitated with the antibodies indicated above (αE5, HA1-specific antibody, 12CA5; αPR, PDGF receptor polyclonal antiserum, 06-131) and were electrophoretically separated on a 7.5% SDS−polyacrylamide gel and visualized by fluorography. Unlabelled, precipitated proteins were added to kinase reaction buffer containing γ-labelled ^{32}P as described in the Materials and methods, and were separated on a 7.5% SDS−polyacrylamide gel and dried down directly following electrophoresis. The first two lanes of the right-hand panel (^{32}P lanes) were exposed for 8 h at at room temperature without an enhancing screen, whereas the last three lanes were exposed for 21 h under the same conditions. Positions of PDGF receptor proteins are indicated on the left-hand side of the gel and molecular weight standards are shown on the right-hand side.

E5 stimulates autophosphorylation of overexpressed PDGF receptor in COS cells

To investigate whether the overexpression of the receptor and the E5 protein in COS cells mimics the biochemical response of the PDGF receptor to E5 expression (Petti *et al.*, 1991), the protein kinase activity associated with the receptor was analyzed in COS cells expressing either the receptor alone or the receptor together with the E5 protein (Figure 6). In the presence or absence of serum, the total levels of precursor and mature forms of the PDGF receptor expressed in COS cells, as determined by metabolic labelling with [^{35}S]methionine and immunoprecipitation with anti-PDGF receptor antibodies, varied significantly depending on whether or not the E5 plasmid was co-transfected (Figure 7; lanes 1 and 2). A 4- to 5-fold decrease of receptor synthesis was consistently observed when PDGF receptor and HA1−E5 plasmids were used in co-transfections as compared with when PDGF receptor DNA was transfected alone. This difference may be due to either the presence of two DNAs in transfections or to the possibility that the E5 expression is causing the activation and subsequent down-regulation of the receptor.

Anti-PDGF receptor immunoprecipitations of extracts of unlabelled, transfected COS cells were done in parallel and assayed for autophosphorylation activity by adding [γ-^{32}P]ATP to the immune complexes in a kinase reaction. Phosphorylated proteins were detected by gel electrophoresis and autoradiography. In the presence of serum, the levels of phosphorylated PDGF receptor reflected the total levels of receptor as measured by ^{35}S-labelling (data not shown). However, in low serum conditions (0.5% FBS), equal levels of phosphorylated mature and precursor forms of the PDGF receptor were detected in the presence or absence of E5 (lanes 3 and 4), even though there were 4- to 5-fold lower levels of receptor protein present in cells co-transfected with E5 and receptor DNAs (lanes 1 and 2). Since the labelling conditions used to measure the amount of PDGF receptor may not necessarily reflect the steady-state levels, we cannot conclude whether the decrease in levels upon E5 expression are due to a decrease in receptor expression or a decrease in receptor half-life. Nevertheless, phosphorylated mature and precursor forms were co-precipitated with antibodies to the HA1−E5 protein, indicating that both phosphorylated forms are associated with the E5−16k complex (lane 5). These results suggest a specific binding and stimulation of autophosphorylation activity of both forms of the PDGF receptor by the HA1−E5 protein, which reflect the biochemical effects induced in cells transformed by E5 (Petti *et al.*, 1991; Petti and DiMaio, 1992).

Discussion

Activation of growth factor receptors appears to be a critical event in E5-induced transformation. For instance, Martin *et al.* (1989) showed that E5 stimulates the phosphorylation of the EGF and CSF-1 receptors in a ligand-independent fashion. The authors observed a decrease in internalization of activated EGF receptors and suggested that E5 may cause activation of normal growth factor receptors in an indirect fashion, specifically by inhibition of internalization of occupied receptors. In a separate study, Petti *et al.* (1991) later demonstrated that E5 stimulates the constitutive activation of endogenous β-type receptor for PDGF. However, no activation of EGF receptor was measured and there were no apparent alterations in either PDGF receptor half-life or receptor levels. Based on these studies and a limited homology between carboxyl-terminal amino acids that are conserved among the fibropapillomaviruses and a short domain on PDGF, these authors proposed that E5 stimulates the activity of the PDGF receptor by binding directly to it as a ligand.

Supporting the finding that E5 stimulates PDGF receptor, Petti and DiMaio (1992) have demonstrated complex formation between this receptor and E5. We have also demonstrated the complex formation of the E5 protein with the PDGF receptor using antibodies directed against either protein and have furthermore demonstrated by co-precipitation that the 16k component of the vacuolar proton pump is present in complexes of E5 and the receptor. Analysis of an E5 mutant revealed that the glutamine residue in the hydrophobic region of E5, previously shown to be necessary for 16k binding, was also necessary for optimal co-precipitation of PDGF receptor. Most surprisingly, an E5 mutant lacking the 14 carboxyl-terminal amino acids (a region postulated to be homologous to PDGF) retained the ability to associate with the PDGF receptor. This mutant also was capable of binding to the 16k protein. These data suggest that E5 binding to the PDGF receptor may be mediated either directly through its hydrophobic sequences or indirectly through its association with the 16k protein.

Several experimental findings now suggest that the E5

carboxyl-terminus does not act as a PDGF ligand. (i) An E5 truncation mutant retains binding to the PDGF receptor, even in the absence of PDGF-like sequences. It would seem highly unlikely that E5 could possess two distinct sites for PDGF receptor interaction. It is more likely that either the E5 carboxyl-terminus functions as a structural domain essential for homo-dimerization or for binding to a yet unidentified cellular protein. Nevertheless, since our studies involved the overexpression of E5 and the PDGF receptor, we cannot completely rule out the possibility that the E5 carboxyl-terminus has a more direct role in activating endogenously expressed receptors in E5 transformed cells. (ii) The limited homology between E5 and PDGF consists of only four amino acids, two of which are the cysteine residues that mediate E5 homodimer formation. One of the remaining two residues (histidine at position 34), when changed to several different amino acids by site directed mutagenesis, had no marked effect on E5 transforming ability (Horwitz *et al.*, 1988). (iii) Amino acids in PDGF-B that are critical for receptor binding map to distinct sites unrelated to the E5 carboxyl-terminus (Östman *et al.*, 1991). In addition, one of the cysteine residues of PDGF that is suspected to be homologous to E5 is dispensable for the biological activity of v-*sis*, the oncogene of simian sarcoma virus (Sauer and Donoghue, 1988; Giese *et al.*, 1990). (iv) E5 can transform mouse keratinocytes that apparently lack PDGF receptor, but contain abundant EGF receptor (Leptak *et al.*, 1991). It is unlikely that the E5 carboxyl-terminus can act as a ligand for both EGF and PDGF receptors.

Our studies clearly define the E5 transmembrane domain as being sufficient for binding to the PDGF receptor. Since E5 did not bind to the other transmembrane receptors tested, our studies also argue for specificity in binding to the PDGF receptor. Several lines of evidence indicate that receptor transmembrane domains exhibit critical regulatory roles in addition to functioning as membrane anchors. For instance, activation of the erythropoeitin receptor by Friend spleen focus-forming virus involves the erythropoeitin receptor transmembrane domain (Zon *et al.*, 1992). Furthermore, previous studies with the *neu* proto-oncogene indicate that transmembrane sequences are essential for the transforming potential of growth factor receptors. A single valine to glutamic acid residue substitution within the transmembrane sequences leads to protein tyrosine kinase activity and signal transduction (Cao *et al.*, 1992). Presumably, this mutation stimulates receptor dimerization, an event critical for receptor activation.

Cumulative data favor at least two novel mechanisms for E5-induced growth factor receptor activation: (i) Homodimers of E5 act to dimerize and activate growth factor receptors. Covalent and non-covalent dimerization of PDGF receptors and other growth factor receptors has been shown to be a hallmark of receptor-mediated cellular responses (Ullrich and Schlessinger, 1990; Li and Schlessinger, 1991). Since E5 is present predominantly as a dimer in transformed cells, the binding of E5−16k to the PDGF receptor may facilitate receptor dimerization and consequent activation. The activation of receptors by antibody-induced cross-linking has been well described and illustrates that there are ligand-independent mechanisms for activating receptors (Veillette, *et al.*, 1989; Eiseman and Bolen, 1992). Interestingly, E5 molecules that have been selectively mutated at the cysteine residues cannot dimerize and are incompetent for cell transformation. Thus, the E5 carboxyl-terminus may fulfil a structural role for homodimer formation. (ii) E5 binding to the 16k protein may modulate its activity as a proton pump. 16k is a strongly hydrophobic, integral membrane protein with four membrane-spanning domains that may have very diverse cellular functions. The protein is ubiquitous and has been isolated from many different subcellular compartments, such as clathrin-coated vesicles, lysosomes, endosomes, Golgi vesicles, synaptic vesicles, the plasma membrane of certain cell types and gap junction-like membrane complexes (Manolson *et al.*, 1985; Uchida *et al.*, 1985; Randall and Sze, 1986; Xie and Stone, 1986; Arai *et al.*, 1987; Sun *et al.*, 1987; Mandel *et al.*, 1988; Leitch and Finbow, 1990; Nelson *et al.*, 1992). Since several of these structures are involved in the processing and transport of growth factor receptors, several mechanisms for receptor activation by complex formation with the E5−16k proteins can be envisioned. For instance, the inhibition of down-regulation of the EGF receptor induced by E5 (Martin *et al.*, 1989) may be due to E5 binding and inhibiting the proton translocating ability of the proton pump in endosomes and lysosomes. A subsequent increase in local pH might prevent receptor down-regulation and cause receptors to be reshuttled to the cell surface, thereby inducing receptor signal amplification. Waters *et al.* (1992) have recently reported that the effect of E5 on EGF receptor-expressing NIH3T3 cells is to stabilize intact EGF−receptor complexes by causing the ligand to bind more tightly to its receptor. In addition, they reported an ~30% decrease in rate constants for degradation of EGF−receptor complexes. The decrease in receptor degradation could potentially reflect E5-induced alterations in endosome and lysosome acidification. Petti *et al.* (1991), however, did not find an increase in PDGF receptor half-life or decrease in receptor down-regulation, suggesting the possibility that E5 may be eliciting pleitropic effects on different growth factor receptors.

Although the EGF receptor is expressed endogenously in COS cells, it did not interact with the E5 protein when it was overexpressed, suggesting that the EGF receptor may not be a target for E5. These results are compatible with previous findings of Petti *et al.* (1991) who have shown that E5 activates the PDGF receptor, but not the EGF receptor, in mouse fibroblasts. However, our binding assays are not compatible with the observation that E5 can transform mouse keratinocytes that apparently lack the PDGF receptor but rather express the EGF receptor (Leptak *et al.*, 1991). It is, however, possible that E5 may transform mouse keratinocytes via binding to alternative receptor complexes (such as the KGF receptor) or that the current binding assays in COS cells are either too stringent or insensitive to detect significant E5−EGF receptor interactions.

The activation of cellular growth factor receptors by the BPV E5 protein may represent a common mechanism by which papillomaviruses induce cell proliferation. Although showing little amino acid homology to the BPV E5 protein, the HPV16 E5 protein is a small and highly hydrophobic protein that appears to stimulate cellular growth by interacting, at least functionally, with the EGF receptor (Leechanachai *et al.*, 1992; Pim *et al.*, 1992). E5 proteins, therefore, appear to effect biological activities through specific hydrophobic interactions within cell membranes. Further characterization of the nature of such protein interactions, as well as identifying additional cellular targets, may provide new insights into the mechanism of receptor activation and signal transduction.

Materials and methods

Cell culture

COS-1 cells (gift of J.Brady) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Plasmid construction

The plasmid pSVL was obtained from Pharmacia Inc. (Piscataway, NJ) and was used to express high levels of protein in COS-1 cells using the SV40 late promoter. PCR techniques were used to construct the E5- and 16k-containing pSVL plasmids. To generate the plasmid pTA6, the HA1–E5 open reading frame (ORF) was amplified from the plasmid pPL15 (Goldstein and Schlegel, 1990) using a 5′ oligonucleotide containing an *Xho*I site and a 3′ oligonucleotide containing a *Bam*HI site. The resulting product was cleaved by restriction enzymes *Xho*I and *Bam*HI and cloned into the unique *Xho*I and *Bam*HI sites of pSVL. pJS17 was generated using the same oligonucleotides that were used to generate plasmid pTA6. However, to generate an HA1–E5 fragment containing a glutamine to glycine substitution at position 17 in the E5 ORF, plasmid pPava 17G was used as a template for PCR amplification (Goldstein *et al.*, 1992). pJS12 was generated by using the same 5′ oligonucleotide as that used to construct pTA6. The 3′ oligonucleotide, however, was complementary to sequences internal to the E5 ORF such that amplification resulted in the generation of an HA1–E5 ORF truncated after the valine residue at position 30. This oligo also contained a *Bam*HI site such that the amplified fragment could be cleaved with *Xho*I and *Bam*HI and cloned into the same sites of pSVL. pTA11 was constructed by PCR amplification of the 16k ORF from plasmid pIC-16k. The 5′ oligonucleotide used in this reaction contained an *Xho*I site, the nucleotide sequence corresponding to the AU1 epitope (underlined sequence), the consensus Kozak sequence (Kozak, 1987) and 12 nucleotides corresponding to the first four amino acids of the 16k ORF (bold type) (TTACATCTCGAGGCCACCATG*GACACCTATCGCTATATA***TCCGAG-GCCAAG**). The 3′ oligonucleotide was complementary to the carboxyl-terminus of the 16k ORF and contained a *Bam*HI site. The amplified AU1–16k fragment was cleaved with *Bam*HI and *Xho*I and ligated into the same sites of pSVL.

The plasmid pSVL-PR was generated by doing a two-step cloning procedure. The cDNA to the β-PDGF receptor (gift of M.Heidaran and S.Aaronson) was first subcloned into the *Eco*RI sites of vector pIC (gift of M.Finbow) to generate the plasmid pIC-PR. pIC-PR was cleaved with *Xba*I at a site in the polylinker of pIC and at nucleotide 4291 of the PDGF receptor cDNA. This excized the entire PDGF receptor ORF, which was then ligated into the unique *Xba*I site of plasmid pSVL to generate pSVL-PR.

DNA transfer and immunoprecipitations

DNA transfections were carried out by the $CaPO_4$–DNA co-precipitation method of Graham and van der Eb (1973). 10 μg of DNA (1 μg test plasmid plus 9 μg pUC18 as carrier) in 500 μl HEPES buffered saline (HBS) were incubated for 30 min following the addition of 50 μl of 1.25 M $CaCl_2$ and subsequently added to 5 ml of DMEM on 100 mm plates of COS cells at 40% confluency. Following an overnight incubation at 37°C, cells were washed once with phosphate-buffered saline (PBS) and glycerol-shocked for 1 min with 2 ml of 15% glycerol in 1×HBS. Cells were then washed three times with PBS and 10 ml of DMEM were added. Transfected cells were incubated for 48 h, after which time they were prepared for metabolic labelling by incubating cells in methionine- and cysteine-free DMEM for 1 h. Cells were labelled with 250 μCi/ml Expre^{35}S^{35}S (NEN), which is a mixture of ^{35}S-labelled methionine (~90%) and cysteine (~10%) for 4 h. Cells were then washed once with PBS and extracts were prepared using a modified radioimmunoprecipitation (RIPA) buffer (20 mM MOPS, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% deoxycholate and 0.1% SDS, pH 7.0) containing 1 μg/ml aprotinin (Boehringer Mannheim) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) as described (Goldstein and Schlegel, 1990). Immunoprecipitations were carried out by adding 4 μl of 12CA5 ascites fluid (gift of I.Wilson), 5 μl of AU1 ascites fluid (gift of B.Jenson), 20 μl of β-type PDGF receptor-specific, rabbit polyclonal serum 06–131 or 06–132 (Upstate Biotechnology, Inc., Lake Placid, NY), 5 μl anti-EGF receptor ascites (RPN-513; Amersham) or 100 μl anti-MHC class I supernatant (antibody W6/32; Barnstable *et al.*, 1978), plus 50 μl of a 1:1 suspension of protein A–Sepharose CL-4B beads (Pharmacia) in PBS to 1 ml extracts of transfected cells. The Tac antigen was precipitated by first incubating 60 μl of protein A–Sepharose beads with 1 ml of supernatant of a hybridoma expressing anti-Tac antibodies (7G7; generous gift of P.Cosson and R.Klausner) and then adding the beads to extracts of COS cells. Following a 1.5 h incubation at 4°C, immune complexes were washed 3–5 times with 1 ml extraction buffer by vortexing for 3–5 min. Sepharose beads were resuspended in 80 μl sample buffer with β-mercaptoethanol, heated at 100°C for 4 min and separated by electrophoresis on either 8 or 14% SDS–polyacrylamide gels. Gels were then fixed with glacial acetic acid–methanol, treated with Enlightening (NEN), dried and exposed to Kodak XAR-5 film for 1–4 days at −70°C, except where indicated.

In vitro kinase assays

Immunoprecipitated PDGF receptor was analyzed *in vitro* for kinase activity essentially as follows: 100 mm plates of COS cells were lysed with 1 ml of RIPA buffer containing PMSF, 1 μg/ml aprotinin, 1 mM sodium orthovanadate and 5 mM sodium fluoride. Immunoprecipitations were performed as described above. Protein A–Sepharose beads were washed three times with 1 ml RIPA, twice with 1% Triton X-100 (Sigma), 50 mM Tris–HCl, pH 8, 2 mM EDTA, 150 mM NaCl, 1 mM sodium orthovanadate and 5 mM sodium fluoride, and once with 50 mM Tris, pH 7.5, 0.05% Triton X-100 and 1 μg/ml aprotinin. Reactions were carried out by adding to the washed beads 50 ml of a solution containing 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 μCi [γ-^{32}P]ATP and 1 μg aprotinin for 25 min at room temperature. Reactions were terminated by spinning down the Sepharose beads in a microcentrifuge, discarding the supernatants and resuspending the beads in 50 ml SDS gel loading buffer. Eluted proteins were analyzed by SDS–PAGE and autoradiography.

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