Transforming signals resulting from sustained activation of the PDGFβ receptor in mortal human fibroblasts

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Summary
The platelet-derived growth factor β receptor (PDGFβR) plays an important role in proliferation and motility of fibroblasts. We have been investigating the effects of sustained PDGFβR activation in mortal human diploid fibroblasts (HDFs), which are typically difficult to transform. We have previously shown that the bovine papillomavirus E5 protein, through its ability to crosslink and constitutively activate the PDGFβR, induces morphological transformation, enhanced growth and loss of contact inhibition (focus formation) in HDFs. Here, we characterized two E5 mutants as being severely defective for focus formation but still competent for enhanced growth, suggesting that proliferation is insufficient for loss of contact inhibition. These E5 mutants were then used in a comparative study to distinguish the PDGFβR signaling intermediates required for the enhanced growth phenotype from those required for focus formation. Our data suggested that a PI 3-kinase (PI3K)-AKT-cyclin D3 pathway, a Grb2-Gab1-SHP2 complex and JNK played a role in the enhanced growth phenotype. However, a SHP2-p66Shc-p190RhoGAP complex and ROCK were implicated exclusively in focus formation. We speculate that a SHP2-p66Shc-p190RhoGAP signaling complex recruited to the activated PDGFβR promotes a distinct Rho-dependent process required for focus formation but not growth of HDFs.

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Key words: PDGF receptor, Signaling, Transformation

Introduction
The platelet-derived growth factor (PDGF) β receptor (PDGFβR; also known as PDGFRB) plays a key role in the proliferation and motility of fibroblasts, vascular smooth muscle cells, capillary endothelial cells and neurons. The binding of dimeric ligand, PDGF-BB, to the extracellular domain of the receptor induces dimerization and activation of the receptor, resulting in autophosphorylation of key tyrosine residues in its cytoplasmic domain. Once phosphorylated, these tyrosine residues become docking sites for important SH2 domain-containing substrates (reviewed by Heldin et al., 1998). These include the 85 kDa regulatory subunit (p85) of phosphoinositide 3-kinase (PI3K), phospholipase Cγ (PLCγ), Src family members, the protein tyrosine phosphatase SHP2, signal transducers and activators of transcription (STATs), the Ras-GTPase activating protein (p120RasGAP), and several signaling adaptors such as Grb2, Shc and Nck. Once recruited to the receptor, these substrates activate signaling pathways leading to cell proliferation, morphological changes and motility.

Sustained ligand-dependent activation of the PDGFβR has oncopgenic effects both in vitro and in vivo. Overexpression of the gene encoding the PDGF-B chain, c-sis, or its viral homolog, v-sis (Doolittle et al., 1983; Waterfield et al., 1983), promotes transformation of rodent fibroblast lines (Beckmann et al., 1988; Clarke et al., 1984). Similarly, uncontrolled expression of PDGF-B resulting in an autocrine loop for PDGFβR receptor activation is thought to play a role in the development of several human cancers, including glioblastomas, fibrosarcomas and menigiomas (Betsholtz et al., 1989; Fleming et al., 1992; Heldin and Westermark, 1999; Hermanson et al., 1992).

Sustained ligand-independent activation of the PDGFβR by the E5 protein of bovine papillomavirus (BPV) also results in transformation. E5 is a 44 amino acid transmembrane protein that dimerizes via two cysteine residues (Cys37 and Cys39) located in its predicted extracellular domain (Burkhardt et al., 1989; Horwitz et al., 1988; Schlegel et al., 1986). E5 is capable of binding to and constitutively activating the endogenous PDGFβR in rodent, bovine and human fibroblasts (Petti and DiMaio, 1992; Petti and DiMaio, 1994; Petti et al., 1991; Petti and Ray, 2000). By itself, E5 can tumorigenically transform rodent fibroblast lines (Bergman et al., 1988; DiMaio et al., 1986; Schiller et al., 1986), and its transforming activity depends on its ability to constitutively activate the PDGFβR (Nilson and DiMaio, 1993; Riese, 2nd and DiMaio, 1995). Like PDGF-BB, E5 binds to the PDGFβR as a dimer and thereby promotes receptor dimerization followed by receptor autophosphorylation and activation (Lai et al., 1998). However, accumulated genetic evidence indicates that E5 binds to the PDGFβR in a unique and ligand-independent manner, via multiple intermolecular transmembrane domain interactions (Klein et al., 1999; Klein et al., 1998; Nappi and Petti, 2002; Nappi et al., 2002; Nilson et al., 1995; Petti et al., 1997).

Previous studies reported on the requirement of particular PDGFβR signaling substrates for transformation of immortal mouse fibroblast lines. Evidence from one study suggested that SHP2 and PLCγ were sufficient for loss of contact inhibition of NIH 3T3 fibroblasts induced
by continuous PDGF expression (Uren et al., 1996). A second study reported that either PLCγ or PI3K was required for PDGF-induced anchorage independent growth (DeMali et al., 1997). A third report suggested that they used mouse fibroblast lines, which are prone to transformation and may not use the signaling pathways that play a role in human cancers. Thus, the PDGFβR signaling substrates identified as being important for transformation in these studies may not be the same as those required for transformation of normal human fibroblasts.

We have been investigating the effect of sustained PDGFβR activation by BPV E5 in young mortal human diploid fibroblasts (HDFs). Compared with rodent fibroblast lines, HDFs represent a more physiologically relevant system to study mechanisms of human cell transformation, as they have not accumulated the genetic alterations that predispose immortal rodent fibroblast lines to transformation. Although HDFs are difficult to transform, we have previously demonstrated that E5, through its ability to constitutively activate the PDGFβR, could induce three transforming phenotypes in HDFs: focus formation (loss of contact inhibition), morphological transformation and continued growth beyond the normal saturation density (Petti and Ray, 2000). Expression of v-Sis in these cells induced a similar set of phenotypes. However, neither E5 nor v-Sis could induce anchorage-independent growth or substantial growth in low serum. Moreover, HDFs expressing E5 or v-Sis eventually undergo apoptosis, which may represent a negative feedback response that prevents full-scale transformation (Petti and Ray, 2000; Zhang et al., 2002).

Here, we investigated the role of specific PDGFβR signaling pathways in E5-induced growth enhancement and focus formation of HDFs. Using partially defective E5 mutants, we demonstrated that enhanced growth could be dissociated from focus formation, suggesting that alleviation of growth control is insufficient for loss of contact inhibition. Further evidence implicated PI3K- and Grb2-mediated pathways in the enhanced growth phenotype and a SHP2-p66Shc-mediated pathway in focus formation.

**Results**

Certain E5 mutants can dissociate focus formation of HDFs from enhanced growth

The previously described E5 mutants W32S and C39S (Fig. 1A) were found to be defective for transformation but still able to bind to and activate the PDGFβR in mouse C127 fibroblasts (Horwitz et al., 1988; Nilson et al., 1995). Here, we examined the effect of these E5 mutants in HDFs in an effort to define more clearly their defect for transformation.

HDFs were made to stably express wild-type or mutant E5 by retroviral mediated gene transfer, and the ability of an E5 protein to bind to and activate the PDGFβR in these cells was first examined.

E5 immunoprecipitation followed by PDGFβR immunoblotting revealed that the W32S and C39S mutants were fully competent for forming a stable complex with the PDGFβR in HDFs (Fig. 1B). PDGFβR immunoprecipitation followed by anti-phosphotyrosine immunoblotting indicated that the W32S and C39S mutants induced substantial tyrosine phosphorylation of both mature and incompletely processed precursor forms of the PDGFβR (PR) (Fig. 1C). Immunoisolates were then subjected to immunoblotting for PDGFβR (PR), E5 or phosphotyrosine (PY) as indicated. Mature (m) and precursor (p) forms of the PDGFβR (PR) are indicated by the arrows on the left. (C) HDFs expressing wild-type (wt) E5, the W32S or C39S mutant, or no viral oncogene (control) were plated at 1.6×10⁵ cells per 60 mm dish and counted in triplicate at various times thereafter. The mean number of cells with the standard error is plotted. (D,E) A focus-forming assay was performed by infecting HDFs with recombinant retroviruses expressing wild-type (wt) E5, C39S, W32S or no viral oncogene (control) and then maintaining cells at confluence as described in the Materials and Methods. In D, Crystal Violet-stained monolayers are shown for visualization of foci. In E, the number of foci induced by E5, C39S and W32S were counted, averaged, corrected for virus titer and expressed as the mean percent (with standard error) relative to the number of foci induced by wild-type E5.

![Fig. 1. The effect of the C39S and W32S E5 mutants in HDFs.](image)

A. The amino acid sequence of the BPV E5 protein showing the positions of the amino acid substitutions present in the W32S and C39S mutants. Boxed region indicates the predicted transmembrane (TM) domain. (B) Extracts of HDFs stably expressing wild-type (wt) E5, W32S, C39S or no viral oncogene (control) were immunoprecipitated (IP) with an anti-E5 or anti-PDGFβR (PR) antiserum. Immunoprecipitates then were subjected to immunoblotting for PDGFβR (PR), E5 or phosphotyrosine (PY) as indicated. Mature (m) and precursor (p) forms of the PDGFβR (PR) are indicated by the arrows on the left. (C) HDFs expressing wild-type (wt) E5, the W32S or C39S mutant, or no viral oncogene (control) were plated at 1.6×10⁵ cells per 60 mm dish and counted in triplicate at various times thereafter. The mean number of cells with the standard error is plotted. (D,E) A focus-forming assay was performed by infecting HDFs with recombinant retroviruses expressing wild-type (wt) E5, C39S, W32S or no viral oncogene (control) and then maintaining cells at confluence as described in the Materials and Methods. In D, Crystal Violet-stained monolayers are shown for visualization of foci. In E, the number of foci induced by E5, C39S and W32S were counted, averaged, corrected for virus titer and expressed as the mean percent (with standard error) relative to the number of foci induced by wild-type E5.
Next, the growth kinetics of HDFs expressing the different E5 proteins was assessed. As shown in Fig. 1C, the wild type E5-, W32S- and C39S-expressing HDFs grew at a similar rate and to a similar saturation density, which was approximately twofold greater than that of control cells not expressing a viral oncoprotein. Therefore, the W32S and C39S mutants were competent for enhancing the growth of HDFs.

Finally, we examined the focus-forming activity of the E5 mutants in HDFs. Although wild-type E5 readily induced focus formation of HDFs, the C39S and W32S mutants were severely defective for this phenotype (Fig. 1D,E). Thus, the W32S and C39S mutants were fully competent for enhanced growth, but were almost completely defective for focus formation. This suggests that another process besides the ability of cells to outgrow their normal saturation density is required for focus formation of HDFs. As these E5 mutants separated enhanced growth from focus formation, we proceeded to use them as tools for distinguishing the PDGFβR signaling pathways involved in these two transforming phenotypes.

**PDGFβR-substrate interactions in the presence of the different E5 proteins**

Given the phenotype of the W32S and C39S mutants, it stands to reason that these mutants somehow disable the PDGFβR from interacting with those substrates that are required for focus formation but not for enhanced growth. Therefore, in an effort to ascertain the PDGFβR substrates involved focus formation of HDFs, we compared the ability of specific substrates to be recruited to the receptor in wild-type E5-, mutant E5- and v-Sis-expressing HDFs.

The in vivo binding of specific substrates to the PDGFβR was determined by co-immunoprecipitation analysis in which PDGFβR immunoprecipitates were analyzed for the presence of various substrates by immunoblotting. As shown in Fig. 2, a substantial amount of PLCγ, p85-PI3K, SHP2, RasGAP and Grb2 could be detected in PDGFβR immunoprecipitates from E5- and v-Sis-expressing HDFs but not from control cells. This indicates that when the PDGFβR is activated by wild-type E5 or v-Sis in HDFs it recruits at a minimum these five substrates. We were unable to detect Nck, Src, Shc or STAT3 co-immunoprecipitating with the PDGFβR in any of the different HDF cell strains (data not shown).

Substrate-receptor binding was reduced in the mutant E5-expressing HDFs, but the magnitude of reduction varied depending on the substrate examined. As shown in Fig. 2, co-immunoprecipitation of p85-PI3K and Grb2 with the PDGFβR was only modestly reduced in the presence of the E5 mutants, while co-immunoprecipitation of PLCγ and RasGAP was reduced to a greater extent. Notably, SHP2 co-immunoprecipitation with the receptor in the presence of the E5 mutants was reduced by the greatest magnitude (by ~80%; Fig. 2). Quantitation of results from multiple experiments was performed and revealed that this trend was reproducible (Fig. 2B). Thus, p85 PI3K and Grb2 showed the greatest amount of binding to the PDGFβR in the presence of the E5 mutants, while SHP2 showed the least amount of binding. Therefore, given the fact that these E5 mutants were fully competent for enhanced growth and greatly defective for focus formation, recruitment of PI3K and Grb2 to the receptor correlated primarily with enhanced growth, while recruitment of SHP2 correlated specifically with focus formation.

**Status of downstream signaling intermediates in HDFs expressing the different E5 proteins**

Next, we examined the activity of signaling intermediates downstream of PI3K and Grb2 in proliferating HDFs expressing the different E5 proteins. Specifically, we ascertained the phosphorylation status of AKT, JNK, c-Jun and ERK1/2 as an indication of their activity in these cells by immunoblot analysis using phospho-specific antibodies (Fig. 3A). Surprisingly, phosphorylation of ERK1/2 in HDFs did not increase in response to expression of wild-type E5, v-Sis or any of the E5 mutants. By contrast, phosphorylation of AKT and JNK was substantially increased in the wild-type E5-, C39S-, W32S- and v-Sis-expressing
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HDFs compared with the control cells. In addition, phosphorylation of c-Jun, a major substrate for JNK, was also increased in these cells, confirming that the activity of JNK was augmented. Thus, increased phosphorylation/activation of JNK and AKT, but not of ERK1/2, correlated with the ability of an E5 protein or v-Sis to enhance the growth of HDFs.

We also examined cyclin D3 levels in proliferating HDFs expressing the different E5 proteins. As shown in Fig. 3B (upper panel), cyclin D3 expression was increased in response to E5, C39S, W32S or v-Sis expression. Moreover, enhanced cyclin D3 expression in the E5-expressing HDFs was inhibited by the PI3K inhibitors LY294002 (LY) or 310 nM wortmannin (W) for 24 hours. Whole-cell lysates were prepared and immunoblotted for cyclin D3 and β-tubulin.

Characterization of SHP2-containing signaling complexes in HDFs expressing the different E5 proteins

As recruitment of SHP2 to the PDGFβR showed the strictest correlation with focus formation of HDFs, we attempted to identify possible downstream effectors of SHP2 that are involved in a pathway for focus formation but not growth. In some systems, SHP2 has been proposed to function as a signaling adaptor because it contains several protein-protein interaction motifs, including two SH2 domains, two phosphotyrosine residues and a proline-rich SH3-binding domain (Poole and Jones, 2005). Therefore, to initially characterize the SHP2-mediated pathway involved in focus formation, we attempted to identify its associated proteins in our different HDF strains.

We first asked whether or not a well-established binding partner for SHP2, Gab1, associates with SHP2 in HDFs expressing the different E5 proteins. Gab1 was originally characterized as a docking protein that binds to Grb2 and is abundantly tyrosine phosphorylated in response to growth factors (Holgado-Madruga et al., 1996). In the context of the ligand-activated PDGFβR, it was shown that once Gab1 is recruited indirectly to the receptor via Grb2 it becomes tyrosine phosphorylated and binds SHP2 (Kallin et al., 2004). To detect a Gab1-SHP2 interaction in our system, we attempted to characterize Gab1 tyrosine phosphorylation and association with SHP2 and Grb2 correlate with the enhanced growth phenotype. SHP2 (A) or Gab1 (B) was immunoprecipitated (IP) from lysates of HDFs expressing no (control) or the indicated viral oncoprotein. Immunoprecipitates were subjected to immunoblotting for Gab1, SHP2, Grb2 or phosphotyrosine (PY) as indicated. For the graphs shown below representative blots, the relative band intensities for the Gab1 and Grb2 blots in A, and the PY and Grb2 blots in B were quantitated using the Image J program and normalized for SHP2 (A) or total Gab1 (B) amounts. These normalized values are expressed as a percentage relative to the amount of co-immunoprecipitation or PY-Gab1 observed in the wild-type E5-expressing HDFs. The mean values from two different experiments with standard error are shown.
in HDFs promotes the binding of SHP2 to Gab1. Tyrosine phosphorylation of Gab1 was also substantially increased in the E5- and v-Sis-expressing HDFs (Fig. 4B), suggesting that it is a substrate for the activated PDGFRβ or an associated tyrosine kinase in these cells. Importantly, a considerable amount of Gab1 associated with SHP2 and was tyrosine phosphorylated in the C39S- and W32S-expressing HDFs (Fig. 4). Thus, given the phenotype of these mutants, Gab1 tyrosine phosphorylation and its association with SHP2 correlated with enhanced growth rather than focus formation of HDFs. HDFs expressing the different E5 proteins were also examined for the presence of Grb2-Gab1 and Grb2-SHP2 complexes by co-immunoprecipitation analysis. Grb2 interactions with Gab1 and SHP2 increased substantially in response to mutant E5, wild-type E5 or v-Sis expression (Fig. 4). Therefore, PDGFRβ-Grb2, Grb2-Gab1, Gab1-SHP2 and Grb2-SHP2 interactions all correlated with enhanced growth rather than focus formation of HDFs. This is consistent with the notion that a Grb2-Gab1-SHP2 signaling complex is recruited to the activated PDGFRβ in HDFs and promotes a signal for enhanced growth.

We also tested the ability of the adaptor protein Shc to interact with SHP2 in HDFs expressing the different E5 proteins. As shown in Fig. 5A, the 66 kDa isoform of Shc (p66Shc) co-immunoprecipitated with SHP2 in the E5- and v-Sis-expressing HDFs but not in control cells. This indicates that sustained PDGFRβ activation promotes a stable association between SHP2 and p66Shc in HDFs. Interestingly, unlike the SHP2-Gab1 interaction, the SHP2-p66Shc interaction was substantially reduced in the presence of the C39S and W32S mutants compared with wild-type E5 (Fig. 5A). Thus, although the presence of a SHP2-Gab1 complex in HDFs correlated with enhanced growth, the presence of a SHP2-p66Shc complex correlated strictly with focus formation and recruitment of SHP2 to the activated PDGFRβ.

The p66Shc isoform differs both structurally and functionally from its 52 and 46 kDa splice variants (reviewed by Pellegrini et al., 2005). p66Shc possesses an additional N-terminal domain, and phosphorylation of Ser36 within this domain is required for its distinctive activities, which include inactivating the Ras/ERK pathway and mediating oxidative stress responses (Migliaccio et al., 1999; Smith et al., 2005; Tiberi et al., 2006). Therefore, we examined the status of p66ShcSer36 phosphorylation in HDFs expressing the different E5 proteins. Shc was immunoprecipitated from cell extracts and then subjected to immunoblotting using a phospho-Ser36-p66Shc-specific antibody. As shown in Fig. 5B, p66Shc-Ser36 phosphorylation was increased substantially in the E5- and v-Sis-expressing HDFs compared with control cells, suggesting that p66Shc is phosphorylated at Ser36 in response to sustained PDGFRβ activation in HDFs. However, no increase in Ser36 phosphorylation was observed in HDFs expressing an E5 mutant (Fig. 5B). Therefore, Ser36 phosphorylation of p66Shc in HDFs correlated with focus formation and SHP2 interactions with the PDGFRβ and p66Shc.

To determine whether SHP2 is required for Ser36 phosphorylation of p66Shc in HDFs, E5-expressing HDFs were transiently transfected with a control or SHP2-specific siRNA and then examined for Ser36 phosphorylation of p66Shc. As shown in Fig. 6A, the SHP2-specific siRNA reduced SHP2 expression as expected, and also reduced Ser36 phosphorylation of p66Shc in the E5-expressing HDFs. This suggests that SHP2 plays a role in phosphorylation of p66Shc at Ser36 in response to PDGFRβ activation. Taken together, our results suggest that once bound to the activated PDGFRβ, SHP2 recruits p66Shc and facilitates its phosphorylation at Ser36.

As it has previously been reported that a Gab1-SHP2 complex is required for downstream activation of JNK (Holgado-Madruga and Wong, 2003), we assessed the requirement of SHP2 for JNK activation in our system using RNA interference of SHP2 expression. Specifically, phosphorylation of JNK was examined...
after transient siRNA-mediated knockdown of SHP2 in the E5-expressing HDFs. As shown in Fig. 6B, phosphorylation of JNK1 was reduced when expression of SHP2 was inhibited. This suggests that SHP2 plays a role in activation of JNK in the E5-expressing HDFs.

To assess the requirement of p66Shc Ser36 phosphorylation in transformation of HDFs, the effect of expressing a nonphosphorylatable p66Shc mutant, S36A, on E5- and v-Sis-induced growth and focus formation was determined. S36A or wild-type p66Shc was introduced and stably expressed in HDFs by retroviral-mediated gene transfer. Increased expression of p66Shc in cells harboring a transgene confirmed that exogenous wild-type p66Shc or S36A was expressed (Fig. 7B). Furthermore, the level of p66Shc phosphorylated at Ser36 increased when wild-type p66Shc but not S36A was overexpressed, confirming the inability of S36A to be phosphorylated at this position (Fig. 7B). First, we determined the effect of wild-type p66Shc or S36A overexpression on enhanced growth induced by E5 or v-Sis. As shown in Fig. 7A, E5 or v-Sis expression resulted in a threefold increase in cell density regardless of whether or not exogenous mutant or wild-type p66Shc was expressed. This suggests that p66Shc does not play a role in the enhanced growth phenotype induced by E5 or v-Sis. We also asked whether overexpressing wild-type or S36A p66Shc could affect the ability of E5 or v-Sis to increase AKT and JNK phosphorylation, which correlated with the enhanced growth phenotype. As shown in Fig. 7B, E5- and v-Sis-induced phosphorylation of AKT and JNK was unaffected by the co-expression of either exogenous wild-type p66Shc or S36A, further confirming the notion that p66Shc does not play a role in signaling events associated with enhanced growth of HDFs. Finally, the focus-forming activity of E5 and v-Sis in HDFs was assessed in the presence or absence of overexpressing exogenous wild-type p66Shc or S36A. As shown in Fig. 7C, overexpression of wild-type p66Shc slightly enhanced focus formation induced by E5 or v-Sis. However, S36A expression reduced the focus-forming activity of these oncogenes by ~40%. Taken together, these results suggest that Ser36 phosphorylation of p66Shc plays a role in focus formation but not in enhanced growth induced by sustained PDGFβR activation in HDFs. It is important to note that the amount of E5- and v-Sis-induced Ser36 phosphorylation of p66Shc did not decrease upon S36A expression (compare S36A with LXSN lanes in Fig. 7B), suggesting that S36A did not inhibit Ser36 phosphorylation of endogenous p66Shc. This implies that inhibition of focus formation by S36A was not at the level of Ser36 phosphorylation.

To initially characterize the signaling events downstream of p66Shc that play a role in focus formation of HDFs, we attempted to identify signaling proteins that interact with p66Shc as a result of sustained PDGFβR activation in these cells. Co-immunoprecipitation analysis revealed that p66Shc formed a stable complex with p190BRhoGAP in E5- and v-Sis-expressing HDFs, but not in control cells (Fig. 8). This indicates that sustained PDGFβR activation in HDFs promotes an interaction between p66Shc and p190BRhoGAP. Furthermore, the p66Shc-p190BRhoGAP interaction was greatly reduced in the C39S- and W32S-expressing HDFs (Fig. 8A), correlating this interaction with focus formation but not enhanced growth of HDFs. Given the role of p190BRhoGAP in inactivating Rho-family GTPases (Vincent and Settleman, 1999), these results raise the possibility that a SHP2-p190BRhoGAP interaction was greatly reduced in the C39S- and W32S-expressing HDFs, (Fig. 8A), suggesting that SHP2 plays a role in activation of JNK in the E5-expressing HDFs.

To determine whether or not p66Shc required Ser36 phosphorylation for its ability to bind to p190B or SHP2, the ability of the S36A p66Shc mutant to interact with these proteins was assessed. Briefly, p66Shc was immunoprecipitated from extracts of HDFs overexpressing wild-type p66Shc or S36A in the presence of E5 or v-Sis and then subjected to E5- or v-Sis-containing retrovirus. Foci were counted and averaged from several experiments. Focus formation is expressed as the mean percent (with standard error) relative to the number of foci formed by the LXSN-HDFs.
immunoblotting. As expected, p190B-p66Shc and SHP2-p66Shc complex formation increased when wild-type p66Shc was overexpressed in the presence of E5 or v-Sis (Fig. 8B). Similar increases in these complexes were observed when S36A was expressed, suggesting that the S36A mutant could interact with p190B and SHP2. This indicates that the ability of p66Shc to interact with SHP2 and p190B does not require Ser36 phosphorylation.

The effect of kinase inhibitors on growth and focus formation of HDFs

The data presented thus far implicate PI3K-AKT-, JNK- and Rho-mediated pathways in the transforming phenotypes induced by sustained PDGFβR activation in HDFs. To determine the requirement of these pathways for E5- and v-Sis-induced enhanced growth and focus formation of HDFs, we assessed the effect of specific kinase inhibitors on these phenotypes. For these experiments we used LY294002 and wortmannin (inhibitors of PI3K), SP600125 (a JNK inhibitor), and Y27632 (an inhibitor of the Rho effector kinase ROCK) at concentrations predetermined to be effective in the E5-expressing HDFs (see Fig. S1 in the supplementary material). First, the effect of these inhibitors on the focus forming activity of E5 or v-Sis in HDFs was determined. As shown in Fig. 9A, all inhibitors reduced focus formation of HDFs induced by E5 or v-Sis. Complete inhibition was achieved with LY294002, while wortmannin, SP600125 and Y27632 inhibited focus formation by ~40-50%. Next, we determined the effect of each inhibitor on the growth kinetics of E5-expressing HDFs. As shown in Fig. 9B,C, LY294002 efficiently arrested the growth of the E5-expressing HDFs, while wortmannin and SP600125 substantially slowed the growth rate of these cells. This suggests that PI3K and JNK activities are required for proliferation of the E5-expressing HDFs. By contrast, the ROCK inhibitor Y27632 had little effect on enhanced growth of the E5-expressing HDFs (Fig. 9D). Thus, PI3K-AKT and JNK pathways are required for both enhanced growth and focus formation of HDFs, while a ROCK signaling pathway is required exclusively for focus formation and not enhanced growth.

Discussion

The work presented here relates specific PDGFβR signaling pathways in normal human fibroblasts to two different aspects of oncogenic transformation, enhanced growth and focus formation. We first described certain partially defective BPV E5 mutants (C39S and W32S) as being competent for enhanced growth but defective
for focus formation of HDFs. This implies that focus formation of these cells requires distinct PDGFβR signaling pathways that are not required for proliferation. The C39S and W32S mutants were less effective than the wild-type E5 protein at inducing PDGFβR tyrosine phosphorylation, even though they were fully capable of interacting with the receptor. We speculate that although the C39S and W32S mutants bind to the PDGFβR, they promote an altered receptor dimer conformation that is suboptimal for receptor autophosphorylation. This altered conformation may also disable the receptor from recruiting those signaling substrates that are required primarily for focus formation.

Accordingly, we performed a comparative analysis of the ability of the PDGFβR to recruit its substrates and activate downstream signaling intermediates in the presence of the different E5 proteins and then correlated specific pathways with focus formation or enhanced growth. First, PI3K- and Grb2-mediated pathways correlated with enhanced growth. The C39S and W32S mutants, like wild-type E5, could promote (1) substantial recruitment of PI3K and Grb2 to the PDGFβR, (2) activation of Akt and JNK, which could serve as downstream effectors of these substrates, (3) a PI-3-kinase-dependent increase in cyclin D3 expression, (4) Grb2 complex formation with Gab1 and SHP2, and (5) Gab1 tyrosine phosphorylation and complex formation with SHP2. Experiments using kinase inhibitors confirmed that PI3K and JNK were required for both focus formation and enhanced growth (Fig. 9). As the activity of these kinases correlated with enhanced growth rather than with focus formation, we propose that they indirectly contribute to focus formation by promoting growth.

It stands to reason that a PI3K-dependent increase in cyclin D3 expression correlated with enhanced growth of HDFs (Fig. 3B). The PI3K-AKT pathway previously has been implicated in increased expression of cyclin D3 and cell proliferation (Spofford et al., 2006; Zhu et al., 2001), and in some systems upregulation of cyclin D3 occurred through AKT-induced activation of mTOR (Feng et al., 2000; Garcia-Morales et al., 2006). Thus, it is reasonable to conclude that PDGFβR stimulation of a PI3K-AKT pathway in HDFs leads to increased expression of cyclin D3, which in turn allows these cells to grow beyond their normal saturation density.

As PDGFβR-Grb2, Grb2-Gab1, Gab1-SHP2 and SHP2-Grb2 complexes were abundant in HDFs expressing the C39S or W32S mutant, we propose that a single complex containing Grb2, Gab1 and SHP2 is recruited to the activated PDGFβR via Grb2 and plays a role in the enhanced growth phenotype. In support of this notion, a previous report indicated that a Grb2-Gab1-SHP2 complex was recruited to the Grb2-binding site on the activated PDGFβR (Kallin et al., 2004). If this is the case in our system, the amount of SHP2 that remained associated with the receptor in the presence of the E5 mutants (~20% of the SHP2 bound to the wild-type E5-activated receptor; Fig. 2B) may represent the pool of SHP2 that is bound to the receptor indirectly via a Grb2-Gab1 complex. Furthermore, several studies have linked Gab1 to downstream activation of JNK (Garcia-Guzman et al., 1999; Sun et al., 2004), and two reports demonstrated that an interaction between Gab1 or Gab2 and SHP2 is required for JNK activation (Holgado-Madruga and Wong, 2003; Yu et al., 2006). These studies, together with our finding that SHP2 was required for JNK1 activation (Fig. 6B), raise the possibility that a Grb2-Gab1-SHP2 complex recruited to the activated PDGFβR relays a signal for activation of JNK rather than ERK1/2 in HDFs. This is consistent with our observation that ERK1/2 was not activated in HDFs expressing E5 or v-Sis (Fig. 3). Taken together, our data suggests that PI3K and a Grb2-Gab1-SHP2 signaling complex recruited to the activated PDGFβR stimulate Akt and JNK signaling pathways, leading to enhanced growth.

Notably, a link was established between recruitment of SHP2 to the activated PDGFβR and focus formation, as receptor binding to SHP2 was greatly impaired in the presence of the W32S or C39S mutant (Fig. 2). When recruited to the activated PDGFβR, SHP2 has been proposed to promote Ras/ERK1/2 signaling either by selectively dephosphorylating the p120RasGAP-binding site on the receptor or by binding to Grb2 (DeMali et al., 1999; Li et al., 1994). However, SHP2 must play a different role in HDFs expressing E5 or v-Sis, as activation of ERK1/2 was not observed in these cells (Fig. 3) and recruitment of SHP2 to the PDGFβR did not inversely correlate with recruitment of p120RasGAP (Fig. 2). Because SHP2 contains several potential protein-protein interaction motifs, an alternative possibility is that SHP2 directly recruited to the PDGFβR plays a positive role in focus formation of HDFs by acting as a signaling adaptor. In support of this notion, we showed that SHP2 formed a stable complex with the 66 kDa form of Shc in response to E5 or v-Sis expression (Fig. 5A). Unlike the SHP2-Gab1 interaction, the SHP2-p66Shc interaction correlated with focus formation and recruitment of SHP2 to the PDGFβR. In addition, we also observed an interaction between p66Shc and p190BRhoGAP in response to sustained PDGFβR activation, and this interaction also correlated with focus formation (Fig. 8). To the best of our knowledge, this is the first report demonstrating the ability of p66Shc to interact with SHP2 and p190B. Thus, we propose that at least two different complexes containing SHP2 are recruited to the activated PDGFβR in HDFs. One is a Grb2-Gab1-SHP2 complex, which is recruited to the receptor via Grb2 and likely to be involved in enhanced growth. The other is a SHP2-p66Shc-p190B complex, which is recruited to the receptor via SHP2 and relays a signal specifically required for focus formation and not growth.

It is possible that the SHP2-p66Shc complex bound to the activated PDGFβR in HDFs recruits and inactivates p190BRhoGAP, resulting in activation of a Rho family GTPase. Others have shown that p190B is a substrate for SHP2 and that SHP2-mediated tyrosine dephosphorylation of p190B inactivates it, leading to subsequent activation of RhoA (Kontaridis et al., 2004; Sordella et al., 2003). We were unable to detect a change in tyrosine phosphorylation of p190B or Rho activity correlating with focus formation in our system (data not shown). Nonetheless, we demonstrated that the activity of ROCK, a downstream effector of Rho, was required for focus formation and not for enhanced growth of HDFs, implying a specific role for Rho in focus formation. It is possible that the fraction of active Rho involved focus formation is located at a specific subcellular site and thus would be difficult to detect by the standard pull-down assay for Rho activity. Therefore, in the E5-expressing HDFs, the SHP2-p66Shc complex may recruit p190B residing at a specialized site for focus formation. SHP2 bound to p66Shc could then dephosphorylate and inactivate p190B, resulting in Rho activation at this site. Experiments are planned to determine more precisely the role of p190B and Rho in focus formation of HDFs.

One intriguing possibility is that RhoA plays a role in focus formation by altering cytokinesis. In a previous report, it was shown that overexpression of a constitutively activated RhoA protein caused improper orientation of the mitotic spindle, thereby altering the plane for cytokinesis such that it was parallel rather than perpendicular to the substrate (Vasiliev et al., 2004). Furthermore,
other studies showed that overactivation of RhoA at sites of cell-cell contact can destabilize adherens junctions (Sahai and Marshall, 2002), which in turn can change the position of the centrosome and alter the plane of cytokinesis (Siegrist and Doe, 2006). Based on these reports, the following scenario could explain the role of Rho in E5-induced focus formation of HDFs: activation of the PDGFβR at sites of cell-cell contact could result in recruitment of a SHP2-p66Shc-p190B complex to the receptor followed by localized inactivation of p190B and activation of RhoA at these sites. This could result in destabilization of cell-cell contacts followed by cleavage furrow formation at these sites. Consequently, cell division would occur parallel to the substrate, resulting in one daughter cell sitting on top of its sister cell. Successive rounds of this process could generate foci of piled up cells.

Our evidence suggests that the Ser36 phosphorylated form of p66Shc plays a specific role in the focus forming phenotype. First, Ser36 phosphorylation of p66Shc correlated with focus formation but not growth (Fig. 5B). Second, the nonphosphorylatable S36A p66Shc mutant impaired focus formation but not growth induced by E5 or v-Sis (Fig. 7). As S36A could still bind to SHP2 and p190B (Fig. 8B), it is possible that it inhibited focus formation by competing with endogenous wild-type p66Shc for binding to SHP2 and p190B, thereby sequestering these proteins in an inactive complex. Focus formation was not completely inhibited by S36A, perhaps because the amount of S36A expressed was insufficient for sequestering all of the available SHP2 and p190B. Therefore, we believe that the Ser36 phosphorylated form of p66Shc is a necessary component of an active signaling complex with SHP2 and/or p190B. For example, the phospho-Ser36 residue may be part of a recognition motif for another signaling protein required for focus formation. Alternatively, as the Ser36 phosphorylated form of p66Shc has been shown to promote the generation of intracellular reactive oxygen species (Migliaccio et al., 1999; Smith et al., 2005; Tiberi et al., 2006), it could act through an oxidant-dependent mechanism to affect the expression or activity of proteins involved in a focus-forming process. Identification of proteins that interact with p66Shc in a Ser36-specific manner in the E5-expressing HDFs will provide further insight into the role of p66Shc in focus formation.

It is important to note that overexpression of wild-type p66Shc did not enable the C39S or W32S mutant to induce focus formation (data not shown). This suggests that an additional pathway is required for focus formation of HDFs. As the C39S and W32S mutants were also defective for promoting recruitment of PLCγ and p120RasGAP to the PDGFβR, signals promoted by one or both of these substrates could also contribute to focus formation of HDFs.

In conclusion, we propose the following model outlining the PDGFβR signaling pathways that contribute to enhanced growth and focus formation of HDFs (Fig. 10): first, PI3K recruited to the activated PDGFβR promotes activation of AKT, which in turn leads to increased expression of cyclin D3 followed by enhanced growth. Grb2 bound to the receptor may also promote a signal for enhanced growth by recruiting Gab1, which in turn becomes tyrosine phosphorylated and recruits SHP2, leading to activation of JNK. Proliferation promoted by PI3K and Grb2 pathways is necessary but insufficient for focus formation. An additional signal provided by SHP2 recruited directly to the PDGFβR is also required for focus formation. Specifically, SHP2 bound to the receptor forms a complex with p66Shc, which in turn binds to p190BRhoGAP and enables SHP2 to inactivate p190B. This would result in activation of Rho, which could stimulate a cytoskeletal remodeling process (such as altered cytokinesis, adhesion or motility) that allows cells to grow in a third dimension. Although some aspects of this model will require further elucidation, we have begun to characterize the signaling pathways downstream of the PDGFβR that play a role in transformation of human cells. These pathways also may be involved in maintenance or dissemination of human tumors in which overstimulation of the PDGFβR has been implicated, and thus may represent pertinent targets for cancer therapy.

Materials and Methods

Cells and cell culture

Young HSF4012 (NHDF4012) human foreskin fibroblasts (HDFs) were purchased from Cambrex (formerly Clonetics) at passage 1. The Phoenix amphotrophic producer cell line was obtained from the American Type Culture Collection (ATCC) with permission from G. Nolan (Stanford University) and was used to produce high titer amphotrophic retrovirus after transient transfection. Cells were maintained as previously described (Petti and Ray, 2000).

Plasmid DNAs

The E5, W32S, C39S and v-sis open reading frames (ORFs) were subcloned from plasmids obtained from Daniel DiMaio (Yale University, New Haven, CT) into the pBabeG418 retroviral vector (which contains the puromycin resistance marker) by standard methods. Plasmids containing the S36A mutant or wild-type p66Shc ORF were obtained from Addgene [plasmids 10972 and 10973, respectively (Nemoto and Finkel, 2002)] and subcloned into the LXSN retroviral vector (which carries the G418 resistance marker).

Stable expression of foreign genes in HDFs

HDF strains stably expressing wild-type E5, mutant E5, v-sis, wild-type p66Shc or the S36A (Ser36 to Ala substitution) p66Shc mutant were established using high titer recombinant retroviruses expressing these genes to infect HSF4012 cells at passage 7 or 8 as described previously (Petti and Ray, 2000). Briefly, transient transfection of plasmid DNA into Phoenix amphotrophic producer cells using the calcium chloride method resulted in production of virus in the tissue culture supernatant. Approximately 2-4×10^5 colony forming units of retrovirus plus 4 μg/ml polybrene was added to ~1.5×10^6 HSF4012 cells. After 1 day, the infected cells were trypsinized and split 1:5. The next day, puromycin (Sigma) or G418 (Gemini) was added to the cells at a final concentration of 0.5 μg/ml or 400 μg/ml, respectively. After 1-2 weeks of selection, stable cell strains were established. Control cells were established using an empty retroviral vector (pBabeG418 or LXSN). HDFs stably co-expressing exogenous wild-type p66Shc or S36A with E5 or v-Sis were established by sequential retroviral infection and selection. Specifically, cells expressing wild-type p66Shc, S36A or LXSN were first established and then subjected to a second round of infection and selection for E5, v-Sis or pBabeG418 expression.
Antibodies and inhibitors
PKA, a rabbit polyclonal antibody recognizing the C-terminal 13 amino acids of the human PDGFβR, and an E5 rabbit polyclonal antiserum recognizing the C-terminal 16 amino acids of the BPV E5 protein were obtained from Daniel DiMaio (Yale University, New Haven, CT). A second PDGFβR rabbit polyclonal antiserum (against the C-terminal domain) was obtained from BD Pharmingen and used for immunoprecipitation in some cases. Antibodies against p-Ser36-p66Shc (UBI), phospho-AKT (pSer 473), total AKT, phospho-ERK1/2 (pThr202/pTyr204), total ERK1/2, anti, anti-phosphotyrosine (P-Tyr-100) and actin antibodies were purchased from Cell Signaling Technology (CST). SH2P2 and p120RasGAP antibodies were purchased from Santa Cruz Biotechnology. A phospho- Ser36-p66Shc-specific antibody was obtained from Calbiochem. All other antibodies were obtained from BD Pharmingen Laboratories. For SHP2 detection, rabbit polyclonal antibody from Santa Cruz (SC-280) was used for immunoprecipitation, while a mouse monoclonal antibody from BD Pharmingen (610621) was used for immunoblotting. LY294002, wortmannin, SP600125 or Y27632 was added to the cells just prior to growth curves
HDFs seeded at an equal density were trypsinized and counted in triplicate using a hemocytometer at various intervals after plating. For Fig. 9C, 2 days after plating, the cells were either left untreated or treated with 20 μM LY294002, 233 nM wortmannin, 10 μM SP600125 or 10 μM Y27632 by adding the inhibitor to the existing medium.

Transient siRNA-mediated knockdown of SHP2
E5-expressing HDFs were transiently transfected with a control or SHP2 siRNA (Santa Cancer Institute, as well as by awards from the Concern Foundation (Young Investigator Award) and Albany Medical College (Bridge Grant).

References
Beckmann, M. P., Betsholtz, C., Heldin, C. H., Westermark, B., Di Marco, E., Di Fiore, P. P., Robbins, K. C. and Aaronson, S. A. (1988). Comparison of biological properties and transforming potential of human PDGF-A and PDGF-B chains. Science 241, 1346-1349.
Bergman, P., Ustav, M., Sedman, J., Moreno-Lopez, J., Vennstrom, B. and Pettersson, U. (1988). The E5 gene of bovine papillomavirus type 1 is sufficient for complete oncogenic transformation. Oncogene. Betsholtz, C., Nister, M., Rorsman, F., Heldin, C. H. and Westermark, B. (1989). Structural and functional aspects of platelet-derived growth factor and its role in the pathogenesis of globomaltosa. Mol. Chem. Neurophysiol. 10, 27-36.
Buckerdorf, A., Willingham, M., Gay, C., Jeang, K. T. and Settleman, J. (1989). The E5 oncoprotein of bovine papillomavirus is asymmetrically localized in Golgi and plasma membranes. Virology 170, 334-339.
Clarke, M. F., Westin, E., Schmidt, D., Jones, S. F., Katner, L., Wong-Staal, F., Gallo, R. C. and Reitz, M. S., Jr (1984). Transformation of NIH 3T3 cells by a human c-sis cDNA clone. Nature 308, 466-467.
DeMali, K. A., Whiteford, C. C., Ueng, T. E. and Kazlauskas, A. (1997). Platelet-derived growth factor-dependent cellular transformation requires either phosphophatase Cgamma or phosphatidylinositol 3 kinase. J. Biol. Chem. 272, 9011-9018.

Buckerdorf, A., Willingham, M., Gay, C., Jeang, K. T. and Settleman, J. (1989). The E5 oncoprotein of bovine papillomavirus is asymmetrically localized in Golgi and plasma membranes. Virology 170, 334-339.
Clarke, M. F., Westin, E., Schmidt, D., Jones, S. F., Katner, L., Wong-Staal, F., Gallo, R. C. and Reitz, M. S., Jr (1984). Transformation of NIH 3T3 cells by a human c-sis cDNA clone. Nature 308, 466-467.
DeMali, K. A., Whiteford, C. C., Ueng, T. E. and Kazlauskas, A. (1997). Platelet-derived growth factor-dependent cellular transformation requires either phosphophatase Cgamm.
Migliaccio, E., Giorgio, M., Mele, S., Pellici, G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L. and Pellici, P. G. (1999). The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* 402, 309-313.

Nappi, V. M. and Petti, L. M. (2002). Multiple transmembrane amino acid requirements suggest a highly specific interaction between the bovine papillomavirus E5 oncoprotein and the platelet-derived growth factor beta receptor. *J. Biol. Chem.* 277, 47149-47159.

Nemoto, S. and Finkel, T. (2002). Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. *Science* 295, 2450-2452.

Nilson, L. A. and DiMaio, D. (1996). Identification of amino acids in the transmembrane and juxtamembrane domains of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. *J. Virol.* 69, 5869-5874.

Pellegrini, M., Pacini, S. and Baldari, C. T. (2005). p66SHC: the apoptotic side of Shc proteins. *Apoptosis* 10, 13-18.

Petti, L. and DiMaio, D. (1994). Stable association between the bovine papillomavirus E5 transforming protein and the endogenous beta receptor for platelet-derived growth factor in mouse C127 cells. *J. Virol.* 68, 3582-3592.

Petti, L. M. and Ray, F. A. (2000). Transformation of mortal human fibroblasts and activation of a growth inhibitory pathway by the bovine papillomavirus E5 oncoprotein. *Cell Growth Differ.* 11, 395-408.

Petti, L., Nilson, L. O. and DiMaio, D. (1991). Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. *EMBO J.* 10, 845-855.

Petti, L. M., Reddy, V., Smith, S. O. and DiMaio, D. (1997). Identification of amino acids in the transmembrane and juxtamembrane domains of the platelet-derived growth factor receptor required for productive interaction with the bovine papillomavirus E5 protein. *J. Virol.* 71, 7318-7327.

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