$\alpha\text{v}\beta 3$ integrin associates with activated insulin and PDGF β receptors and potentiates the biological activity of PDGF

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Integrin-mediated cell attachment modulates growth responses and growth factors regulate cell attachment. Moreover, both cell attachment to extracellular matrix and mitogenic signaling by growth factors are necessary for the proliferation of most types of normal cells, suggesting that integrin and growth factor receptor signaling pathways meet at some downstream point. We report here that a small, highly tyrosine-phosphorylated fraction of PDGF β and insulin receptors co-immunoprecipitates with the $\alpha\text{v}\beta 3$ integrin from cells. The integrin association requires growth factor stimulation of the receptors. Several signaling molecules that are known to be associated with activated growth factor receptors were present in the $\alpha\text{v}\beta 3$ integrin complexes. Mitogenicity and chemotaxis induced by PDGF-BB were enhanced in cells plated on the $\alpha\text{v}\beta 3$ ligand vitronectin compared with cells plated on the $\beta 1$ integrin ligand collagen. Thus, the engagement of the $\alpha\text{v}\beta 3$ integrin in cell–matrix interactions appears to coordinate an intense response to growth factors, helping to explain the importance of this integrin for tissue regeneration, angiogenesis and tumor metastasis.

Keywords: $\alpha\text{v}\beta 3$ integrin/cell attachment/cell–matrix interaction/growth factors/PDGF β

Introduction

Several lines of evidence indicate that integrin-mediated signaling processes synergize with growth factor responses. First, many of the signaling molecules associated with tyrosine kinase growth factor receptors, such as Src and phosphatidylinositol 3' kinase, are also present in integrin complexes at focal adhesions (Schlaepfer *et al.*, 1994; Clark and Brugge, 1995). Secondly, most types of normal cells require integrin-mediated attachment to extracellular matrix to be able to respond to growth factor stimulation, or even to survive (reviewed in Frisch and Ruoslahti, 1997). Bypassing this anchorage requirement is a hallmark of malignantly transformed cells; it enables malignant cells to leave their original tissue position and invade and metastasize. Thirdly, growth factors often induce cell migration, which is an integrin-dependent process (Klemke *et al.*, 1994).

The integrin signaling pathways that affect growth

factor responses may be either pathways shared by many integrins, or they may be specific for a few integrins. In some cases, a signal appears to be specific for one particular integrin. Activation of the focal adhesion kinase (FAK) is a pathway shared by several integrins. It seems to be particularly important in anchorage dependence (Frisch *et al.*, 1996; Xu *et al.*, 1996). Moreover, several growth factor receptors have been shown to be present in isolated focal adhesion complexes and in other membrane structures containing aggregated integrins (Plopper *et al.*, 1995; Miyamoto *et al.*, 1996).

The pathways that are known to be restricted to individual integrins include up-regulation of the anti-apoptosis protein Bcl-2 by the $\alpha 5 \beta 1$ integrin (Zhang *et al.*, 1995), activation of the adaptor protein Shc by $\alpha 6 \beta 4$ (Mainiero *et al.*, 1995) and, presumably by an unrelated process, by certain other integrins (Wary *et al.*, 1996). Moreover, the $\alpha\text{v}\beta 3$ integrin interacts with the PDGF and insulin receptor signaling pathways (Bartfeld *et al.*, 1993; Vuori and Ruoslahti, 1994).

That the $\alpha\text{v}\beta 3$ integrin is somehow linked to PDGF signaling was first suggested by the finding of Bartfeld *et al.* (1993) that a 190 kDa tyrosine-phosphorylated protein could be immunoprecipitated together with $\alpha\text{v}\beta 3$ from PDGF-BB-stimulated cells. The identity of the 190 kDa protein is not known. An involvement of $\alpha\text{v}\beta 3$ in insulin signaling was discovered when IRS-1, a cytoplasmic signal transduction mediator of the insulin (and insulin-like growth factor, IGF) receptors, was found to co-immunoprecipitate with $\alpha\text{v}\beta 3$ (Vuori and Ruoslahti, 1994). IRS-1 is tyrosine-phosphorylated by the activated insulin (and IGF) receptor tyrosine kinases (RTK) and as a result binds a number of downstream signaling molecules (White and Kahn, 1994). The mitogenic activity of insulin is enhanced in cells that have adhered to a substrate (such as vitronectin) through $\alpha\text{v}\beta 3$ (Vuori and Ruoslahti, 1994).

The $\alpha\text{v}\beta 3$ integrin serves as a receptor for a wide variety of proteins containing the Arg-Gly-Asp (RGD) recognition sequence (Ruoslahti, 1996). This integrin has been associated with a number of processes related to cell migration, such as angiogenesis, metastasis and wound healing (Gehlsen *et al.*, 1992; Leavesley *et al.*, 1992; Bhattacharya *et al.*, 1995; Filardo *et al.*, 1995; Friedlander *et al.*, 1995; Jones *et al.*, 1996).

We have undertaken an analysis of the association of the $\alpha\text{v}\beta 3$ integrin with the insulin and PDGF signaling pathways. We report here that both the insulin and PDGF β receptors co-immunoprecipitate with $\alpha\text{v}\beta 3$ and that the receptor molecules associated with the integrin represent a highly phosphorylated and highly activated subfraction of the insulin and PDGF receptors. These results may explain the enhanced activity of insulin and PDGF in cells that have attached to a substrate through $\alpha\text{v}\beta 3$.

Fig. 1. The $\alpha_v\beta_3$ integrin becomes associated with tyrosine-phosphorylated proteins upon growth factor stimulation. (A) Phosphotyrosine blot of immunoprecipitates (IP) obtained with anti- $\alpha_5\beta_1$ and anti- $\alpha_v\beta_3$ antisera from unstimulated, insulin-stimulated and PDGF-BB-stimulated NIH 3T3 cells. (B) Phosphotyrosine blot of an anti- $\alpha_v\beta_3$ immunoprecipitate (IP) dissolved and reprecipitated with anti- $\alpha_v\beta_3$, anti-insulin receptor IgG or anti-PDGF receptor IgG.

Results

The $\alpha_v\beta_3$ integrin becomes associated with tyrosine-phosphorylated proteins upon growth factor stimulation

Anti- $\alpha_v\beta_3$ integrin immunoprecipitates from NIH 3T3 mouse fibroblasts stimulated with insulin or PDGF each contained several tyrosine-phosphorylated proteins, whereas none was seen in immunoprecipitates obtained with anti- $\alpha_5\beta_1$ integrin antibodies from the same cells (Figure 1A). The main tyrosine-phosphorylated components obtained from the insulin-stimulated cells migrated at 170 and 100 kDa; the 170 kDa protein was found to be insulin receptor substrate-1 (IRS-1), which has been shown to associate with $\alpha_v\beta_3$ (Vuori and Ruoslahti, 1994). The 100 kDa protein was identified as the insulin receptor (IR) β chain by immunoprecipitation of SDS-dissociated $\alpha_v\beta_3$ complexes with anti-IR antibodies, followed by blotting with anti-phosphotyrosine (anti-pY) antibodies (Figure 1B).

In agreement with previous results, the main tyrosine-phosphorylated component in $\alpha_v\beta_3$ immunoprecipitates from PDGF-stimulated cells migrated at 190 kDa (pp190). Its size and PDGF-dependent phosphorylation suggested that it could be the PDGF β receptor (PDGF β -R). Indeed, immunoprecipitation of dissociated $\alpha_v\beta_3$ complexes with anti-PDGF β -R and subsequent immunoblotting of the

$\alpha_v\beta_3$ associates with the PDGF receptor

Fig. 2. Deglycosylation of PDGF β -R from $\alpha_v\beta_3$ complexes and anti-PDGF β -R immunoprecipitates produces the same core protein. (A) $\alpha_v\beta_3$ -associated pp190 and anti-PDGF β -R immunoprecipitated with two different antibodies from PDGF-BB-stimulated NIH 3T3 cells were deglycosylated with PNGaseF or left untreated. The precipitates were run on an SDS gel and immunoblotted with anti-pY. (B) The blot in (A) was stripped and reprobed with anti-PDGF β -R #1. The anti PDGF β -R positive band in the $\alpha_v\beta_3$ immunocomplex runs slightly more slowly than the anti-PDGF β -R positive band in the two anti-PDGF β -R immunoprecipitates, but aligning the blots in (A) and (B) showed that it migrated identically with the part of the PDGF β -R band that was detected with anti-pY.

precipitated material with anti-pY identified a band at the PDGF β -R position (Figure 1B).

To confirm the identity of pp190 as PDGF β -R we compared the $\alpha_v\beta_3$ -associated pp190 with authentic PDGF β -R after removal of carbohydrates with endoglycanase PNGaseF. pp190 and immunoprecipitated PDGF β -R underwent a similar change in electrophoretic mobility after deglycosylation (Figure 2A), providing additional evidence that the $\alpha_v\beta_3$ -associated protein is PDGF β -R. Immunoblotting with anti-PDGF β -R revealed a band in the $\alpha_v\beta_3$ complex that migrated slightly behind the band in total PDGF β -R detected with the same antibody (Figure 2B), but aligned exactly with the phosphorylated PDGF β -R fraction detected with anti-pY. These data indicate that a fraction of PDGF β -R associates with $\alpha_v\beta_3$.

A small fraction of RTKs is tyrosine-phosphorylated upon stimulation with ligand

Probing for the presence of IR β and PDGF β -R in the $\alpha_v\beta_3$ complexes revealed surprisingly little receptor protein relative to the amount of phosphotyrosine in the samples. This, and the electrophoretic mobility of the $\alpha_v\beta_3$ -associated PDGF β -R described above, suggested that the $\alpha_v\beta_3$ -associated growth factor receptor might represent a particularly highly phosphorylated subset of receptor molecules. We therefore set out to investigate whether growth factor stimulation might induce a highly activated subfraction of the receptor tyrosine kinases. We took advantage of the fact that activated PDGF β -R binds to p120 Ras-GAP (p21 Ras GTPase activating protein) through the Ras-GAP SH2 domains (Valius *et al.*, 1995). Ras-GAP immunoprecipitates, therefore, contain only activated PDGF β -R.

Immunoblotting revealed PDGF β -R in both anti- $\alpha_v\beta_3$ and anti-Ras-GAP immunocomplexes. The receptor migrated identically with the $\alpha_v\beta_3$ -associated PDGF β -R and both were slightly slower than the bulk of the

Fig. 3. The $\alpha v\beta 3$ -associated pp190 and pp120Ras-GAP-associated PDGF β -R are similar. (A) Anti- $\alpha v\beta 3$, anti-pp120Ras-GAP and anti-PDGF β -R immunoprecipitates were prepared from PDGF-BB-stimulated NIH 3T3 cells and deglycosylated with PNGaseF, or left untreated. The precipitates were run on an SDS gel and immunoblotted with anti-PDGF β -R#1. (B) The blot in (A) was stripped and reprobed with anti-pY. The amount of protein applied in lanes 5–8 was twice the amount in lanes 1–4. The 165 kDa band in the anti-PDGF β -R immunoprecipitates corresponds to incompletely glycosylated PDGF β -R core protein that is not present at the cell surface and therefore is not tyrosine-phosphorylated upon PDGF-BB stimulation. After deglycosylation a single band at 135 kDa is seen. Anti-PDGF β -R antibody #1 precipitates more phosphorylated PDGF β -R than anti-PDGF β -R antibody #2. Immunoblotting showed that the anti-pY-reactive 120 kDa band in the anti Ras-GAP immunoprecipitates corresponds to tyrosine-phosphorylated pp120 Ras-GAP.

PDGF β -R (Figure 3A). Deglycosylation confirmed the identity of the Ras-GAP-associated PDGF β -R. Part of the tyrosine-phosphorylated band at 190 kDa in the anti-Ras-GAP immunocomplexes shifted to the 135 kDa position (Figure 3). The residual 190 kDa protein in the Ras-GAP immunoprecipitates was identified immunologically as pp190 Rho-GAP (data not shown), which is known to associate with pp120 Ras-GAP (Settleman *et al.*, 1992). As a cytoplasmic protein, Rho-GAP would not be expected to change mobility in the SDS-PAGE upon treatment with an endoglycosidase.

To characterize further the highly phosphorylated fraction of both IR and PDGF β -R, anti-IR and anti-PDGF β -R immune complexes from insulin-stimulated and PDGF-stimulated cells were dissociated, split in two equal parts and one half was reprecipitated with anti-PDGF β -R or anti-IR and the other with anti-pY. Immunoblotting with anti-pY showed that the IR β band contained a similar amount of phosphotyrosine in each immunoprecipitate (Figure 4A). However, while the IR β protein was readily detected with anti-IR immunoblotting in the anti-IR fraction, only a trace was detected in the anti-pY fraction (Figure 4B). The anti-pY fraction from cells treated with insulin in the presence of the tyrosine phosphatase inhibitor pervanadate contained readily detectable IR β protein (Figure 4A and B), showing that the anti-IR antibody can bind highly phosphorylated IR β . PDGF β -R complexes similarly contained a small, highly anti-pY reactive fraction (Figure 4C and D). These data show that growth factor stimulation causes an uneven tyrosine phosphorylation of PDGF β -R and IR such that a small subfraction becomes highly tyrosine-phosphorylated, while the bulk of the receptors remains non-reactive with anti-phosphotyrosine.

Fig. 4. A subfraction of IR and PDGF β -R becomes tyrosine-phosphorylated upon stimulation of cells with the growth factor ligands. (A) Anti-pY blot of IR that was immunoprecipitated from insulin-stimulated NIH 3T3 cells with anti-IR, dissociated with SDS and reprecipitated (Re-IP) with either anti-IR or anti-pY. (B) The same blot stripped and reprobed with anti-IR. In lanes 3 and 4, the cells were pretreated for 3 min with 1 mM pervanadate before stimulation with insulin. (C) Anti-pY blot of PDGF β -R that was immunoprecipitated from PDGF-BB-stimulated NIH 3T3 cells by using anti-PDGF β -R #1, dissociated with SDS and reprecipitated with either anti-PDGF β -R #1 or anti-pY. (D) The same blot as in (C), was stripped and reprobed with anti-PDGF β -R #1.

$\alpha v\beta 3$ -associated PDGF β -R is complexed with downstream signaling molecules

Because tyrosine phosphorylation enables the PDGF β -R to bind and activate signal transfer molecules containing SH2 domains, we investigated the presence of such molecules in the $\alpha v\beta 3$ complexes. Anti-PDGF β -R and anti- $\alpha v\beta 3$ precipitated an identical set of tyrosine-phosphorylated proteins from PDGF-stimulated Rat 1 cells (Figure 5A). PLC- γ , Ras-GAP, the p85 subunit of the PI3 kinase as well as the tyrosine phosphatase SHP2 were detected by immunoblotting in both the anti-PDGF β -R and anti- $\alpha v\beta 3$ immune precipitates (Figure 5B). Despite its lower content of PDGF β -R protein, the $\alpha v\beta 3$ -associated PDGF β -R contained as much of these downstream signaling molecules as the bulk of PDGF β -R precipitated

Fig. 5. $\alpha v\beta 3$ -associated PDGFβ-R is complexed with downstream signaling molecules. (A) Anti-pY blot of immunoprecipitates obtained from PDGF-BB-stimulated Rat 1 cells with anti-PDGFβ-R #1, anti- $\alpha v\beta 3$ and non-immune serum. (B) The blot in (A) was stripped and reprobed with the indicated antibodies.

with the anti-PDGFβ-R. Thus, the PDGFβ-R fraction that is associated with $\alpha v\beta 3$ binds downstream signaling proteins and, therefore, appears to represent a particularly active subfraction of the total PDGFβ-R population.

The $\alpha v\beta 3$ -growth factor receptor complexes form after stimulation with growth factor

We next determined whether growth factor stimulation was necessary for the association of the growth factor receptors with the integrin. While IR was readily detected in anti- $\alpha v\beta 3$ complexes from insulin-stimulated Rat 1 cells, only traces were seen without insulin (Figure 6A). Similar results were obtained for the PDGFβ-R (Figure 6B). These data suggest that activated IR and PDGFβ-R become complexed with $\alpha v\beta 3$ only after stimulation of the receptors. That this is the case was further corroborated by surface biotinylation of Rat 1 cells stably transfected with EGF-receptor. Only after PDGF stimulation did a 190 kDa band appear in the $\alpha v\beta 3$ immune precipitates. Bands corresponding to αv and $\beta 3$ in anti-PDGFβ-R precipitates were also seen only after PDGF treatment, not in EGF-stimulated cells (Figure 6C). No EGF-receptor was found in the $\alpha v\beta 3$ complexes from EGF-stimulated cells (not shown).

Fig. 6. Stimulation of IR and PDGFβ-R is required for their association with $\alpha v\beta 3$. (A) Anti- $\alpha v\beta 3$ and anti- $\alpha 5\beta 1$ immunoprecipitates from insulin-stimulated and non-stimulated Rat 1 fibroblasts were separated on an SDS gel and immunoblotted with anti-IR. (B) The same experiment as in (A) with PDGF-BB-stimulated Rat 1 cells and blotted with anti-PDGFβ-R #1. (C) Surface biotinylated Rat 1 cells transfected with human EGF-receptor were stimulated with PDGF-BB or EGF, cell extracts were precipitated with anti- $\alpha v\beta 3$ or anti-PDGFβ-R #1, the precipitates were separated on SDS gels, and biotinylated proteins were detected with avidin-peroxidase (Avidin-PO) conjugate. A 190 kDa band was detected in anti- $\alpha v\beta 3$ immunoprecipitates after PDGF-BB-stimulation, but not without it. Similarly, two bands co-migrating with the αv and $\beta 3$ integrin subunits were present in the anti-PDGFβ-R immunoprecipitates from the PDGF-BB-stimulated cells, but not from the EGF-stimulated cells.

The activity of PDGF-BB is potentiated on an $\alpha v\beta 3$ ligand substrate

Previous results have shown that insulin is only a weak mitogen in the absence of cooperation from $\alpha v\beta 3$, but becomes nearly as active as serum (generally the strongest mitogen) in cells plated on the $\alpha v\beta 3$ ligand, vitronectin (Vuori and Ruoslahti, 1994). We have found the same to be the case with PDGF-BB (Figure 7A). Moreover, the ability of PDGF-BB to stimulate cell motility was far higher on vitronectin than on type I collagen, which is not a ligand for $\alpha v\beta 3$ (Figure 7B).

The enhanced biological activity of PDGF signaling in cells plated on vitronectin was also reflected in Erk1/2 activity. PDGF-BB-stimulated FS-fibroblasts cultured on vitronectin contained more phospho-Erk1/2 and Erk1/2

Fig. 7. Mitogenic signaling of PDGF-BB and cell migration to PDGF is potentiated on an $\alpha v \beta 3$ ligand substrate. **(A)** Incorporation of [3 H]thymidine into DNA. Thymidine incorporation into DNA was determined in human foreskin fibroblasts (FS) plated on vitronectin (solid bars) or on collagen (open bars). The cells were cultured in 10% FCS/DMEM containing glutamine and antibiotics (lanes 1 and 2), serum-starved and left unstimulated (lanes 3 and 4), or stimulated with PDGF-BB (lanes 5 and 6). Error bars indicate the standard error of three determinations. **(B)** PDGF-BB-stimulated cell migration on vitronectin and collagen. Cell motility on vitronectin ($\circ$, $\bullet$) or on collagen ($\square$, $\blacksquare$) in the absence (open symbols) or presence (solid symbols) of PDGF-BB was measured. The results at each time point are the mean cell number of 32 randomly selected high-magnification microscopic fields. **(C)** Foreskin fibroblasts were plated on vitronectin (VN) or type I collagen (Col) and stimulated as indicated. After 10 or 20 min, total cell lysates were prepared, separated on an SDS gel and blotted onto PVDF membranes. The blots were probed with anti-phospho-Erk1/2 and stripped and reprobed with anti-Erk1/2 to detect Erk1/2 protein. Phosphorylated PDGF β -R in the same blot was detected as a band that appeared upon PDGF-stimulation by stripping and reprobing with anti-pY. **(D)** Erk1/2 proteins were immunoprecipitated from lysates of foreskin fibroblasts treated as in (C) and subjected to a kinase assay with PhasI protein as a substrate. The products were separated on an SDS gel and subjected to autoradiography. The difference between cells plated on vitronectin and collagen was statistically significant ($n = 6$, t -test; $P < 0.01$).

kinase activity than the same cells cultured on type I collagen (Figure 7C and D). The difference was most obvious 20 min after PDGF-BB addition. Tyrosine phosphorylation of the PDGF β -R itself was not dependent on the substrate in several experiments (Figure 7C). Moreover, $\alpha v \beta 3$ and PDGF β -R co-immunoprecipitated both on collagen and vitronectin (not shown). These data indicate that PDGF-BB signaling is dependent on the integrin a cell uses for attachment to a matrix and that the matrix and the integrin influence signal transduction downstream of the PDGF β -R.

Discussion

The main findings in this work are that only a small subset of each of IR and PDGF β -R is tyrosine-phosphorylated upon growth factor stimulation, that this subset can associate with the $\alpha v \beta 3$ integrin, and that PDGF activity is enhanced in cells that are plated on an $\alpha v \beta 3$ ligand.

In addition to the 190 kDa protein and IRS-1 described previously in association with $\alpha v \beta 3$ (Bartfeld *et al.*, 1993; Vuori and Ruoslahti, 1994), we detected several other

tyrosine-phosphorylated proteins in $\alpha v \beta 3$ immunocomplexes from cells stimulated with insulin or PDGF-BB. In insulin-stimulated cells, one of these proteins was shown to be the β -subunit of IR. We had not found IR β associated with $\alpha v \beta 3$ in our earlier study, because the anti-pY antibodies used in that work react poorly with IR β .

The 190 kDa phosphoprotein that we observed associated with $\alpha v \beta 3$ in PDGF-stimulated cells was identified as PDGF β -R. Several lines of evidence, including immunoprecipitation of the protein with two anti-PDGF β -R antibodies and its size, support the identification. Moreover, deglycosylation of the $\alpha v \beta 3$ -associated 190 kDa component showed that most of it is likely to represent PDGF β -R, because all detectable anti-pY-reactivity shifted to the deglycosylated PDGF β -R position.

The deglycosylation analysis also allowed us to discriminate between glycosylated transmembrane proteins and non-glycosylated cytoplasmic signaling molecules. We showed that the anti-phosphotyrosine-reactive 190 kDa band in the anti-Ras-GAP immunocomplex consists of two co-migrating proteins, the transmembrane protein PDGF β -R and the cytoplasmic phosphoprotein pp190Rho-GAP.

Accordingly, we could exclude the presence of significant amounts of pp190Rho-GAP in α v β 3 complexes.

There are several reports of hitherto unidentified 190 kDa phosphoproteins in PDGF-stimulated cells, found by immunoprecipitating with anti-FAK (Chen and Guan, 1996), anti- α v β 3 (Bartfeld *et al.*, 1993) or anti-caveolin (Liu *et al.*, 1996) antibodies. In each case, these proteins have been reported not to be reactive with anti-PDGF β -R antibodies. Our results show that only a small fraction of the PDGF β -R becomes tyrosine-phosphorylated upon PDGF-stimulation and that the small amount of protein in this subfraction is difficult to detect with anti-PDGF β -R. These results suggest that it will be necessary to study whether the 190 kDa proteins might, at least partly, represent a highly phosphorylated subfraction of the PDGF β -R. Further study will also be needed to determine how the non-uniform phosphorylation of IR and PDGF β -R is brought about. One interesting possibility is that the integrin association might protect the receptors against dephosphorylation by phosphatases, thus maintaining high receptor activity.

The association of activated IR and PDGF β -R with integrins was specific for the α v β 3 integrin. We never observed significant amounts of any tyrosine-phosphorylated proteins in anti- β 1 integrin immunocomplexes. A similar integrin specificity has been noted previously in integrin association with IRS-1 and with the 190 kDa protein of Bartfeld *et al.* (1993), shown here to consist primarily of PDGF β -R. Because all α v β 3 antibodies we tested did not co-precipitate growth factor receptors, we cannot completely exclude that the several anti- β 1 antibodies used in this study might not have been able to recognize possible β 1 integrin-growth factor receptor complexes. However, the functional data showing enhanced insulin (Vuori and Ruoslahti, 1994) and PDGF activity (this work) in cells plated on vitronectin support a specific interaction between α v β 3 integrin and the two growth factor pathways.

The nature of the connection between the α v β 3 integrin and the growth factor receptors remains to be elucidated, but the interaction may take place via the actin cytoskeleton. We have found that both α v β 3 and the activated PDGF β -R are associated with a cytoskeletal (NP-40 insoluble) fraction in PDGF-stimulated cells (M.Schneller and E.Ruoslahti, manuscript in preparation). Since the receptors associated with α v β 3 only after stimulation with the appropriate growth factor, it is likely that phosphotyrosine-dependent interactions with SH2 or PTB domains are involved. The SH2 or PTB domains of the various IR and PDGF β -R-associated signaling molecules may also bind directly to the α v β 3 cytoplasmic domain. Indeed, we have shown that a recombinant Shc PTB domain can bind to the cytoplasmic tail of β 3 integrin in which a tyrosine residue of the two NPXY sequences is phosphorylated (M.Schneller and E.Ruoslahti, unpublished results). Phosphorylation of the tyrosine in both of these sequences in the β 3 cytoplasmic tail has recently been demonstrated (Law *et al.*, 1996). However, we consider an involvement of β 3 phosphorylation unlikely, because we have not seen significant phosphorylation of β 3 in the cells used in these experiments.

PDGF β -R was tyrosine-phosphorylated to the same approximate degree on vitronectin and type I collagen in

several experiments, excluding the possibility that the collagen substrate might have in some way interfered with the binding of PDGF with its receptor. Thus, the integrin-matrix interaction seems to influence the efficiency of PDGF signaling at a level downstream of the PDGF receptor. As integrins engaged in substrate adhesion become clustered in focal adhesions, growth factor receptors may co-cluster with α v β 3, and the clustering may enhance signaling efficiency of the growth factor receptors. The presence of other kinases and kinase substrates at high concentrations in focal adhesions may also enhance growth factor activity. In contrast, engagement of the integrin in cell attachment was not needed for the PDGF β -R association.

The strong mitogenic and chemotactic effects ascribed to PDGF (Heldin, 1992) have been observed using cells cultured in the presence of serum. As vitronectin is the main adhesion protein in fetal calf serum, such cells were, in effect, cultured on vitronectin (Hayman *et al.*, 1985). Thus, these *in vitro* experiments may be representative of those *in vivo* situations where α v β 3 provides a co-stimulus for PDGF signaling. As we show here, the co-stimulation enhances PDGF β -R activity. Interestingly, vitronectin is released by platelets together with PDGF at sites of platelet activation (Seiffert and Schleef, 1996). Moreover, in type I collagen or laminin, a cryptic RGD becomes available for α v β 3 binding as a result of proteolysis associated with inflammation and tumor invasion (Sonnenberg *et al.*, 1990; Montgomery *et al.*, 1994). Thus, it is possible that α v β 3 plays a supportive role in pathophysiological events involving PDGF. The α v β 3 integrin is expressed in angiogenic blood vessels (Friedlander *et al.*, 1995), in certain metastatic cells (Gehlsen *et al.*, 1992; Leavesley *et al.*, 1992) and in migrating smooth muscle cells (Liaw *et al.*, 1995; Jones *et al.*, 1996). Moreover, the synergistic effect of α v β 3 engagement on PDGF signaling may be pharmacologically relevant when PDGF is used as a wound healing agent (Pierce *et al.*, 1989); it may be necessary to provide the α v β 3 co-signal to achieve an optimal PDGF effect.

Materials and methods

Antibodies

Anti- α v β 3 and α 5 β 1 integrin antibodies were polyclonal antisera (Suzuki *et al.*, 1986; Vuori and Ruoslahti, 1994). Anti-Ras-GAP monoclonal antibody and polyclonal anti-PDGF β -R (#1) anti-IR and anti-Erk1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-PDGF β -R (#2) and monoclonal anti-PLC- γ were from UBI (Lake Placid, NY). The monoclonal anti-p85 PI3K, anti-SHP2 and anti-Rho-GAP were from Transduction Labs (Lexington, KY). Polyclonal affinity-purified anti-phosphotyrosine antibodies were a gift from Dr E.Pasquale (this institute).

Immunoprecipitations and immunoblotting

NIH 3T3 and Rat 1 cells were grown to 90% confluency in 10% FCS in DMEM containing glutamine and antibiotics. A culture dish with about 5×10^6 cells was starved in medium without serum for 16 h and then stimulated with either 40 ng/ml PDGF-BB, 100 nM insulin or 100 ng/ml EGF (Sigma, St Louis, MO) for 3 min or left unstimulated. After 3 min the cells were lysed in NP-40 buffer [1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10% glycerol, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.1 U/ml), leupeptin (10 μ g/ml) and pepstatin A (4 μ g/ml)], and then centrifuged for 10 min at 15 000 g. The supernatants were precleared with Gammabind Sepharose G (Pharmacia, Uppsala, Sweden) for 1 h and then incubated with the antibodies for 2 h. Integrins were immunopre-

precipitated with various antibodies (Vuori and Ruoslahti, 1994). The complexes were precipitated with Gammabind Sepharose G for 1 h, the Sepharose beads were washed three times with the lysis buffer and then boiled for 5 min in SDS-PAGE sample buffer containing 50 mM DTT.

The eluted samples were separated on 4–12% SDS-PAGE precast gels (Novex, San Diego, CA) and electroblotted to PVDF membranes. The blots were probed with anti-phosphotyrosine antibodies followed by detection of bound antibodies with protein A-horseradish peroxidase conjugate and enhanced chemiluminescence (ECL). For reprecipitation analysis the Gammabind Sepharose beads were boiled for 5 min in 2% SDS buffered with 50 mM Tris-HCl, pH 7.5, the supernatants were diluted 1:20 in NP-40 buffer and proteins were precipitated with various antibodies and Gammabind Sepharose. Proteins were eluted, separated and probed for phosphotyrosine as described above.

Surface biotinylation was carried out using the membrane-impermeable biotinylation reagent Sulfo-NHS-biotin (Pierce, Rockford, IL). Adherent cells in a culture dish on ice were overlaid with 1 mg/ml sulfo-NHS-biotin in PBS for 20 min. After washing the cells once with PBS, the residual NHS groups were reacted with 0.1 M glycine/PBS on ice for 10 min. After washing, the cells were lysed in NP-40 lysis buffer, and immunoprecipitation, electrophoresis and blotting were carried out as described above. The membranes were probed with avidin-peroxidase (Sigma, St Louis, MO) at 1:5000 and developed with ECL.

Cell mitogenicity and mobility assay

After overnight starvation in 0.3% FCS/DMEM, human foreskin fibroblasts (Coriell, Camden, NJ) were transferred to 24-well plates (1×10^5 cells/well) that had been coated with 10 µg/ml of vitronectin [purified after the method of Yatohgo *et al.* (1988) or type I collagen; Collaborative Biomedical Products, Bedford, MA]. Cell adhesion was shown to be equal at this concentration of vitronectin and type I collagen (data not shown). Cells were stimulated at a final concentration of 40 ng/ml PDGF-BB in DMEM and thymidine incorporation was measured (Vuori and Ruoslahti, 1994). Controls showed that adhesion to vitronectin was inhibited by anti- $\alpha v \beta 3$ but not anti- $\beta 1$, and adhesion to collagen was inhibited by anti- $\beta 1$ antibodies (Suzuki *et al.*, 1986; Vuori and Ruoslahti, 1994). Cells were $\geq 85\%$ viable on both vitronectin and collagen after 24 h of serum-starvation. Similar to mouse and rat fibroblasts, a tyrosine-phosphorylated 190 kDa band was found to co-precipitate with the $\alpha v \beta 3$ integrin in human fibroblasts following PDGF stimulation (data not shown).

Cell motility assay was performed using a modified Boyden chamber. The undersurface of polycarbonate filter was coated with 10 µg/ml of vitronectin or type I collagen. Lower chambers were filled with DMEM containing 0.1% BSA, or with DMEM containing 0.1% BSA and 40 ng/ml of PDGF-BB. Human foreskin fibroblasts (1×10^5 cells) were added to the upper chambers in DMEM containing 0.1% BSA. The cells were incubated at 37°C for the indicated times, after which the membranes were fixed and stained with 0.5% toluidine blue. The number of cells that had migrated to the lower surface were counted. Four high-magnification microscopic fields were analyzed per well; all experiments were performed in quadruplicate.

Measurement of extracellular signal-regulated kinase activation

Human foreskin fibroblasts were grown to 90% confluency in 10% FCS in DMEM containing glutamine and antibiotics and starved in medium without serum for 16 h. Cells were then trypsinized, washed in the presence of trypsin-inhibitor and resuspended in medium without serum. The cells were re-plated on six-well plates (1×10^5 cells/well) that had been coated with 10 µg/ml of vitronectin or type I collagen. After 6 h the cells were stimulated with 40 ng/ml PDGF-BB for 20 min, washed in PBS and then lysed in NP-40 lysis buffer and centrifuged for 10 min at 15 000 g. Erk1/2 activities were measured in two different ways. First, by separating the lysates by SDS-PAGE followed by blotting and probing with antibodies against the activated forms of Erk1/2 (New England Biolabs, Beverly, MA). Total Erk1/2 was then estimated by stripping the blots and reprobing with anti-Erk1/2 antibodies (Santa Cruz Biotechnology). Second, Erk1/2 was immunoprecipitated with anti-Erk2 antibodies and with Gammabind Sepharose G, and washing of the Gammabind twice with lysis buffer and once with kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂). Erk1/2 activity bound to Gammabind was measured by incubation with kinase reaction buffer containing 2 µCi [γ -³²P]ATP and 5 µg Phas I substrate protein (Promega, Madison, WI) for 20 min at room temperature, followed by analysis by SDS-PAGE. Quantification was performed using NIH Image 1.54.

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