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Stimulation of tyrosine phosphorylation of distinct proteins in response to antibody-mediated ligation and clustering of α_3 and α_6 integrins

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SUMMARY

The interaction of cells with components of the extracellular matrix through their integrin receptors results in the stimulation of tyrosine phosphorylation of several proteins, suggesting that these receptors play a key role in signal transduction. Here we report that antibody-mediated ligation and clustering of $\alpha_3\beta_1$ and $\alpha_6\beta_1/\alpha_6\beta_4$ integrins resulted in the stimulation of tyrosine phosphorylation of proteins that are specific for each heterodimer. Thus, ligation and clustering of the $\alpha_3\beta_1$ integrin on human prostate carcinoma cells (PC-3) and human umbilical vein endothelial cells (HUVEC) with anti-α_3 antibodies resulted in the stimulation of tyrosine phosphorylation of a 55 kDa protein. In contrast, ligation and clustering of the $\alpha_6\beta_1$ integrin on these cells with anti-α_6 antibody resulted in the dramatic stimulation of tyrosine phosphorylation of a 90 kDa protein in addition to a 52 kDa protein, and ligation and clustering of $\alpha_5\beta_1$ on HUVEC did not result in the apparent stimulation of tyrosine phosphorylation of any proteins. Clustering with anti-β_1 antibodies triggered the tyrosine phosphorylation of all of these proteins, whereas ligation and clustering of PC-3 cells with an anti-β_4 antibody resulted in the tyrosine phosphorylation of a distinct 62 kDa protein. Since the PC-3 cells express both $\alpha_6\beta_1$ and $\alpha_6\beta_4$, these data suggest that these two receptors can transduce distinct signals. All of the phosphorylations could be inhibited by treating the cells with Genistein, a tyrosine kinase inhibitor. Antibody-mediated ligation and clustering of integrins on the two types of cells did not result in the stimulation of tyrosine phosphorylation of pp125 focal adhesion kinase, although this was observed upon cell attachment and spreading on fibronectin, laminin and anti-α_3 monoclonal antibody. Collectively, these data demonstrate that cross-linking of different integrin heterodimers can stimulate tyrosine kinase activities, leading to the phosphorylation of distinct proteins, which are also different from those observed when cells are allowed to spread on a matrix.

Key words: clustering, tyrosine phosphorylation, integrin, signal transduction

INTRODUCTION

Integrins are a family of heterodimeric cell surface receptors composed of one α and one β subunit, each of which has a large extracellular domain, a single transmembrane domain, and usually a short cytoplasmic tail (Hynes, 1992; Ruoslahti and Pierschbacher, 1987). The 14 distinct α subunits and 8 β subunits can associate in various combinations to form integrin heterodimers with a variety of ligand binding specificities (Hynes, 1992). The ligands of the integrins can be components of the extracellular matrix (ECM), e.g. fibronectin, laminin, collagen and vitronectin (Hynes, 1992; Dedhar, 1990; Ruoslahti and Pierschbacher, 1987), or they can be other integral plasma membrane proteins (Hynes, 1992; Springer, 1990) including other integrins (Symington et al., 1993).

Many different studies, utilizing various cell types, have demonstrated that integrins can function as signal transducers of extracellular stimuli which modulate cell proliferation, regulate cell differentiation and induce cell migration. Thus, adhesion of suspension-arrested fibroblasts to fibronectin induces c-*fos* and c-*myc* expression (Dike and Farmer, 1988), and adhesion of keratinocytes to fibronectin results in cell-cycle withdrawal and inhibition of terminal differentiation (Adams and Watt, 1989). Myoblast differentiation and myotube formation require β_1 integrin function (Menko and Boettiger, 1987) as does the interleukin-1-induced osteoblastic differentiation of osteosarcoma cells (Dedhar, 1989). Furthermore, the expression of various integrins is upregulated during neuronal differentiation (Dedhar et al., 1991, 1993a; Rossino et al., 1990). Engagement of the $\alpha_5\beta_1$ integrin in fibroblasts also results in an induction of the expression of collagenase and stromelysin genes (Werb et al., 1989). Perhaps the strongest evidence for signal transduction via integrins comes from work carried out with T-lymphocytes and monocytes. Adherence of $CD4^+$ T cells to fibronectin via $\alpha_4\beta_1$ or $\alpha_5\beta_1$ integrins (Nojima et al., 1990; Shimizu et al., 1990), or to laminin via the $\alpha_6\beta_1$ integrin (Shimizu et al., 1990), provides co-stimulatory signals for CD3-dependent T-cell proliferation. Engagement of the β_2 integrin, LFA-1, with ICAM-1 or with anti-LFA monoclonal antibodies also demonstrates that these receptors act as co-stimulators of T-cell activation (van Seventer et al., 1991a,b). Monocyte adhesion to various

ECM proteins can differentially regulate the levels and types of monocyte-adherence-derived (MAD) inflammatory genes. Furthermore, monoclonal antibody engagement of β_1 integrins on monocytes also results in the stimulation of expression in the inflammatory mediating genes (Yurochko et al., 1992).

Although the intracellular signalling pathways induced by integrin occupation have not been well characterized as yet, preliminary evidence from various laboratories has demonstrated that the activation of the Na^+/H^+ antiporter (Schwartz et al., 1991; Juliano and Haskill, 1993), calcium influx and mobilization (Juliano and Haskill, 1993), activation of the transcription factors NF-κB (Juliano and Haskill, 1993) and AP1 (Yamada et al., 1991) and stimulation of tyrosine phosphorylation (Guan et al., 1991; Kornberg et al., 1991) are involved. Adhesion of various types of cells on different ECMs can result in the tyrosine phosphorylation of paxillin, a component of the actin cytoskeleton (Burridge et al., 1992), as well as of pp125 focal adhesion kinase (FAK), a tyrosine kinase which is a substrata for $pp60^{src}$ and is found in focal adhesion plaques (Schaller et al., 1992; Kornberg et al., 1992; Guan and Shalloway, 1992; Burridge et al., 1992).

Monoclonal antibody-mediated integrin ligation and clustering has also been utilized to investigate integrin-mediated signalling events. Thus, the clustering of the fibronectin receptor integrin, $\alpha_5\beta_1$, in fibroblasts results in an increase in the intracellular pH (Schwartz et al., 1991), and the clustering of $\alpha_3\beta_1$ with either anti-α_3 or anti-β_1 monoclonal antibodies in human KB cells results in the stimulation of tyrosine phosphorylation of $pp125^{FAK}$ (Kornberg et al., 1991, 1992). The clustering of $\alpha_4\beta_1$ on T-lymphocytes, on the other hand, results in the stimulation of phosphorylation of an as yet unidentified 105 kDa protein (Nojima et al., 1992). The stimulation of the tyrosine phosphorylations of $pp125^{FAK}$ and the pp105 proteins do not appear to be specific for antibody-mediated integrin clustering, as the phosphorylation of both these proteins is also stimulated upon cell adhesion to the appropriate ECM substrata (Nojima et al., 1992; Kornberg et al., 1992; Burridge et al., 1992; Guan and Shalloway, 1992). Recently it has been shown that the activation of the $\alpha_2\beta_1$ integrin on T-lymphoblastic cells with activating anti-β_1 and anti-α_2 monoclonal antibodies results in the stimulation of tyrosine phosphorylation of 47 and 52 kDa proteins, as well as the activation of $p21^{ras}$ (Kapron-Bras et al., 1993).

The present study addresses the question of whether specific signals may be transmitted by different integrin heterodimers. We provide evidence for the stimulation of tyrosine phosphorylation of distinct 55, 52, 90 and 62 kDa proteins upon ligation and clustering of the $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins, respectively, in human prostate carcinoma cells (PC-3) and human umbilical vein endothelial cells (HUVEC). These proteins are different from $pp125^{FAK}$ and p105, and their tyrosine phosphorylation stimulation is specific for individual integrins.

MATERIALS AND METHODS

Materials

Monoclonal antibodies against the integrins α_3, (P1B5), α_5 (P1D6), and β_4 (3E1) were purchased from Telios Pharmaceuticals, La Jolla, CA, as were human fibronectin and laminin. The anti-α_6 (JIB5) and anti-β_1 (A11B2) monoclonal antibodies were a kind gift from Dr Caroline Damsky, University of Southern California, San Francisco. The anti-β_1 (DH-12) antibody was a kind gift from Dr Cassiman, Belgium. The mouse monoclonal antibody (J143), which was raised against α_3, was obtained from Dr Albino (New York, NY). The anti-phosphotyrosine antibody, PY-20, was purchased from ICN Immunobiologicals, Costa Mesa, CA. Horseradish peroxidase, fluorescein isothiocyanate (FITC) and tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated and unconjugated secondary antibodies, and anti-mouse and anti-rat IgGs (H + L), were from Jackson ImmunoResearch Laboratories (West Grove, PA). The polyclonal (BC3) antibody against the pp125 focal adhesion kinase was a kind gift from Dr Parsons, Virginia. ECL western blotting reagents were obtained from Amersham Corp., Oakville, Ontario. Protein A-Sepharose and poly-L-lysine were purchased from Sigma (St Louis, MO). Genistein, a tyrosine kinase inhibitor, was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and *o*-phospho-L-tyrosine was obtained from Boehringer Mannheim (Laval, Quebec).

Cells and growth conditions

PC-3 human prostate carcinoma cells obtained from American Type Culture Collection (ATCC) were cultured in DMEM (high glucose), supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, UT). Human umbilical vein endothelial cells (HUVEC), obtained from Clonetics Corp., San Diego, CA, were cultured in MCDB 131 medium (JRH Biosciences, Lenesca, KS) supplemented with 10% FBS, 5 ng/ml human recombinant basic fibroblast growth factor (Upstate Biotechnology, Inc.), 1 μg/ml hydrocortisone (Sigma), 10 ng/ml epidermal growth factor (Upstate Biotechnology, Inc.), and 10 units/ml heparin (porcine, Sigma). For routine subculturing of cells, monolayers were washed with PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.3) and detached with EDTA (5 mM).

Preparation of detergent lysates from cells plated on various substrata

Tissue culture plates (35 mm) were coated overnight with laminin (5 μg/ml), fibronectin (20 μg/ml), or poly-L-lysine (20 μg/ml) at 4°C. The coated plates were washed twice with PBS, and blocked with 2 mg/ml BSA in PBS for 2 hours at 37°C. Cells were harvested from culture and resuspended in serum-free DMEM, or MCDB131 containing 2 mg/ml BSA and plated onto the ECM-coated plates at 1.5×10^6 cells/plate. In some cases 0.5 mM sodium vanadate was added at this point. Control, unplated cells were kept in suspension in a 1.5 ml tube. The cells were incubated at 37°C and at various times thereafter the medium was removed and the cells were washed twice with cold PBS. Cell lysis was carried out in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA, 2 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 0.5 mM sodium vanadate, and 10 mg/ml nitrophenylphosphate). The cell lysates were then passed through (8 times) a syringe with a 25 gauge needle and centrifuged for 10 minutes at 4°C in a microfuge. Protein concentrations of the supernatants were determined using the BioRad Protein Assay (Bio-Rad, Richmond, CA).

SDS-PAGE and western blotting

Cell lysates or immunoprecipitates were electrophoresed on 6% or 7.5% SDS-polyacrylamide gels under reducing (5% 2-mercaptoethanol) or non-reducing conditions by the method of Laemmli (1970). Prestained molecular mass markers (Bio-Rad) were myosin (205 kDa), β-galactosidase (116 kDa), bovine serum albumin (80 kDa), and ovalbumin (50 kDa). The resolved proteins were electrophoretically transferred onto PVDF membranes (ImmobilonP, Millipore, Bedford, MA) (Towbin et al., 1979), and the phosphotyrosine-containing proteins were detected using an anti-phosphotyrosine antibody (PY-20) together with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse or rabbit anti-mouse) and an Enhanced Chemiluminescence (ECL) detection system (Amersham Corp.) used as per the instructions of the manufacturers.

Control blots were probed with HRP-conjugated secondary antibody alone, in the absence of the anti-phosphotyrosine antibody.

Immunoprecipitations

Lysate supernatants containing equivalent amounts of protein were immunoprecipitated with anti-FAK antibodies. The lysates were incubated overnight at 4°C with the primary antibody. Protein A-Sepharose was added and after a 2 hour incubation the immune complexes were washed several times with RIPA buffer (see above). The immune complexes were eluted with SDS-PAGE sample buffer and the supernatants analyzed by SDS-PAGE as described above.

Integrin clustering and immunofluorescence

PC-3 and HUVEC cells were harvested from culture and allowed to grow on 35 mm tissue culture plates overnight at 37°C at an initial cell density of 7.5×10^5 cells/plate. For antibody-mediated clustering, the cells were washed twice with ice-cold serum-free DMEM- (for PC-3 cells), or MCDB- (for HUVEC cells) containing 20 mM Hepes buffer, pH 7.2, and 0.5 mM sodium vanadate. The cells were then incubated for 30 minutes at 4°C with the appropriate anti-integrin primary antibodies (approximately 50 μg/ml) or control immunoglobulins diluted in the serum-free medium. The primary antibodies were then removed and the plates were rinsed twice with ice-cold medium. The cells were then further incubated with the appropriate secondary antibodies (100 μg/ml) for 10 minutes at 37°C (clustering). Control plates (no clustering) were incubated with medium alone. The plates were then placed on ice, the secondary antibody was removed, and the cells were lysed with the lysis buffer described above. For inhibition of tyrosine kinase activity, the cells were exposed to 60 or 100 μM Genistein (Akiyama et al., 1987; Markovits et al., 1989) for 1.5 hours prior to the addition of the primary antibody. Genistein was also present during the clustering process. For immunofluorescence visualization of clustering, the cells were treated exactly as above except that they were grown on glass coverslips and the secondary antibody used for clustering was conjugated with FITC.

RESULTS

Antibody-mediated ligation and clustering of $\alpha_3\beta_1$ and $\alpha_6\beta_1/\beta_4$ integrins on PC-3 and HUVEC cells results in tyrosine phosphorylation of distinct proteins

In order to determine whether direct ligation and cross-linking (clustering) of integrins results in the stimulation of tyrosine phosphorylation of proteins, integrins $\alpha_5\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ were ligated and clustered with anti α-subunit- or anti-β-subunit-specific monoclonal antibodies, followed by appropriate secondary antibodies as described in Materials and Methods. As shown in Fig. 1, antibody cross-linking of various integrins on HUVEC resulted in the clustering of these integrins. Clustering was achieved with each of the antibodies used for the analysis of tyrosine phosphorylation, and no significant differences were observed in the type or extent of clustering with the different antibodies.

We next examined whether clustering of the integrins on HUVEC or PC-3 cells resulted in the stimulation of tyrosine phosphorylation. As shown in Fig. 2A, in HUVEC clustering with an anti-α_6 antibody (J1B5) resulted in the tyrosine phosphorylation of a 52 kDa protein, as well as a 90 kDa protein, as determined by western blot analysis of cell extracts with an anti-phosphotyrosine antibody (PY-20). Surprisingly, the tyrosine phosphorylation of the 52 kDa protein was stimulated, as compared to IgG controls, with only the primary anti-integrin antibody, in the absence of the induction of clustering with the secondary antibody. The tyrosine phosphorylation of the 90 kDa protein was stimulated only after cross-linking and clustering of the integrins upon addition of a secondary antibody. As can be seen in Fig. 2A, neither the 52 kDa nor the 90 kDa proteins became phosphorylated on tyrosine when the $\alpha_5\beta_1$ integrin was clustered with an anti-α_5 antibody (PID5). The 53 kDa protein that became phosphorylated was not specific for clustering of $\alpha_5\beta_1$, since it is also seen in the cells that were incubated with non-specific mouse or rat IgG plus secondary antibody. The differences in tyrosine phosphorylation that occurred upon cross-linking with anti-α_5 and anti-α_6 antibodies cannot be explained by differences in the extent of clustering, as immunofluorescence detection reveals them to be quite similar (Fig. 1B compared to 1C).

In PC-3 cells, clustering of the $\alpha_3\beta_1$ integrin with an anti-α_3 antibody (P1B5) resulted in the tyrosine phosphorylation of a 55 kDa protein (Fig. 2B). This response was seen with the primary antibody alone, but was substantially enhanced upon the induction of clustering with the secondary antibody. Phosphorylation of a 55 kDa protein was also stimulated when PC-3 cells were clustered with another anti-α_3 antibody, J143 (data not shown). Clustering of the α_6 integrins in PC-3 cells leads to the phosphorylation of the 52 and 90 kDa proteins, as it did in HUVEC. Western blot analysis of identical samples from PC-3 cells with the HRP-conjugated secondary antibody alone, i.e. in the absence of the primary anti-phosphotyrosine antibody, did not result in the detection of the 52 kDa, 55 kDa or 90 kDa proteins. As well, these proteins were not observed when the primary anti-phosphotyrosine antibody was pre-incubated with *o*-phosphotyrosine before it was added to the blot (data not shown). These controls confirm the specific detection of tyrosine-phosphorylated proteins.

Since α_6 can associate with either of two β subunits, β_1 or β_4, and since PC-3 cells express both heterodimers on their cell surface (Dedhar et al., 1992, 1993b), we wanted to determine which α_6 heterodimer was responsible for the observed induction of tyrosine phosphorylation. As shown in Fig. 2C, clustering with anti-β_1 antibody (DH12) resulted in the stimulation of tyrosine phosphorylation of the 52 kDa, 55 kDa, as well as the 90 kDa proteins. Once again the tyrosine phosphorylation of the 52 kDa and 55 kDa proteins was stimulated with the primary anti-β_1 antibody alone, whereas the tyrosine phosphorylation of the 90 kDa protein was stimulated only upon clustering with the secondary antibody. Clustering with the anti-β_1 antibody did not result in an enhancement of phosphorylation of the 55 kDa protein, compared to stimulation with primary antibody alone, as was observed upon clustering with the anti-α_3 antibody. Clustering with another anti-β_1 antibody (AIIB2) gave similar results (data not shown).

Clustering of the $\alpha_6\beta_4$ integrin on PC-3 cells with an anti-β_4 antibody (3E1) resulted in the stimulation of phosphorylation of yet another distinct 62 kDa protein, as shown in Fig. 2C. The stimulation of tyrosine phosphorylation of this protein was dependent on cross-linking with the secondary antibody and did not occur with the primary antibody alone. Since clustering with the anti-β_4 antibody did not stimulate phosphorylation of the 90 kDa protein, the induction of tyrosine phosphorylation of this protein appears to be a unique property of the $\alpha_6\beta_1$ integrin, whereas the phosphorylation of the 62 kDa protein appears to be unique to the β_4 subunit.

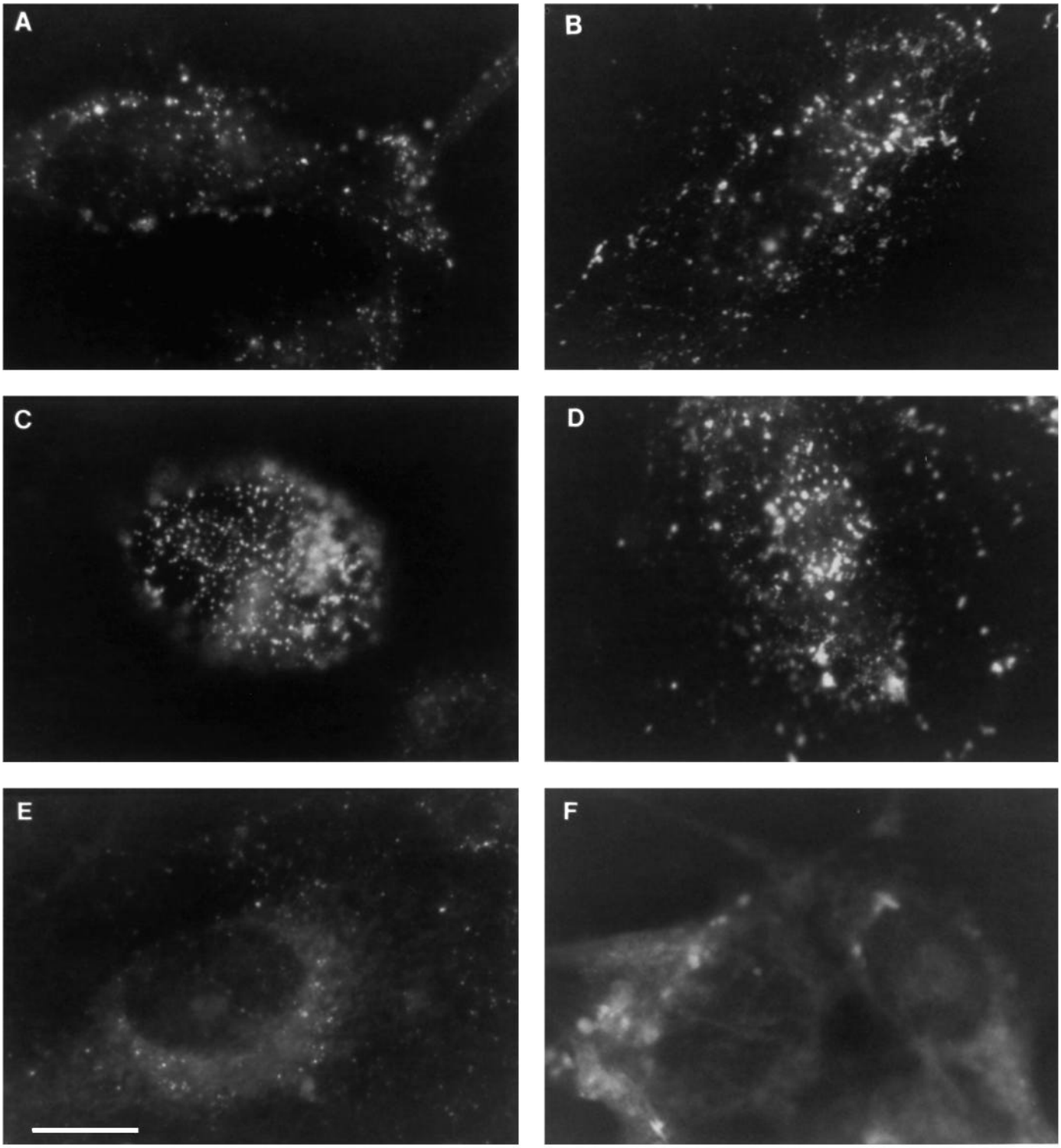

Fig. 1. Immunofluorescence visualization of antibody-mediated clustering of integrins on HUVEC. The integrins were clustered utilizing subunit-specific monoclonal antibodies and the appropriate FITC-conjugated secondary antibodies as described in Materials and Methods. For microscopy, the coverslips were mounted on microscope slides with Gel/Mount (Biomeda Corp., Foster City, CA) and visualized using a Zeiss Axioscop microscope under oil immersion, and photographed with Kodak T-Max 400 film. Bar, 10 μm. (A) Clustered with mouse anti-human α_3 monoclonal antibody (P1B5); (B) clustered with mouse anti-human α_5 monoclonal antibody (P1D6); (C) clustered with rat anti-human α_6 monoclonal antibody (J1B5); (D) clustered with mouse anti-human β_1 monoclonal antibody (DH12); (E) clustered with anti-human β_4 monoclonal antibody (3E1); (F) treated in the absence of primary antibody (control).

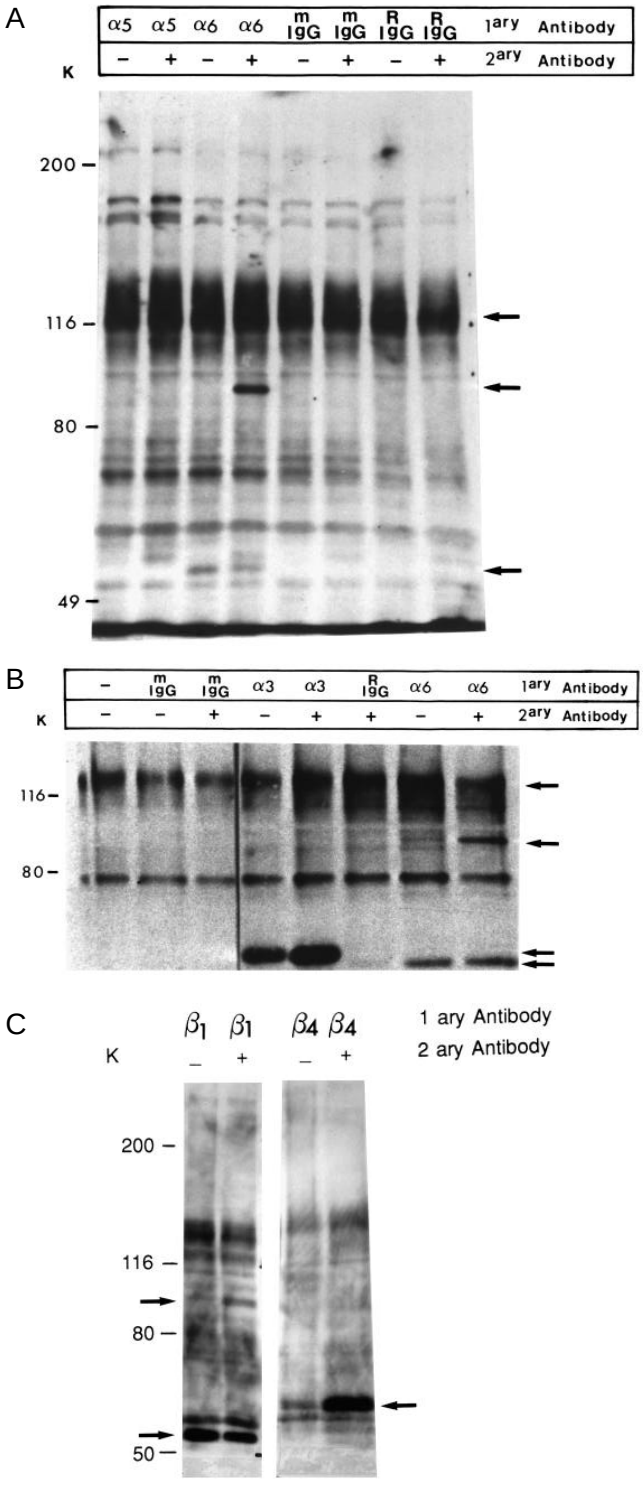

Fig. 2. Stimulation of tyrosine phosphorylation in response to antibody-mediated integrin clustering on HUVEC (A) and PC-3 cells (B) and (C). The cells were treated with the 3 indicated primary antibodies at 4°C followed by cross-linking with the appropriate secondary antibodies at 37°C as described in Materials and Methods. The lysates were analyzed by SDS-PAGE under reducing conditions and western blotted with an anti-phosphotyrosine monoclonal antibody (PY-20). The blots were visualized using Enhanced Chemiluminescence (ECL). The antibodies utilized are described in Materials and Methods. K, kDa.

Anti-integrin antibody-induced tyrosine phosphorylation results from the activation of tyrosine kinase activity

The stimulation of the tyrosine phosphorylation of the various proteins upon antibody-mediated ligation and clustering of integrins could come about as a result of activation of tyrosine kinase activity or of inhibition of tyrosine phosphatase activity. In order to distinguish between these two alternatives, tyrosine kinase activity was inhibited prior to and during the clustering experiment by treating PC-3 cells with the tyrosine kinase inhibitor, Genistein (Akiyama et al., 1987; Markovits et al., 1989). As shown in Fig. 3, tyrosine phosphorylation is inhibited by Genistein (60 μM). The enhanced tyrosine phosphorylation of the 55 kDa protein induced upon clustering with anti-α_3 antibody (P1B5) is inhibited by Genistein (Fig. 3A). The anti-α_6 antibody-induced tyrosine phosphorylation of the 52 kDa protein is also completely inhibited upon treatment of cells with Genistein, as is the tyrosine phosphorylation of the 90 kDa protein, which is phosphorylated upon anti-α_6 antibody-mediated clustering (Fig. 3B). Tyrosine phosphorylation induced upon clustering with anti-β_1 and anti-β_4 antibodies was also inhibited by Genistein (data not shown). These results demonstrate that either antibody ligation, or cross-linking and clustering of the integrins, results in the stimulation of tyrosine kinase activity and thus the tyrosine phosphorylation of specific proteins.

The effect of Genistein on integrin clustering was investigated to ensure that the observed inhibition of tyrosine phosphorylation was not simply due to inhibition of clustering. As such, PC-3 cells were pre-treated with Genistein in an identical manner, followed by addition of anti-β_1 (DH12) antibody, and the appropriate rhodamine-conjugated secondary antibody. Immunofluorescence detection revealed that there is no difference in the ability of anti-β_1 antibodies to ligate and cluster integrin receptors in the untreated cells (Fig. 4C,D) versus the Genistein-treated cells (Fig. 4A,B).

Anti-integrin antibody-mediated ligation and clustering does not stimulate tyrosine phosphorylation of pp125^{FAK} in HUVEC and PC-3 cells

As can be seen from Figs 2 and 3, antibody-ligation and clustering of $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ resulted in the stimulation of tyrosine phosphorylation of distinct proteins of 52 kDa, 55 kDa, 62 kDa and 90 kDa. A pp125 tyrosine kinase has recently been characterized and found to localize in focal adhesion plaques (Schaller et al., 1992). This kinase, called focal adhesion kinase (pp125^{FAK}), is highly phosphorylated in v-*src*-transformed cells and has been demonstrated to become phosphorylated in response to fibronectin in various cell types (Burridge et al., 1992; Guan and Shalloway, 1992). We did not observe any apparent stimulation of tyrosine phosphorylation of proteins in the range of 120-130 kDa, but because Kornberg et al. (1991, 1992) have reported stimulation of tyrosine phosphorylation of pp125^{FAK} in response to $\alpha_3\beta_1$ clustering, we wanted to confirm whether or not FAK phosphorylation was also stimulated upon integrin clustering in our system. Thus, pp125^{FAK} was immunoprecipitated from cell lysates after anti-integrin antibody ligation and clustering and its tyrosine phosphorylation was analyzed by western blotting with anti-phos-

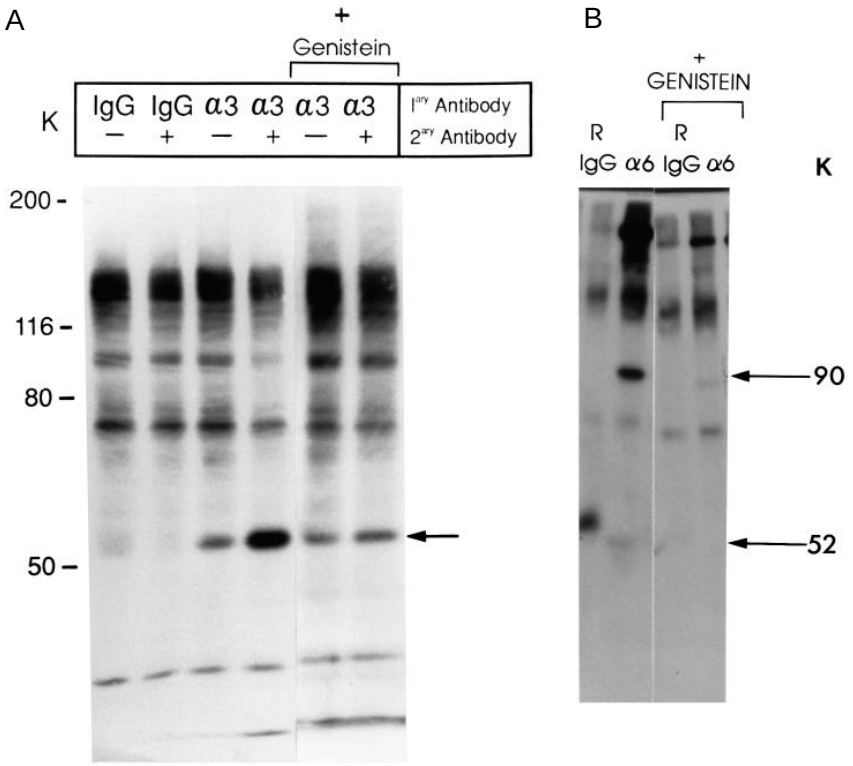

Fig. 3. Effect of the tyrosine kinase inhibitor, Genistein, on integrin ligation and clustering-induced tyrosine phosphorylation. PC-3 cells were treated for 1.5 hours with Genistein (60-100 μM) prior to the addition of the antibodies. Tyrosine phosphorylation was analyzed as described in Materials and Methods. (A) Effect of Genistein on tyrosine phosphorylation of the 55 kDa protein upon anti-α_3 antibody ligation (primary antibody only), and clustering (primary and secondary antibody). (B) Tyrosine phosphorylation of the 90 kDa and 52 kDa proteins in response to anti-α_6 antibody-mediated clustering (i.e. primary and secondary antibodies) in the absence and presence of Genistein. K, kDa.

photyrosine antibody. As shown in Fig. 5, there was no apparent stimulation of tyrosine phosphorylation of pp125^{FAK} upon integrin ligation and clustering with any of the antibodies in HUVEC, and similar results were obtained with PC-3 cells (data not shown). These experiments were carried out on cells that were well spread after having been plated overnight, which could explain the significant constitutive levels of pp125^{FAK} phosphorylation observed (Figs 2, 3 and 5). In order to ensure that this constitutive phosphorylation was not obscuring any additional signals, antibody-mediated ligation and clustering of α_3 and β_1 integrins was carried out in PC3 cells that were maintained in suspension. Even under these conditions, no increase in phosphorylation of pp125^{FAK} was seen, although the lower molecular mass proteins did become phosphorylated (data not shown). The data obtained so far suggest that antibody-mediated clustering of integrins on the surfaces of PC3 and HUVEC cells resulted in integrin-heterodimeric-specific tyrosine phosphorylation of distinct proteins (50-60 Kda and 90 kDa), and that at least in these cell types, the phosphorylation of pp125^{FAK} was not stimulated under these conditions. We therefore wanted to determine whether attachment and spreading on anti-integrin antibodies resulted in the stimulation of tyrosine phosphorylation of pp125^{FAK}. Indeed, as shown in Fig. 5B plating of PC3 cells on anti-α_3 antibody-coated plates resulted in the stimulation of tyrosine phosphorylation of a 125 kDa protein, likely to be FAK. The identity of the 220-230 kDa phospho-protein is not known at present. Tyrosine phosphorylation of 50-60 kDa proteins was not, however, stimulated relative to the IgG control. These results indicate that antibody-mediated clustering of integrins results in the inducution of a different signalling pathway, i.e. stimulation of tyrosine phosphorylation of distinct integrin-dependent proteins, whereas cell spreading, whether on anti-integrin antibodies or ECM substrata (see below), results in the stimulation of tyrosine phosphorylation of pp125^{FAK} and other as yet undefined proteins distinct from the 50-60 kDa and 90 kDa proteins.

Cell attachment and spreading of HUVEC and PC-3 cells results in the stimulation of tyrosine phosphorylation of pp125^{FAK} and pp130

Since we did not observe pp125^{FAK} phosphorylation after integrin clustering in PC3 cells or HUVEC, we examined whether this protein becomes phosphorylated when the cells are allowed to interact with the ECM substrata laminin or fibronectin, compared to non-adherent cells or those allowed to interact with poly-L-lysine. After various incubation time periods on the substrata or in suspension, the cells were lysed in situ and the proteins were analyzed by SDS-polyacrylamide gel electrophoresis and western blotting with an anti-phosphotyrosine antibody. As shown in Fig. 6A the tyrosine phosphorylation of two proteins (130 kDa and 125 kDa) was stimulated when PC-3 cells were allowed to attach to the ECM proteins. In the endothelial cells, the extent of phosphorylation was weaker and the lower molecular mass protein (pp125) was phosphorylated to a greater extent than the pp130 protein. The extent of phosphorylation in PC-3 cells was similar for all substrata, whereas for HUVEC, the extent of phosphorylation of pp125 was greater when plated on laminin and fibronectin as compared to poly-L-lysine (Fig. 6A). In contrast to antibody-mediated clustering, adhesion of cells to extracellular matrix proteins did not result in the phophorylation of the 50-60 kDa or 90 kDa proteins, either at the 60 minute timepoint shown in Fig. 6A, or at a 10 minute timepoint (data not shown). Although the presence of sodium vanadate in the lysis buffer

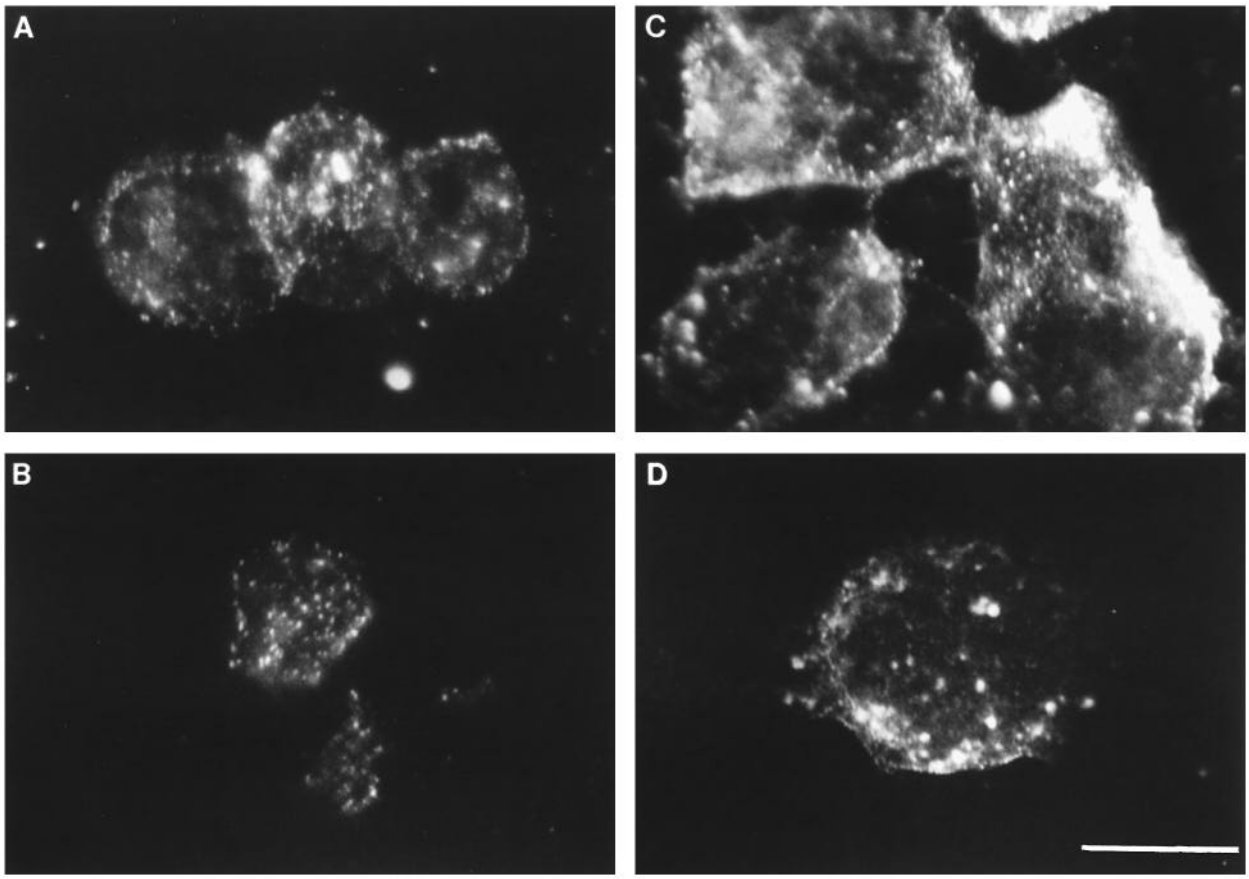

Fig. 4. Effect of Genistein on integrin clustering induced by anti-β_1 antibodies in PC-3 cells, as detected by immunofluorescence labelling. Cells were treated with 100 μM Genistein for 1.5 hours before incubation with antibodies. Clustering and detection of fluorescence was carried out as described in Fig. 1 and Materials and Methods. (C and D) Untreated cells; (A and B) Genistein-treated cells. Bar, 10 μm.

was critical, similar results were obtained whether vanadate was present or absent during cell adhesion.

As shown in Fig. 6B, immunoprecipitation with an anti-FAK antibody (BC3) followed by anti-phosphotyrosine western blotting demonstrated that the pp125 tyrosine-phosphorylated protein observed in Fig. 6A is pp125^{FAK}. It is interesting to note that, whereas the extent of tyrosine phosphorylation of pp125^{FAK} is similar in PC-3 cells plated on both ECM substrata, pp125^{FAK} was phosphorylated to a greater extent in HUVEC plated on fibronectin, followed by laminin, and to the lowest extent on poly-L-lysine. Morphological examination of the cells demonstrated that the extent of tyrosine phosphorylation correlated with the extent of cell spreading (data not shown). The identity of the 200 kDa phosphorylated protein co-immunoprecipitating with the anti-FAK antibody is not known at present.

DISCUSSION

The stimulation of protein tyrosine phosphorylation plays an important role in the transduction of signals via many plasma membrane receptor systems. Recently, signal transduction induced by the extracellular matrix has also been demonstrated to involve tyrosine phosphorylation, specifically of a 125 kDa protein, pp125^{FAK} (Burridge et al., 1992; Schaller et al., 1992; Guan and Shalloway, 1992). As cell surface receptors, it is likely that integrins can transduce signals presented to them in various different forms and that the mode of presentation may trigger different intracellular responses. Thus, intact ECM proteins such as fibronectin and laminin may trigger different responses from those elicited by fibronectin and laminin fragments. In addition, ligation of different regions of the extracellular domains of integrins may also transduce different signals. For example, fragments of fibronectin, or Arg-Gly-Asp-containing synthetic peptides, can induce the expression and secretion of metalloproteinases in synovial fibroblasts, whereas intact fibronectin is ineffective in inducing these genes (Werb et al., 1989). It is interesting that anti-integrin antibodies (to $\alpha_5\beta_1$) were also able to elicit this response (Werb et al., 1989). Similar findings have been reported for fragments of laminin versus intact laminin. Here, we have described the stimulation of tyrosine phosphorylation of different proteins upon cell attachment to ECM components and upon direct integrin ligation and clustering. Thus, we report here the stimulation of tyrosine phosphorylation of novel proteins (52 kDa, 55 kDa, 62 kDa and 90 kDa) upon anti-integrin antibody ligation and/or clustering. The phosphorylation of these proteins, which are integrin heterodimer-specific, is not stimulated when cells are allowed to attach and spread on intact ECM substrata (fibronectin and laminin). Conversely, the tyrosine phosphorylation of pp125^{FAK} is not stimulated in the

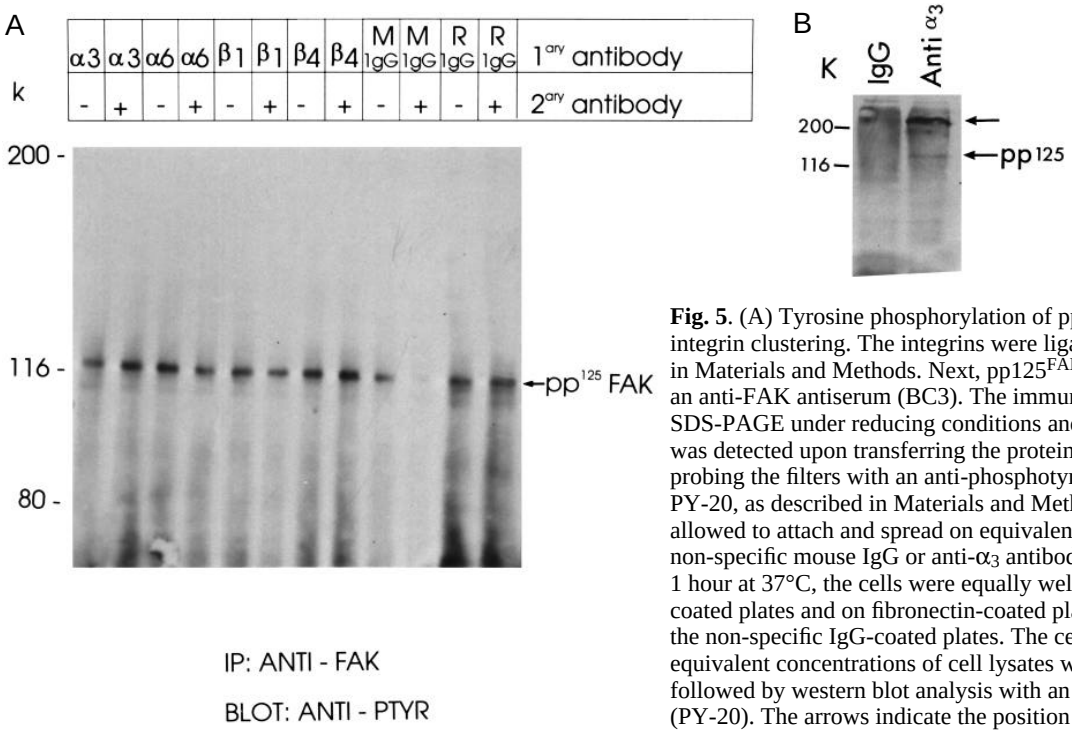

Fig. 5. (A) Tyrosine phosphorylation of pp125^{FAK} is not stimulated upon integrin clustering. The integrins were ligated and clustered as described in Materials and Methods. Next, pp125^{FAK} was immunoprecipitated with an anti-FAK antiserum (BC3). The immunoprecipitates were analyzed by SDS-PAGE under reducing conditions and the tyrosine phosphorylation was detected upon transferring the proteins onto PVDF membranes and probing the filters with an anti-phosphotyrosine monoclonal antibody, PY-20, as described in Materials and Methods. **(**B) PC3 cells were allowed to attach and spread on equivalent concentrations (50 μg/ml) of non-specific mouse IgG or anti-α_3 antibody (P1B5). After incubation for 1 hour at 37°C, the cells were equally well spread on the anti-α_3 antibody-coated plates and on fibronectin-coated plates, but remained rounded on the non-specific IgG-coated plates. The cells were then lysed and equivalent concentrations of cell lysates were analyzed by SDS-PAGE followed by western blot analysis with an anti-phosphotyrosine antibody (PY-20). The arrows indicate the position of two proteins whose phosphorylation was stimulated on anti-α_3, pp125 and pp230.

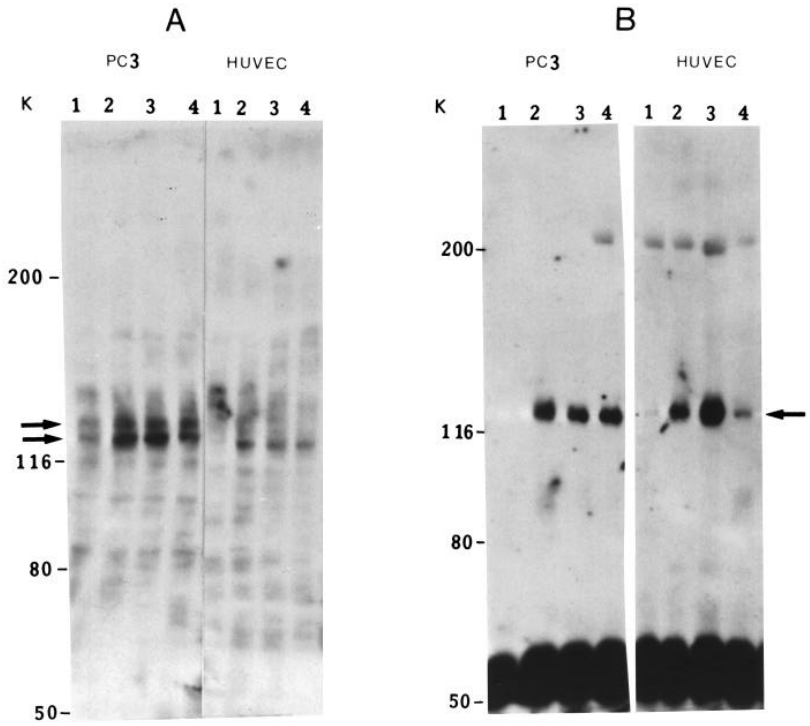

Fig. 6. Effect of cell attachment to extracellular matrix components on tyrosine phosphorylation of pp125 focal adhesion kinase. PC-3 human prostate carcinoma or human umbilical vein endothelial cells were incubated at 37°C for one hour in suspension (lane 1), or on non-tissue culture plates coated with laminin (lane 2), fibronectin (lane 3) or poly-L-lysine (lane 4). (A) At the end of the incubation period the cells were lysed and equivalent concentrations of cell lysates were analyzed by SDS-PAGE followed by western blot analysis with an anti-phosphotyrosine antibody (PY-20). The arrows indicate the positions of two tyrosine-phosphorylated proteins of 130 kDa and 125 kDa. (B) Equivalent concentrations of lysates described in A were immunoprecipitated with an antiserum (BC3) prepared against recombinant pp125 focal adhesion kinase (FAK). The immunoprecipitates were analyzed by SDS-PAGE under reducing conditions followed by western blot analysis with an anti-phosphotyrosine antibody (PY-20). The arrow indicates the position of the tyrosine-phosphorylated pp125^{FAK}.

PC-3 and HUVEC upon integrin clustering but only when the cells spread on ECM substrata or on anti-integrin antibodies. In human KB cells the tyrosine phosphorylation of FAK is stimulated upon clustering with antibodies to the α_3 and β_1 subunits, but not with antibodies to α_2, α_5 and α_6 (Kornberg et al., 1992). The phosphorylation of other proteins in KB cells after clustering was not reported (Kornberg et al., 1992). On the other hand, in platelets cross-linking of the $\alpha_{IIb}\beta_3$ integrin complex with antibodies, or binding of fibrinogen to the same integrin, both resulted in the stimulation of tyrosine phosphorylation of several proteins of 50-68 kDa and 140 kDa, but not of pp125^{FAK} (Huang et al., 1993). Phosphorylation of pp125^{FAK} was observed only after agonist-induced platelet aggregation or spreading of platelets on a fibrinogen matrix,

conditions under which phosphorylation of the 50-68 kDa and 140 kDa proteins was not seen (Huang et al., 1993). It therefore appears that phosphorylation of pp125^{FAK} and other proteins after integrin cross-linking is cell type-specific and that distinct signalling events are mediated by integrin occupancy and cell spreading. Thus, depending on the cell type, the phosphorylation of the 52-62 kDa and 90 kDa proteins may reflect initial signalling events mediated by integrin ligation and clustering, whereas the phosphorylation of pp125^{FAK} is stimulated subsequent to cell adhesion and spreading.

The proteins whose tyrosine phosphorylation is stimulated in the PC-3 and HUVEC upon integrin clustering appear to be integrin heterodimer-specific. Thus, the stimulation of tyrosine phosphorylation of the 52 kDa and 90 kDa proteins is specific for the $\alpha_6\beta_1$ heterodimer, whereas clustering with anti-β_4, but not with anti-β_1 antibody, stimulates the phosphorylation of a distinct 62 kDa protein. Furthermore, clustering of the $\alpha_3\beta_1$ integrin results in the stimulation of a 55 kDa protein, whereas clustering of $\alpha_5\beta_1$ did not result in a detectable stimulation of tyrosine phosphorylation. Our results with the tyrosine kinase inhibitor, Genistein, demonstrate that the induced tyrosine phosphorylations result from the activation of tyrosine kinase activity, since pretreatment of the cells with Genistein abrogates the tyrosine phosphorylation. Burridge and colleagues have recently demonstrated that treatment of cells with Genistein results not only in an inhibition of pp125^{FAK} phosphorylation but also in the inhibition of formation of focal adhesion plaques (Burridge et al., 1992). In our experiments, Genistein did not affect antibody-mediated clustering, suggesting that the observed tyrosine phosphorylation events occur subsequent to integrin ligation and clustering, and represent true signal-transduction events.

We have not identified any of the tyrosine-phosphorylated proteins as yet, although, based on molecular masses, the 52-62 kDa proteins could be members of the src family of non-receptor tyrosine kinases, or other tyrosine phosphorylated proteins implicated in the pathway of p21^{ras} activation (Rozakis-Adcock et al., 1992; Egan et al., 1993). Indeed, we have recently demonstrated that anti-integrin antibody-mediated activation of β_1 integrins on T-lymphoblastic Jurkat cells results in the activation of p21^{ras} and stimulation of tyrosine phosphorylation of 47-52 kDa proteins (Huang et al., 1993). The 52-62 kDa proteins described here, therefore, may be similar to the Jurkat cell proteins whose tyrosine phosphorylation is stimulated by β_1 integrin-activating antibodies, as well as to those described in platelets (Huang et al., 1993) after $\alpha_{IIb}\beta_3$ cross-linking or binding to fibrinogen. Thus, the induction of conformationally active integrins as well as cross-linking and clustering of integrins may result in the stimulation of tyrosine phosphorylation of these distinct 50-60 kDa proteins.

In conclusion, we have identified distinct proteins whose tyrosine phosphorylation is stimulated upon antibody-mediated ligation and/or clustering of the $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins in an integrin-specific manner. Future studies will be directed towards identification of these proteins and their role(s) in integrin-mediated signalling.

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