Altered Localization and Cytoplasmic Domain-binding Properties of Tyrosine-phosphorylated β_1 Integrin

Mats W. Johansson,*‡ Elisabeth Larsson,§ Björn Lüning,§ Elena B. Pasquale,* and Erkki Ruoslahti*

*La Jolla Cancer Research Foundation, La Jolla, California 92037; ‡Department of Physiological Botany, University of Uppsala, S-752 36 Uppsala, Sweden; and §Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-10691 Stockholm, Sweden

Abstract. We describe a novel approach to study tyrosine-phosphorylated (PY) integrins in cells transformed by virally encoded tyrosine kinases. We have synthesized a peptide (PYβ_1 peptide) that represents a portion of the cytoplasmic domain of the β_1 integrin subunit and is phosphorylated on the tyrosine residue known to be the target of oncogenic tyrosine kinases. Antibodies prepared against the PYβ_1 peptide, after removal of cross-reacting antibodies by absorption and affinity purification, recognized the PYβ_1 peptide and the tyrosine-phosphorylated form of the intact β_1 subunit, but did not bind the nonphosphorylated β_1 peptide, the nonphosphorylated β_1 subunit or other unrelated tyrosine-phosphorylated proteins. The anti-PYβ_1 antibodies labeled the podosomes of Rous sarcoma virus-transformed fibroblasts, but did not detectably stain nontransformed fibroblasts. The localization of the tyrosine phosphorylated β_1 subunit appeared distinct from that of the β_1 subunit. Adhesion plaques were stained by the anti-β_1 subunit antibodies in Rous sarcoma virus-transformed fibroblasts plated on fibronectin, whereas neither podosomes nor adhesion plaques were labeled on vitronectin or on uncoated plates. Anti-phosphotyrosine antibodies labeled podosomes, adhesion plaques and cell–cell boundaries regardless of the substratum. One of the SH2 domains of the p85 subunit of phosphatidylinositol-3-kinase bound to the PYβ_1 peptide, but not to the nonphosphorylated β_1 cytoplasmic peptide. Other SH2 domains did not bind to the PYβ_1 peptide. These results show that the phosphorylated form of the β_1 integrin subunit is detected in a different subcellular localization than the nonphosphorylated form and suggest that the phosphorylation on tyrosine of the β_1 subunit cytoplasmic domain may affect cellular signaling pathways.

INTEGRINS are a family of transmembrane proteins composed of an α and β subunit. Many of the integrins are receptors for extracellular matrix proteins and mediate cell adhesion to matrices (for reviews, see' Ruoslahti, 1991; Hynes, 1992). The β_1 integrin subunit combines with several α subunits to form receptors for extracellular matrix proteins such as fibronectin, laminin, and collagens. The cytoplasmic domains of the integrins are thought to bind cytoskeletal and other intracellular components. The β_1 integrin subunit has been shown to interact with talin (Horwitz et al., 1986) and α-actinin (Otey et al., 1990, 1993) in vitro. Furthermore, the cytoplasmic tail is necessary and sufficient for β_1 integrins to localize in adhesion plaques (Solowska et al., 1989; LaFlamme et al., 1992). Cell transformation by virally encoded tyrosine kinases has been shown to cause increased phosphorylation on tyrosine of the β_1 subunit cytoplasmic domain (Hirst et al., 1986).

The transforming protein of Rous sarcoma virus (RSV),[1] $p60^{v\text{-}src}$, is a cytoplasmic tyrosine kinase with constitutive activity (Hunter and Sefton, 1980). Fibroblasts transformed by RSV express high levels of $p60^{v\text{-}src}$, which causes extensive protein tyrosine phosphorylation of cellular proteins (Sefton et al., 1980). It is thought that the phosphorylation on tyrosine of critical substrates leads to the rounded morphology, disorganization of the cytoskeleton and decreased adhesiveness to the substratum that are characteristic of RSV-transformed cells. The search for substrates involved in the morphological alterations of RSV-transformed cells has led to the identification of a number of cytoskeletal components that are phosphorylated on tyrosine at higher levels in transformed fibroblasts than in normal fibroblasts. These cytoskeletal proteins include vinculin (Sefton et al., 1981),

Address all correspondence to Dr. Erkki Ruoslahti, La Jolla Cancer Research Foundation, 10901 North Torrey Pines Rd., La Jolla, CA 92037. Tel: (619) 455-6480. Fax: (619) 455-0181.

1. *Abbreviations used in this paper*: DMF, dimethylformamide; FMOC, 9-fluorenylmethyloxycarbonyl; PI-3-kinase, phosphatidylinositol-3′-kinase; PLC, phospholipase C; PY, tyrosine phosphorylated; RSV, Rous sarcoma virus; SH2, *src* homology 2; t-boc, t-butyloxycarbonyl; TFA, trifluoracetic acid.

talin (Pasquale et al., 1986), integrins (Hirst et al., 1986), paxillin (Glenney and Zokas, 1989), tensin (Davis et al., 1991), and, as has been shown more recently, cadherins and catenins (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993). However, in most cases these cytoskeletal proteins are phosphorylated on tyrosine at low stoichiometry in RSV-transformed cells.

To demonstrate that tyrosine phosphorylation of structural proteins leads to morphological alterations, even if it occurs at low stoichiometry, the specific subcellular localization and binding properties of the tyrosine-phosphorylated proteins need to be examined and compared to those of the nonphosphorylated proteins. The localization of the tyrosine-phosphorylated molecules in structures that are crucial to maintain the cytoskeletal organization and a normal morphology, such as cell–substrate adhesion sites, would support the importance of tyrosine-phosphorylation in generating a transformed phenotype. These experiments are in principle straightforward, but have been hampered by the lack of antibodies capable of recognizing specifically the tyrosine-phosphorylated forms of proteins. Here we describe the use of a tyrosine-phosphorylated peptide (PYβ_1), corresponding to a region of the cytoplasmic domain of the β_1 subunit, to generate antibodies specific for the tyrosine-phosphorylated β_1 integrin subunit. The residue that is phosphorylated in the peptide represents a consensus site for tyrosine-phosphorylation in the cytoplasmic domain of the β_1 integrin subunit and, thus, is thought to represent the tyrosine which is phosphorylated in RSV-transformed fibroblasts (Tamkun et al., 1986; Argraves et al., 1987; Tapley et al., 1989; Hynes, 1990).

We have used these anti-PYβ_1 antibodies to show that the tyrosine-phosphorylated β_1 subunit is concentrated in the podosomes of RSV-transformed NIH 3T3 cells, a localization distinct from both that of β_1 integrins and phosphotyrosine in general. We discuss the possible function of tyrosine phosphorylated β_1 integrins in the podosomes, which are round, dynamic adhesion structures also known as rosettes and believed to be associated with invasiveness (David-Pfeuty and Singer, 1980; Tarone et al., 1985; Marchisio et al., 1987; Ruoslahti and Giancotti, 1989). We also present evidence that the tyrosine phosphorylated β_1 subunit may specifically interact with phosphatidylinositol (PI)-3-kinase.

Materials and Methods

Synthetic Peptides

The tyrosine-phosphorylated peptides KWDTGENPIY(P)KSAVTT (PYβ_1) and KWDTANNPLY(P)KEATST(PYβ_3), corresponding to parts of the cytoplasmic domains of the human β_1 subunit (Argraves et al., 1987) and β_3 subunit (Fitzgerald et al., 1987) subunits, respectively, were synthesized manually by solid phase technique (Larsson et al., 1993). A 9-fluorenylmethyloxycarbonyl (FMOC) protocol was used in which the FMOC group was deblocked with morpholine (20% in dimethylformamide [DMF]), hydroxyl groups were protected as t-butyl ethers or esters, the ϵ-amino group in lysine as a t-butyloxycarbonyl (t-Boc) derivative and the phosphorylated tyrosine as a dibenzyl ester. The synthesis was initiated on a threonine t-butyl ether Sasrin resin. Coupling of the amino acids was achieved by means of preformed 1-hydroxybenzotriazole esters in a threefold excess in DMF and the completion of the reaction was monitored by a ninhydrin method (Sarin et al., 1981). ^{31}P and ^{1}H NMR spectra were measured on all phosphorylated compounds in a JEOL 270 MHz spectrometer and peptides were characterized by amino acid analysis, HPLC, and gasphase microsequencing.

After removal of the last FMOC group the side chain protected peptide was released from the resin by trifluoracetic acid (TFA), 1% in CH_2Cl_2, and was then desalted on Sephadex G15 in 0.1 M NH_4HCO_3 and lyophilized. The protected PYβ_1 peptide gave a ^{31}P NMR signal at −5.8 ppm. Attempts to remove the benzyl protecting groups through hydrogenolysis (Pd/C, 50 psi) reduced the tyrosine and removed the phosphate. It was found, however, that the benzyl groups could be removed concomitantly with the t-butyl groups by means of TFA. The protected peptide (272 mg), TFA (5 ml), indole (0.2 g), and *p*-cresol (0.2 g) were stirred at 0°C under nitrogen for 1 h. The peptide was then precipitated with dry ether at −60°C, taken up in 10% aqueous acetic acid, lyophilized, and then desalted over a Sephadex G15 column giving the pure phosphopeptide in a yield of 33 mg having ^{31}P NMR signal at −2.9 ppm. In a sample that had not been fully deprotected an NMR signal corresponding to the monobenzyl derivative of the peptide was observed at −4.2 ppm. The three positions of the ^{31}P NMR signal were consistent with the appearance of the benzyl signals in the ^{1}H NMR spectra. The tyrosine-phosphorylated β_3 subunit peptide (PYβ_3) was synthesized essentially as peptide PYβ_1, giving in all steps similar ^{31}P NMR signals. After the TFA deblocking procedure followed by desalting 77 mg PYβ_3 peptide was obtained. Nonphosphorylated β_1 integrin peptide KKKEKEKMNAKWDTGENPIYKSAVTTVNPKYEGK and β_3 integrin peptide KFEEERARAKWDTANNPLYKEATSTFTNITYRGT were synthesized at the Protein Chemistry Facility at the La Jolla Cancer Research Foundation (La Jolla, CA).

Proteins

Phosphotyrosine-BSA was prepared by coupling 4 mg phosphotyrosine to 5 mg BSA using glutaraldehyde as described (Wang, 1991). Vitronectin purified from human plasma according to Yatohgo et al. (1988) was a gift from Dr. Bianca Tomasini-Johansson, University of Uppsala (Uppsala, Sweden), human plasma fibronectin was obtained from the Blood Transfusion Service of the Finnish Red Cross (Helsinki, Finland) and calf intestine alkaline phosphatase from Boehringer (Indianapolis, IN).

Antibodies

1 mg PYβ_1 peptide in 300 μl PBS with 50 μM Na_3VO_4 was mixed with 12.5 μl of 1 mg/ml methylated BSA (Benoit et al., 1982) and 300 μl Freund's complete adjuvant and injected subcutaneously into a New Zealand white female rabbit. Incomplete adjuvant was used for booster immunizations. The antiserum was made 1 mM Na_3VO_4 and precipitated with $(NH_4)_2SO_4$ at 40% saturation; the precipitation and the subsequent steps were performed at 4°C. The precipitate was dissolved in half of the original volume of PBS with 1 mM Na_3VO_4, dialyzed towards PBS-1 mM Na_3VO_4 and absorbed by passing through a column of BSA–agarose and a column of the nonphosphorylated β1 peptide coupled to CNBr–Sepharose according to the manufacturer's instructions (10 mg peptide/ml resin). Na_3VO_4 was added to the serum to inhibit the phosphotyrosine phosphatases that may be present and would cause the dephosphorylation of the tyrosine phosphorylated reagents that were incubated with the serum (e.g., the PYβ_1 on the affinity-column). This flowthrough was mixed with a phosphotyrosine-rich protein aggregate suspension (10 μl per ml of the antibody solution). The phosphotyrosine proteins were prepared from a 10 ml culture of v-*abl*–expressing *Escherichia coli* as described (Wang, 1991). After incubation on a rocker overnight, this mixture was centrifuged at 10,000 *g* for 10 min. The supernatant was applied to a column of PYβ_1 peptide coupled to CNBr–Sepharose (2 mg peptide/ml resin) and antibodies were eluted with 0.1 M glycine–HCl, pH 2.5, containing 1 mM Na_3VO_4. The eluate was neutralized with a 1/10 vol of 1 M Tris–HCl, pH 8.0. This procedure yielded ~900 μg affinity-purified anti-PYβ_1 antibodies from 40 ml antiserum. Affinity-purified rabbit anti-phosphotyrosine (anti-PY) antibodies (Wang, 1991) and rabbit antisera against cytoplasmic peptides of the α_v (Freed et al., 1989) and β_1 (Giancotti and Ruoslahti, 1990) integrin subunits have been described. Anti-β_1 subunit antibodies were affinity-purified on a column of the peptide coupled to Sepharose. Mouse anti-$p60^{v-src}$ mAb was from Oncogene Science (Uniondale, NY).

Cells

RSV-transformed and nontransformed mouse NIH 3T3 fibroblasts were gifts from Dr. Janice Buss, Iowa State University, and were cultured in DME (GIBCO, Grand Island, NY) with 10% FCS (Tissue Culture Biologicals, Tulare, CA) and glutamine pen-strep (Irvine Scientific, Santa Ana, CA). v-*abl* expressing *E. coli* (Wang, 1991) were grown in LB medium with 50 μg/ml ampicillin.

Other Materials

CNBr-Sepharose and protein A-Sepharose were from Pharmacia (Piscataway, NJ), goat anti-rabbit IgG-peroxidase conjugate from BioRad (Richmond, CA), octylglucoside from Calbiochem (La Jolla, CA), precast SDS-PAGE gels from Novex (San Diego, CA), diaminobenzidine from Cappel and [^{32}P]phosphoric acid from NEN. Other reagents were from Sigma Chem. Co. (St. Louis, MO). Biotinylated PhosphoBlot™ SH2 (*src* homology 2) domain reagents were from Oncogene Science (Uniondale, NY). The SH2 domains used are: the human p60$^{v\text{-}src}$ SH2 domain, the bovine phospholipase C (PLC)γ-1 amino-terminal SH2 domain and the bovine carboxy-terminal SH2 domain of the p85 subunit of PI-3-kinase. Avidin and biotinylated horseradish peroxidase were from Clontech (Palo Alto, CA).

ELISA

Microtiter plate wells were coated overnight at room temperature with 100 μl of 10 μg/ml peptide solutions in PBS containing 0.5% glutaraldehyde, pH 7.4, or with 100 μl cell lysates as described below. After coating, wells were washed three times with PBS, blocked for 2 h with PBS containing 3% BSA and 1 mM Na_3VO_4, washed again with PBS, then incubated with 100 μl primary antibody in PBS with 0.05% Tween 20 and 1 mM Na_3VO_4 for 4 h, washed three times with PBS-Tween, incubated with anti-rabbit IgG-horseradish peroxidase conjugate 1:1,000 in PBS-Tween-Na_3VO_4 for 2 h, and washed again three times with PBS-Tween and then three times with PBS. Finally, peroxidase was assayed with 0.4 mg/ml ortho-phenyldiamine and 0.01% H_2O_2 in a buffer of 24.3 mM citric acid and 51.4 mM Na_2HPO_4 and the result read at 450 nm.

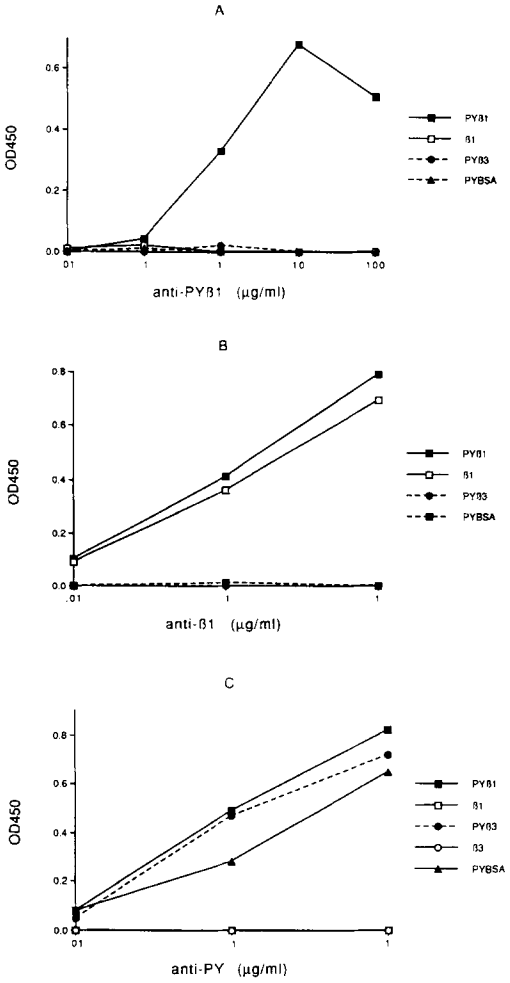

Figure 1. Binding of anti-integrin and anti-phosphotyrosine antibodies to integrin cytoplasmic domain peptides in ELISA. ELISA was performed with affinity-purified antibodies against the PYβ_1 cytoplasmic peptide (*A*), the β_1 cytoplasmic peptide (*B*), or phosphotyrosine (anti-PY) (*C*) in microtiter wells coated with PYβ_1, β_1, PYβ_3, or β_3 peptides at 10 μg/ml or with 10 μg/ml phosphotyrosine as a BSA conjugate (*PYBSA*). Antibodies against tyrosine-phosphorylated β_1 (anti-PYβ_1) recognize only the PYβ_1 peptide and not the nonphosphorylated β_1 peptide or other tyrosine-phosphorylated substrates.

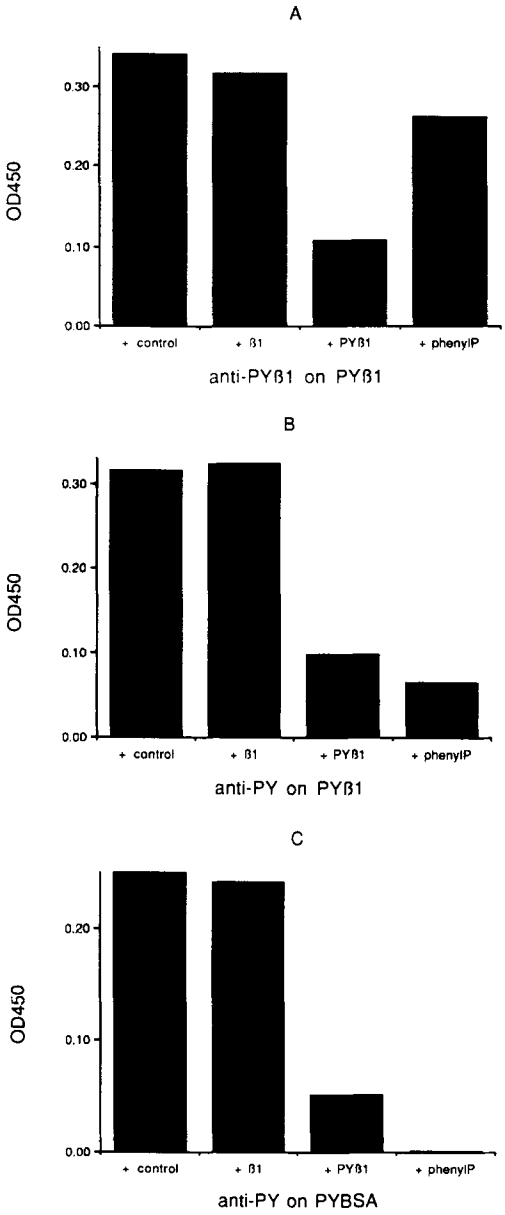

Figure 2. Specific inhibition of the binding of anti-PYβ_1 antibodies by the PYβ_1 peptide in ELISA. ELISA was performed with affinity-purified anti-PYβ_1 subunit antibodies at 10 μg/ml in microtiter wells coated with 10 μg/ml of the PYβ_1 peptide (*A*), or anti-phosphotyrosine antibodies at 1 μg/ml in microtiter wells coated with 10 μg/ml of the PYβ_1 peptide (*B*), or with anti-phosphotyrosine antibodies at 1 μg/ml in wells coated with 10 μg/ml of phosphotyrosine as a BSA conjugate (PYBSA) (*C*). Buffer only (*control*), the indicated peptide at 100 μg/ml or 40 mM phenylphosphate were added in the primary antibody solution.

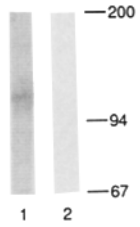

Figure 3. Immunoprecipitation with anti-PYβ_1 peptide antibodies of cell-surface-iodinated integrin from RSV-transformed and nontransformed mouse NIH 3T3 fibroblasts. RSV-transformed (lane *1*) and nontransformed (lane *2*) cells were surface iodinated and lysed. Lysates were immunoprecipitated with anti-PYβ_1 by using 1:10 dilution of absorbed antiserum.

Binding Assay with SH2 Domains

Microtiter plates were coated and blocked with peptides as described above for ELISA experiments. After blocking, wells were incubated with 100 μl biotinylated SH2 domain reagent (5 μg/ml in PBS, 0.05% Tween 20 containing 1 mM Na_3VO_4 and 5 mM DTT) for 1 h at room temperature. The wells were washed three times with PBS-Tween, incubated with a mixture of avidin (1:250) and biotinylated horseradish peroxidase (1:250) in PBS-Tween-Na_3VO_4 for 30 min, washed three times with PBS and finally developed as above.

^{125}I Surface Labeling of Cells

RSV-transformed cells were treated for 15 min with 1 mM Na_3VO_4 and 2 mM H_2O_2 (Volberg et al., 1992) in serum-free DME, washed twice with cold PBS-100 μM Na_3VO_4, once with PBS, and then harvested and iodinated as described (Pytela et al., 1985), except that cells were lysed in 50 mM octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_3VO_4 TBS, pH 7.4, containing protease inhibitors as above.

Immunoprecipitation

Lysates of iodinated cells were immunoprecipitated as described (Giancotti and Ruoslahti, 1990), except that 1 mM Na_3VO_4 was present throughout and immunoprecipitates were washed only with octylglucoside buffer (and not in SDS). Immunoprecipitates were analyzed by SDS-PAGE in a 6% gel and visualized by autoradiography.

Immunofluorescence

Cells were harvested with trypsin-EDTA, diluted 1:10 in DME-glutamine-pen-strep-10% FCS and added to noncoated glass coverslips or to coverslips coated for 2 h at room temperature with 10 μg/ml vitronectin or fibronectin in PBS. After 24 h cells were fixed for 10 min in 3% paraformaldehyde, 60 mM sucrose, 50 μM Na_3VO_4 in PBS, washed twice with PBS and permeabilized with 0.5% NP-40, 1 mM Na_3VO_4, in PBS for 15 min at room temperature. Cells were washed again twice with PBS, once with PBS-3% BSA and incubated with primary antibody in PBS-3% BSA-1 mM Na_3VO_4 for 1 h, washed as before, incubated with anti-rabbit IgG-rhodamine 1:150 in TBS-3% BSA-1 mM Na_3VO_4 for 45 min and finally washed three times with PBS before observation.

Phosphatase Treatment of Cells and PYβ_1 Peptide

Microtiter plate wells coated with peptide solutions or cells fixed and permeabilized for immunofluorescence were treated with 100 U/ml alkaline phosphatase in supplied dephosphorylation buffer (Boehringer Mannheim) for 24 h at 37°C and then washed with PBS and PBS-BSA and incubated with antibodies or SH2 domains as above. Control cells received only dephosphorylation buffer containing 1 mM Na_3VO_4.

Results

Specificity and Reactivity of Anti-tyrosine-phosphorylated β_1 Integrin Subunit Antibodies

The crude antiserum from a rabbit immunized with the PYβ_1 peptide contained reactivities to both the PYβ_1 peptide and the nonphosphorylated β_1 cytoplasmic peptide as well as to phosphotyrosine. However, absorption of the antiserum on nonphosphorylated β_1 subunit peptide and on a mixture of tyrosine phosphorylated proteins, followed by affinity purification on a PYβ_1 peptide column, yielded antibodies specific for the PYβ_1 peptide. In ELISA experiments, these antibodies recognized only the tyrosine-phosphorylated β_1 peptide and not the nonphosphorylated β_1 peptide or other tyrosine-phosphorylated substrates, such as the related PYβ_3 peptide or phosphotyrosine coupled to BSA (Fig. 1 *A*). In addition, dephosphorylation of the PYβ_1 peptide by alkaline phosphatase greatly reduced (by about 70%) the binding of the antibodies (not shown). In contrast, conventional anti-β_1 subunit cytoplasmic peptide antibodies (Giancotti and Ruoslahti, 1990) recognized β_1 peptides regardless of phosphorylation (Fig. 1 *B*) and, similarly, an anti-β_3 subunit cytoplasmic peptide antiserum recognized the PYβ_3 peptide (not shown). As expected, anti-phosphotyrosine antibodies (Wang, 1991) recognized the tyrosine-phosphorylated β_1 and β_3 peptides as well as phosphotyrosine coupled at BSA, but not the nonphosphorylated peptides nor BSA (Fig. 1 *C*). The results with anti-phosphotyrosine antibodies also confirmed that the PYβ_1 and PYβ_3 peptides indeed contain phosphorylated tyrosine residues and that the phosphotyrosine-BSA coupling was effective.

The specificity of the anti-PYβ_1 antibodies was further demonstrated by the inhibition observed with the PYβ_1 peptide in ELISA experiments and by the lack of inhibition with the nonphosphorylated β_1 peptide (Fig. 2 *A*). Moreover, phenylphosphate only marginally inhibited the anti-PYβ_1 antibodies (Fig. 2 *A*), whereas it inhibited the binding of the anti-phosphotyrosine antibodies to phosphotyrosine-

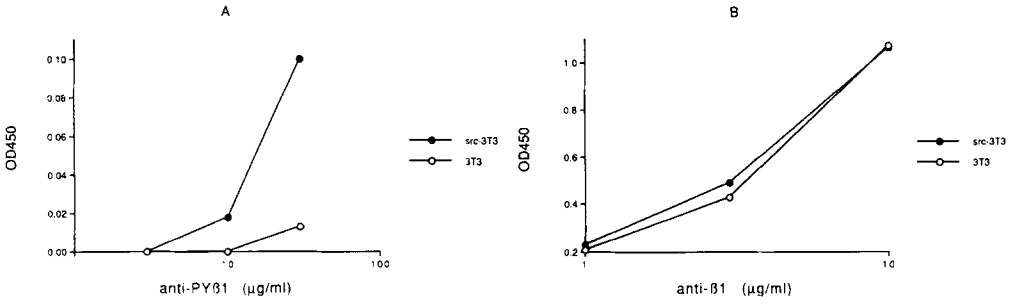

Figure 4. Binding of anti-PYβ_1 antibodies to lysates of RSV-transformed (src-3T3) and nontransformed mouse NIH 3T3 (3T3) fibroblasts in ELISA. Cells were washed three times with cold PBS containing 100 μM Na_3VO_4 lysed on the plate on ice in 50 mM octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and TBS, pH 7.4. The two lysates were adjusted to the same protein concentration. ELISA was performed with affinity-purified anti-PYβ_1 (*A*) or anti-β_1 (*B*) antibodies as described in Materials and Methods. The lysates were coated onto microtiter wells diluted 1:50 in TBS which contained 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

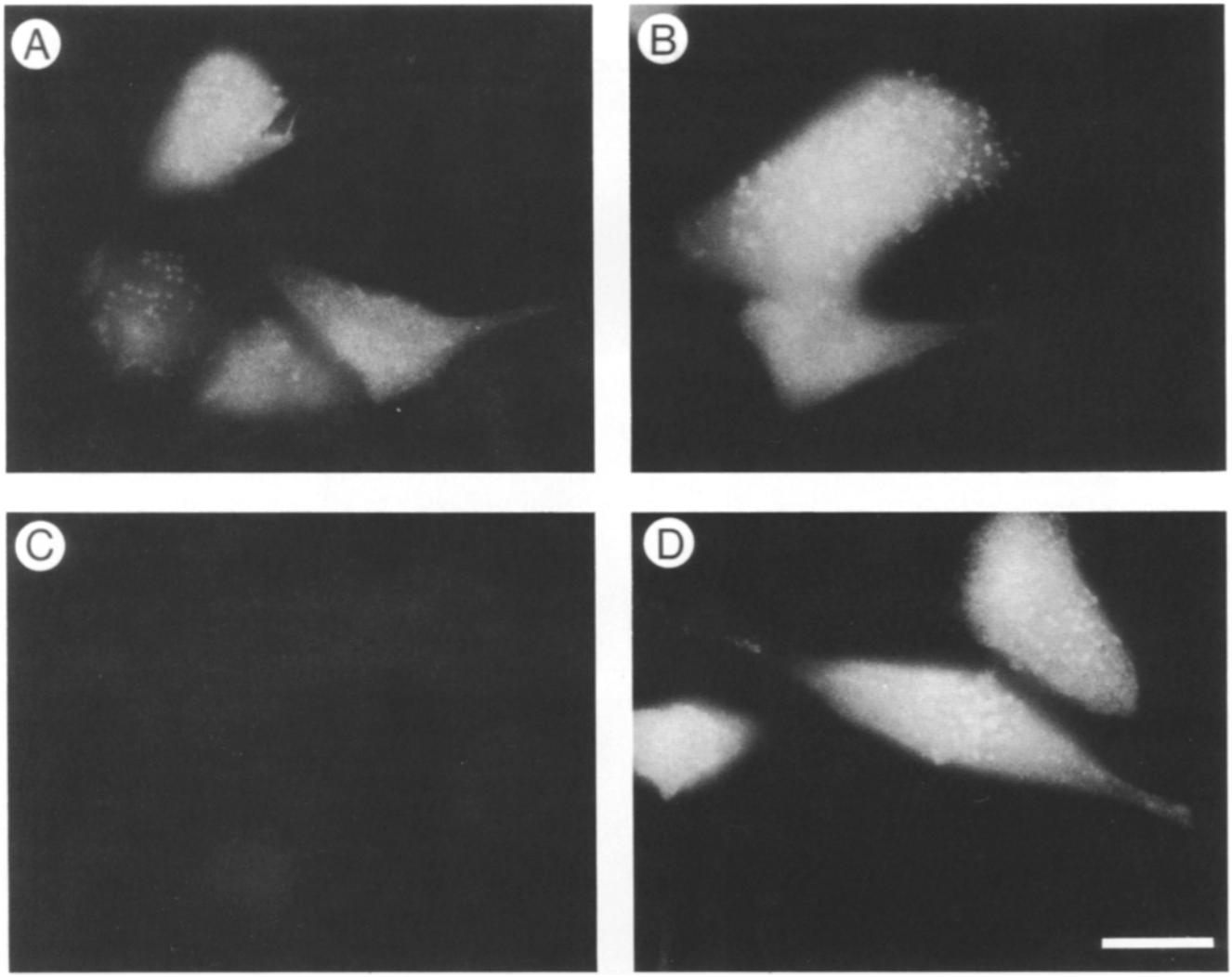

Figure 5. Immunofluorescent localization of the tyrosine phosphorylated β_1 integrin subunit. Immunofluorescence was performed with 100 μg/ml of affinity-purified anti-PYβ_1 antibodies, using RSV-transformed mouse NIH 3T3 fibroblasts plated on noncoated glass. No peptide (*A*), β_1 (*B*), or PYβ_1 (*C*) peptides at 500 μg/ml, or 40 mM phenylphosphate (*D*) were added to the primary antibody solution to assess the specificity of the staining. Bar, 20 μm.

coupled BSA and to the PYβ_1 peptide (Fig. 2, *B* and *C*). The anti-phosphotyrosine antibodies were also inhibited by the PYβ_1, peptide (Fig. 2, *B* and *C*), again confirming the presence of phosphotyrosine on this peptide.

Detection of Tyrosine-phosphorylated β_1 Subunit in RSV-transformed Fibroblasts

Anti-PYβ_1 antibodies immunoprecipitated the β_1 subunit from surface iodinated RSV-transformed mouse NIH3T3 fibroblasts (Fig. 3, lane *1*), but not from nontransformed cells (Fig. 3, lane *2*). They also reacted ~10-fold more strongly in ELISA experiments with lysates of RSV-transformed cells than with lysates on nontransformed cells (Fig. 4 *A*). These results indicate that the β_1 integrin subunit is tyrosine-phosphorylated in RSV-transformed NIH 3T3 fibroblasts, but not, or only at a very low level, in normal NIH 3T3 fibroblasts. Transformation by RSV does not affect the overall levels of β_1 subunit, as judged by immunoprecipitation from lysates of surface iodinated cells (not shown) and by ELISA on the cell lysates with anti-β_1 (Fig. 4 *B*).

Distinct Localization of Tyrosine-phosphorylated β_1 Integrin Subunit in the Podosomes of RSV-transformed Fibroblasts

We used the new anti-PYβ_1 antibodies to determine the localization of the tyrosine phosphorylated β_1 subunit in cells plated on noncoated glass. In addition to some diffuse staining, distinct staining of podosomes was observed in RSV-transformed NIH 3T3 fibroblasts (Fig. 5 *A*). The specificity of the anti-PYβ_1 antibody staining was shown by inhibition experiments; the PYβ_1 peptide specifically inhibited the staining of podosomes as well as the diffuse staining (Fig. 5 *C*, compare to *A*), whereas the nonphosphorylated β_1 peptide (Fig. 5 *B*) or phenylphosphate (Fig. 5 *D*) did not inhibit.

In contrast with the specific staining observed in RSV-

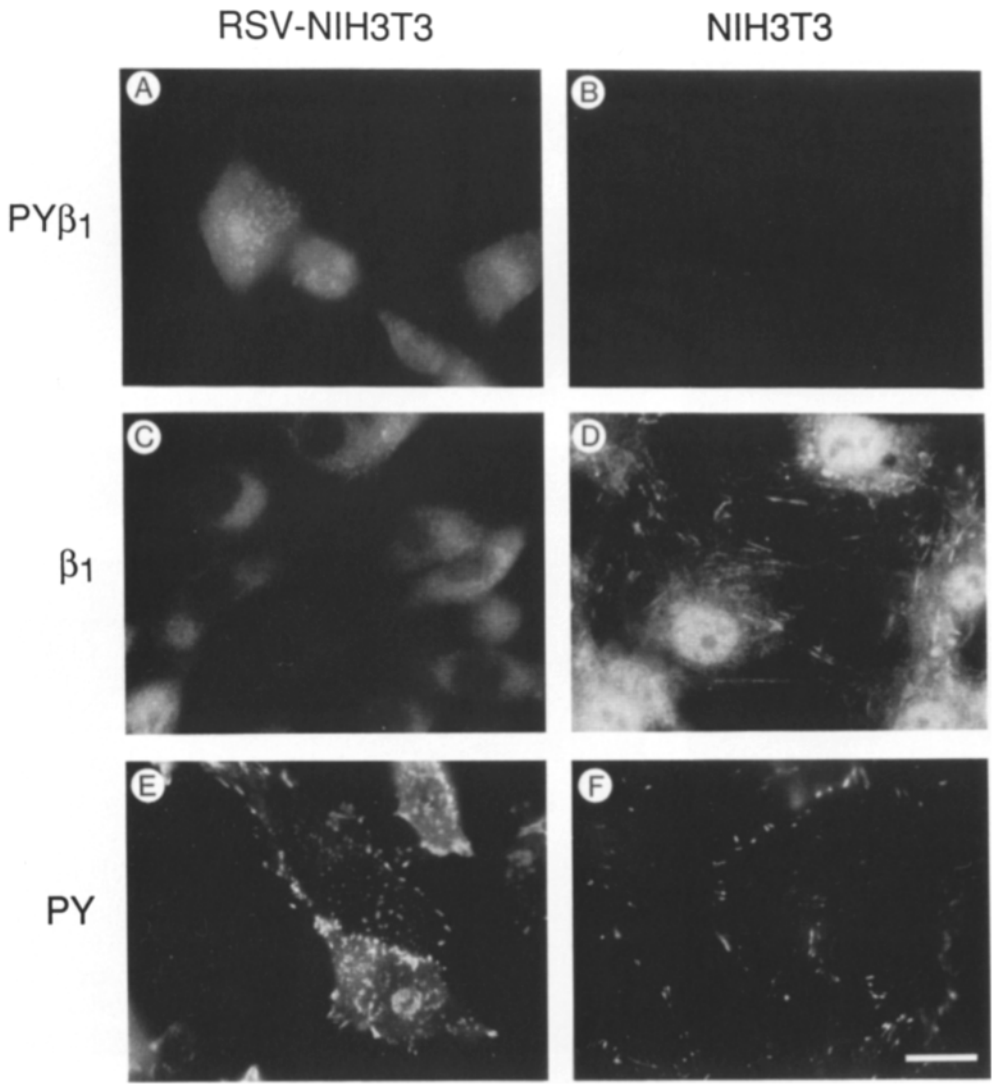

Figure 6. Immunofluorescent localization of tyrosine-phosphorylated β_1 integrin subunit (PYβ_1), β_1 integrin subunit (β_1), and phosphotyrosine (PY) in normal (NIH3T3) and RSV-transformed (RSV-NIH-3T3) fibroblasts plated on noncoated glass. Immunofluorescence was performed with 100 μg/ml of affinity-purified anti-PYβ_1 antibodies, 10 μg/ml affinity-purified anti-β_1 antibodies, and 10 μg/ml affinity-purified anti-phosphotyrosine antibodies. Bar, 20 μm.

Table I. Immunofluorescent Staining of RSV-transformed and Nontransformed Mouse NIH 3T3 Fibroblasts with Anti-tyrosine Phosphorylated β_1 Integrin Subunit, Anti-β_1 Integrin Subunit, Anti-phosphotyrosine, and Anti-p60$^{v\text{-}src}$ Antibodies

Antibody	RSV-transformed cells on noncoated or vitronectin-coated substrate	RSV-transformed cells on fibronectin-coated substrate	Nontransformed cells on noncoated or vitronectin-coated substrate	Nontransformed cells on fibronectin-coated substrate
Anti-tyrosine phosphorylated β_1 subunit	Podosomes diffuse staining	Podosomes diffuse staining	Not detected	Not detected
Anti-β_1 subunit	Diffuse staining	Adhesion plaques, diffuse staining	Adhesion plaques	Adhesion plaques
Anti-phosphotyrosine	Podosomes, adhesion plaques, cell–cell boundaries	Podosomes, adhesion plaques, cell–cell boundaries	Adhesion plaques	Adhesion plaques
Anti-p60$^{v\text{-}src}$	Podosomes, adhesion plaques	Podosomes, adhesion plaques	Not detected	Not detected

This table summarizes the data shown in Fig. 6 and Fig. 7 as well as data not shown. Immunofluorescence microscopy was performed with affinity-purified antibodies against β_1 integrin subunit cytoplasmic peptide (10 μg/ml), PYβ_1 (100 μg/ml) and phosphotyrosine (10 μg/ml) or with 10 μg/ml mouse anti-p60$^{v\text{-}src}$ antibodies as described in Materials and Methods.

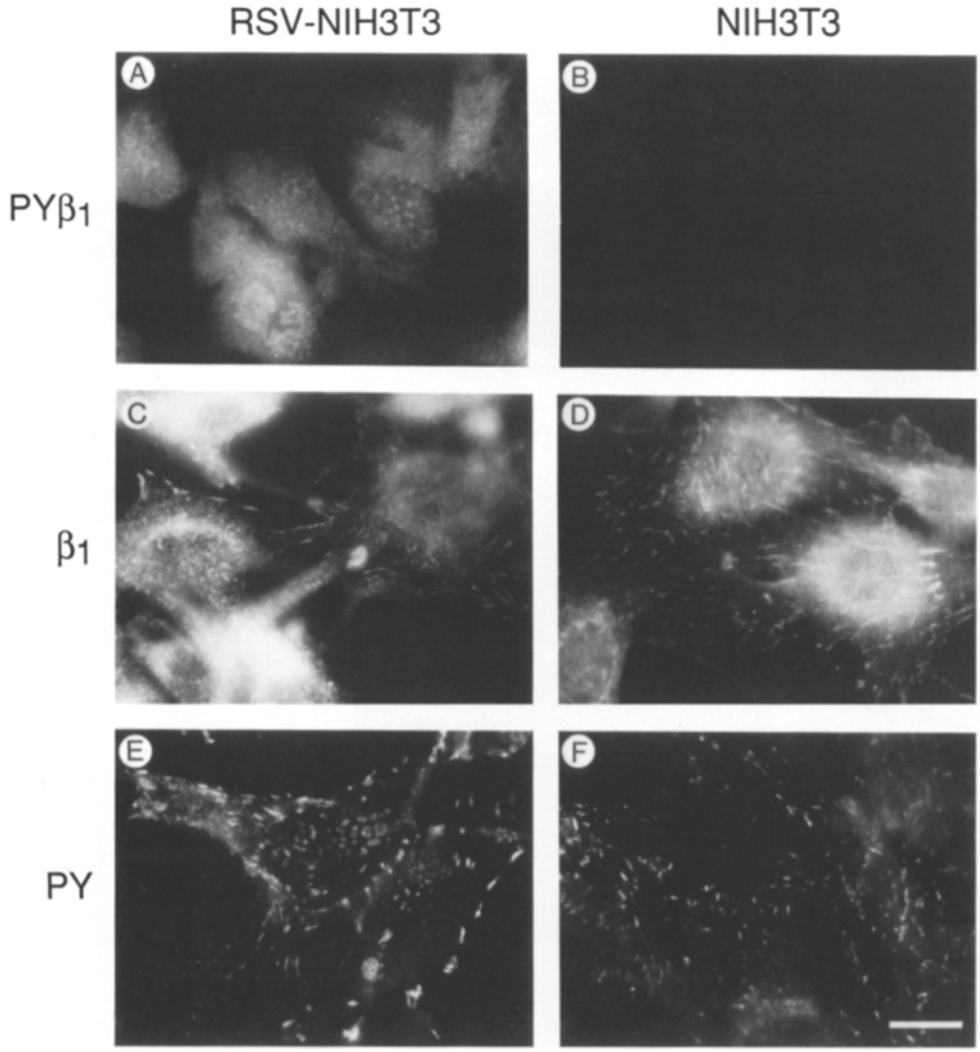

Figure 7. Immunofluorescent localization of tyrosine-phosphorylated β_1 integrin subunit (PYβ_1), β_1 integrin subunit (β_1) and phosphotyrosine (PY) in normal (NIH3T3) and RSV-transformed (RSV-NIH-3T3) fibroblasts plated on fibronectin-coated glass. Immunofluorescence was performed with 100 μg/ml of affinity-purified anti-PYβ_1 antibodies, 10 μg/ml affinity-purified anti-β_1 antibodies and 10 μg/ml affinity-purified anti-phosphotyrosine antibodies. Bar, 20 μm.

transformed fibroblasts (Figs. 5, *A*, *C*, and *D* and Fig. 6 *A*), no anti-PYβ_1 staining above background was obtained in nontransformed fibroblasts (Fig. 6 *B*). Conventional anti-β_1 integrin subunit antibodies labeled diffusely RSV-transformed NIH 3T3 fibroblasts plated on noncoated glass (Fig. 6 *C*) and stained adhesion plaques in nontransformed fibroblasts (Fig. 6 *D*). Anti-phosphotyrosine antibodies stained podosomes, adhesion-plaque–like structures (Fig. 6 *E*) and cell–cell boundaries (Table I) in the transformed fibroblasts, while adhesion plaques were primarily stained in nontransformed fibroblasts (Fig. 6 *F*). Results indistinguishable from those obtained with cells plated on noncoated glass (Figs. 5 and 6) were obtained on a vitronectin substrate (Table I).

In Fig. 7 the staining patterns of anti-PYβ_1, anti-β_1 and anti-phosphotyrosine antibodies were examined in cells plated on a fibronectin substrate. In RSV-transformed fibroblasts plated on fibronectin β_1 integrin subunit immunoreactivity was concentrated in focal adhesions (Fig. 7 *C*), while on noncoated glass (Fig. 6 *C*) and on vitronectin (Table I) only diffuse staining was observed. Thus, the anti-PYβ_1 antibody staining is distinct from the localization of both β_1 subunit and phosphotyrosine immunoreactivities. The localization of p60$^{v\text{-}src}$ was also examined for comparison (Table I). p60$^{v\text{-}src}$ was found both in podosomes and in adhesion plaques in the RSV-transformed NIH3T3 fibroblasts. Control experiments showed that the nonphosphorylated β_1 peptide greatly reduces anti-β_1 staining and phenylphosphate completely inhibits anti-phosphotyrosine antibody staining, as does the PYβ_1 peptide (not shown). As expected, the nonphosphorylated β_1 peptide had no effect on anti-phosphotyrosine staining (not shown). These inhibition patterns are consistent with the ELISA results shown in Fig. 2. The results with anti-β_1, anti-phosphotyrosine and anti-p60$^{v\text{-}src}$ antibodies are in agreement with results obtained by others using transformed and nontransformed fibroblasts (Maher et al., 1985; Tarone et al., 1985; Chen et al., 1986; Giancotti et al., 1986; Singer et al., 1988).

Even though the anti-PYβ_1 antibody staining was not inhibited by phenylphosphate, the PYβ_1 epitope does indeed encompass a phosphate group, as demonstrated by the absence of anti-PYβ_1 staining of podosomes after treatment of the fixed and permeabilized cells with alkaline phosphatase (Fig. 8 *B*, compare with *A*). Anti-phosphotyrosine staining was similarly abolished by the alkaline phosphatase treat-

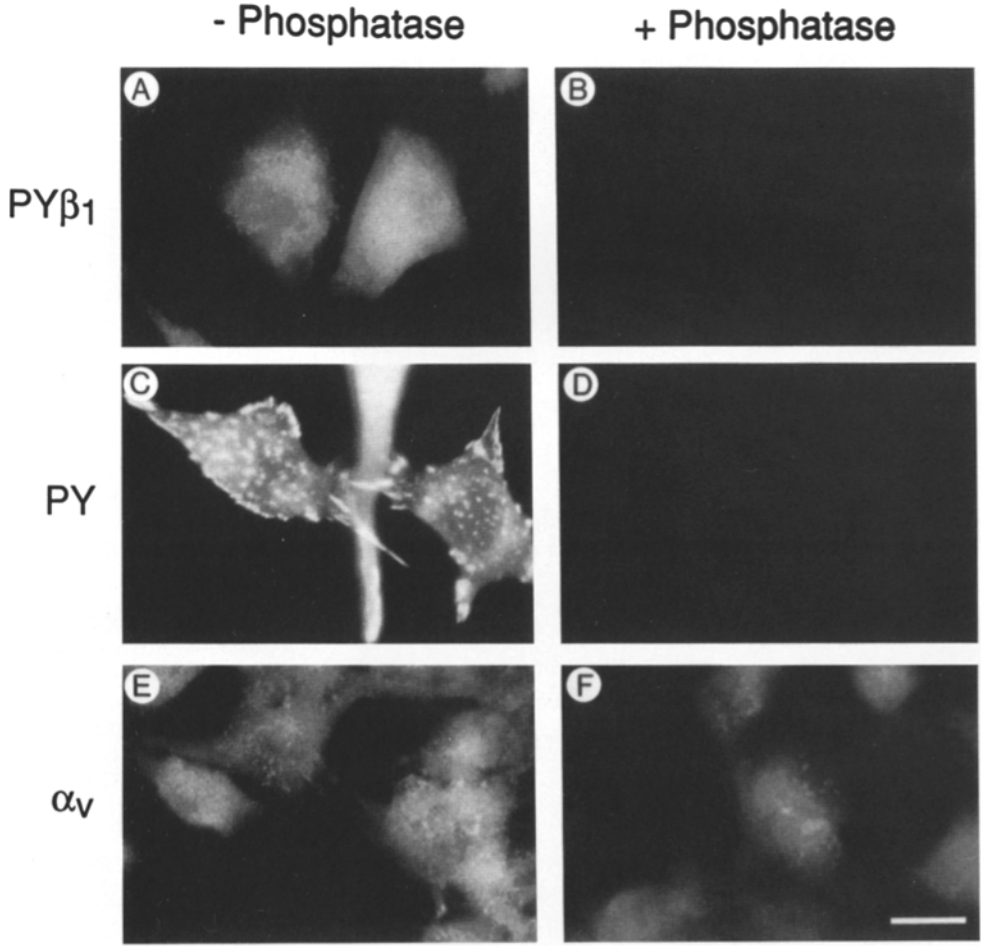

Figure 8. Abolition of anti-PYβ_1 and anti-phosphotyrosine antibody immunofluorescent staining by phosphatase treatment. Immunofluorescence was performed using phosphatase-treated (*B*, *D*, and *F*) or nontreated (*A*, *C*, and *E*) fixed and permeabilized RSV-transformed NIH 3T3 fibroblasts. The antibodies used are: 100 μg/ml of affinity-purified anti-PYβ_1 antibodies (*A* and *B*), 10 μg/ml of affinity-purified anti-phosphotyrosine antibodies (*C* and *D*) and anti-α_v integrin antiserum at a dilution of 1:100 (*E* and *F*). Bar, 20 μm.

ment (Fig. 8 *D*, compare to *C*). Staining of podosomes with anti-α_v integrin subunit antibodies confirmed that the podosome structure itself was not destroyed by the phosphatase treatment (Fig. 8, *E* and *F*).

Binding of SH2 Domains to Tyrosine-phosphorylated β_1 Integrin Subunit Peptide

Phosphorylated tyrosine residues in proteins often represent recognition sites for the SH2 domains of cytoplasmic signaling proteins (Koch et al., 1991). To examine the possibility that tyrosine phosphorylation of the β_1 subunit cytoplasmic domain may create a binding site for specific SH2 domains, we tested the recombinant SH2 domains of several proteins in a binding assay. As shown in Fig. 9 *A*, we found that the carboxy-terminal SH2 domain of the p85 subunit of PI-3 kinase binds to the PYβ_1 peptide and, to a lower degree, to the related PYβ_3 peptide. In contrast, the SH2 domains of $p60^{v\text{-}src}$ and PLCγ-1 did not bind to PYβ_1. No binding was observed to the β_1 and β_3 nonphosphorylated peptides. Furthermore, treatment of the PYβ_1 peptide with alkaline phosphatase greatly reduced the binding of the p85 subunit SH2 domain (Fig. 9 *B*).

Discussion

We have successfully synthesized a phosphopeptide that mimics the tyrosine-phosphorylated cytoplasmic domain of the β_1 integrin subunit, prepared antibodies to it that specifically recognize the phosphorylated β_1 subunit, and shown with these antibodies that tyrosine-phosphorylated β_1 integrins are concentrated in different subcellular structures than nonphosphorylated β_1 integrins. Our results also show that an SH2 domain of PI-3-kinase binds to the phosphorylated β_1 peptide.

Our study indicates that polyclonal antibodies specific for various tyrosine-phosphorylated substrates can be useful tools, for example, for immunolocalization studies. Antibodies specific for the phosphorylated form of a particular protein have previously been raised against serine or threonine phosphorylated peptides (Czernik et al., 1991). In addition, while our manuscript was in preparation, a polyclonal antibody made against a phosphopeptide comprising a phosphorylated tyrosine residue of $P185^{neu/erbB\text{-}2}$ was described (Bangalore et al., 1992). This antibody was also used in immunofluorescence microscopy experiments; however, the subcellular localization of the phosphorylated protein was not distinct from that of the nonphosphorylated protein or from phosphotyrosine in general.

Our first efforts to produce the PYβ_1 phosphopeptide involved a conventional solid phase synthesis according to an FMOC protocol, followed by a global phosphorylation of unblocked tyrosine in the resin-bound peptide. The ^{31}P NMR signal of the deblocked peptide was, however, later shown to have an incorrect chemical shift. We subsequently

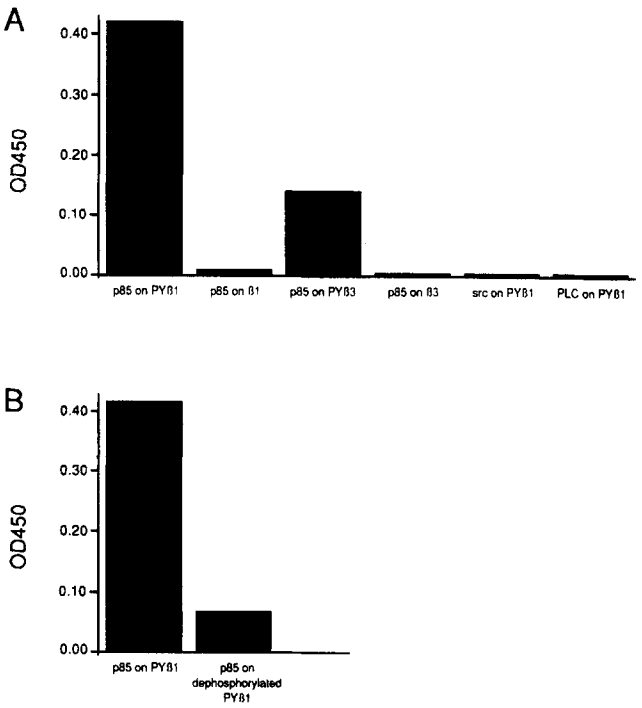

Figure 9. Binding of SH2 domains to the PYβ_1 and PYβ_3 peptides. The binding assay was performed with 5 μg/ml of the biotinylated SH2 domains of the p85 subunit of PI-3-kinase, p60^{src} or PLCγ in microtiter wells coated with PYβ_1, β_1, PYβ_3, or β_3 peptides at 10 μg/ml (*A*) and with PYβ_1 or PYβ_1 dephosphorylated by treatment with alkaline phosphatase (*B*).

adopted a method employing a benzyl-protected phosphotyrosine in the solid phase synthesis. This method proved to be successful when the removal of the FMOC group in each step was made with morpholine; attempts to use other bases led to loss of benzyl groups and premature termination of the synthesis (Kitas et al., 1991).

The antibodies we prepared against the β_1 subunit cytoplasmic phosphopeptide recognize the phosphotyrosine residue in the context of the surrounding β_1 subunit amino acid sequence. This conclusion is supported by the finding that the antibodies did not recognize phosphotyrosine nor other tyrosine-phosphorylated peptides, such as the PYβ_3 peptide. The antibodies prepared against the PYβ_1 subunit peptide recognized the intact tyrosine-phosphorylated β_1 subunit in RSV-transformed mouse NIH 3T3 fibroblasts, as shown by ELISA and by surface iodination followed by immunoprecipitation. Phosphate labeling and immunoprecipitation confirmed that the β_1 subunit is phosphorylated in RSV-transformed fibroblasts. These experiments showed that the anti-PYβ_1 antibodies were suitable for studying the subcellular localization of tyrosine-phosphorylated β_1 subunit.

The overall distribution of phosphotyrosine-containing proteins had been studied in normal and transformed cells (e.g., Maher et al., 1985; Tarone et al., 1985), but the subcellular distribution of the tyrosine-phosphorylated β_1 subunit was unknown (result not shown). With the anti-PYβ_1 peptide antibodies, the phosphorylated β_1 subunit could be detected in RSV-transformed 3T3 fibroblasts without growing the cells in the presence of the phosphotyrosine phosphatase inhibitor vanadate. In contrast, significant levels of β_1 subunit phosphorylation could previously be detected after immunoprecipitation only in cells labeled in the presence of vanadate (Hirst et al., 1986; Hynes, 1990; Haimovich et al., 1991). Although the tyrosine-phosphorylated β_1 subunit represents only a minor fraction of the total β_1 subunit (Hirst et al., 1986), it is concentrated in the podosomes, where it is likely to have specific functions.

Podosomes are dot-shaped sites of close contact with the substratum found in some transformed cells and in nontransformed cells derived from the bone marrow, including osteoclasts (Tarone et al., 1985; Marchisio et al., 1987; see Burridge et al., 1988 for a review on podosomes). Podosomes have been reported to contain high levels of cytoskeletal proteins such as actin, α-actinin, fimbrin, vinculin, and talin. Phosphotyrosine containing molecules, some of which have been identified as p60$^{v\text{-}src}$ substrates, are also concentrated in podosomes (Tarone et al., 1985; Kanner et al., 1991; Wu et al., 1991). Several characteristics distinguish the podosome adhesion structures from adhesion plaques, including their rapid assembly and disassembly, which may be caused by the low levels of integrins and lack of ECM components associated with podosomes, and may confer migratory and invasive properties to cells. The localization in podosomes of the tyrosine phosphorylated β_1 subunit, observed both on vitronectin and fibronectin substrates, is distinct from the distribution of the β_1 subunit, which in transformed cells is diffusely distributed on a vitronectin substrate or on uncoated glass (Chen et al., 1986; Giancotti et al., 1986; Ruoslahti and Giancotti, 1989; Hynes, 1990) and concentrated in adhesion plaques on a fibronectin substrate (Chen et al., 1986; and Table I). Thus, one consequence of β_1 subunit phosphorylation appears to be the substratum-independent localization in podosomes.

The β_1 subunit primarily associates with the α_5 and α_v subunits in the RSV-transformed NIH 3T3 cells (not shown). However, because of the low quantity of the tyrosine-phosphorylated β_1 subunit, we have not been able to identify its companion α subunit. The α_5 subunit localizes diffusely in the RSV-transformed fibroblasts and accumulates in the adhesion plaques of cells attached on fibronectin (not shown), whereas we detected α_v in the podosomes. This suggests that in the podosomes PYβ_1 is present in complex with α_v. The $\alpha_v\beta_1$ integrin is a fibronectin receptor (Vogel et al., 1990), but unlike $\alpha_5\beta_1$ it does not support the assembly of a fibronectin matrix (Zhang et al., 1993). Tyrosine-phosphorylated $\alpha_v\beta_1$ integrin is probably a weaker fibronectin receptor, because phosphorylation has been found to reduce the fibronectin binding of a chicken integrin complex (Horwitz et al., 1986). Local weak fibronectin binding at the podosomes may be important for the degradation of fibronectin that takes place at this site in RSV-transformed cells (Chen et al., 1985; Mueller and Chen, 1991).

The tyrosine-phosphorylated β_1 subunit isolated from RSV-transformed chicken fibroblasts has a lower ability to interact with fibronectin or talin than integrin from normal cells (Tapley et al., 1989). For this reason, β_1 subunit phosphorylation on tyrosine may contribute to the abnormal phenotype of RSV-transformed cells. Consistent with this possibility, β_1 subunit tyrosine-phosphorylation was not de-

tected in chicken fibroblasts transformed by a virus encoding a mutant $p60^{v\text{-}src}$. Cells infected with this mutant virus retain surface fibronectin and have a relatively normal morphology (Horvath et al., 1990).

The tyrosine kinase $p60^{v\text{-}src}$, which presumably is responsible, directly or indirectly, for integrin phosphorylation, is localized in podosomes as well as in adhesion plaques in RSV-transformed cells (Chen et al., 1985; and Table I). However, the tyrosine-phosphorylated β_1 subunit is detected in podosomes, but not in adhesion plaques. It is not clear why the β_1 subunit in the adhesion plaques is not phosphorylated by the $p60^{v\text{-}src}$ present there, since in vitro both the β_1 subunit and a synthetic peptide containing the consensus site for β_1 tyrosine phosphorylation are phosphorylated on tyrosine by $p60^{v\text{-}src}$ (Tapley et al., 1989). A number of explanations are possible: Some factor necessary for integrin phosphorylation may be missing in the plaques or there may be locally high phosphotyrosine phosphatase activity. Alternatively, $p60^{v\text{-}src}$ may phosphorylate β_1 in the plaques, but once phosphorylated, the integrin diffuses away from the adhesion plaques because of a decreased interaction with talin (Tapley et al., 1989). Finally, we cannot exclude the possibility that tyrosine phosphorylated β_1 subunit is present in adhesion plaques, but not accessible to the antibodies. However, this explanation is unlikely since the nonphosphorylated β_1 subunit is detected in adhesion plaques with antibodies to its cytoplasmic domain. Furthermore, it was recently shown that a β_1 subunit mutant in which the critical cytoplasmic domain tyrosine residue had been replaced by glutamic acid had a decreased localization in adhesion plaques of 3T3 cells (Reszka et al., 1992), suggesting that the plaque localization may be abolished by introduction of a negative charge, such as that of a phosphate group, at this site. It is also possible that β_1 subunit phosphorylation on tyrosine could convert adhesion plaques into podosomes; podosomes are highly dynamic structures that assemble and disassemble rapidly (Burridge et al., 1988).

The function of tyrosine-phosphorylated β_1 subunit in the podosomes is unknown. As discussed above, previous reports suggest that phosphorylation on tyrosine of its cytoplasmic domain impairs the binding of the β_1 subunit to the cytoskeletal proteins that normally interact with it, such as talin and vinculin. Our results suggest that an SH2 domain of the p85 subunit of PI-3-kinase has affinity for the tyrosine phosphorylated β_1 integrin subunit. Although the sequences of the cytoplasmic domains of β_1 and β_3 integrins do not conform to the canonical consensus sequence recognized by the p85 subunit SH2 domains, exceptions have been reported (Miura et al., 1994). While an in vivo association remains to be demonstrated, it is conceivable that the phosphorylation of the β_1 integrin subunit in RSV-transformed cells may act to recruit PI-3-kinase to a critical subcellular localization. PI-3-kinase phosphorylates several phosphoinositide membrane lipids at the D-3 position of the inositol ring. Thus, binding to tyrosine-phosphorylated β_1 subunit would bring PI-3-kinase in contact not only with its substrates, but also with $p60^{v\text{-}src}$. PI-3-kinase is a substrate of $p60^{v\text{-}src}$ and it is likely to be activated by tyrosine phosphorylation of its 85-kD subunit (Cantley et al., 1991). Interestingly, the increased concentration of D-3 phosphorylated phosphoinositides resulting from the activation of PI-3-kinase, may be responsible for the disorganization of actin filament observed in cells treated with growth factors and in transformed cells (Cantley et al., 1991). Further work will be necessary to evaluate the intriguing hypothesis that the phosphorylated β_1 subunit in the podosomes may, in analogy with phosphorylated growth factor receptors, recruit cytoplasmic signaling molecules.

We thank Katie Thompson for help with the immunizations, Dr. Janice Buss for cell lines, and Dr. Bianca Tomasini-Johansson for vitronectin.

This work was supported by grants CA 42507 and CA 28896 and Cancer Center support grant CA 30199 (to E. Ruoslahti) and by a grant from the Swedish National Board for Technical Development (to B. Lüning). M. W. Johansson was supported by a postdoctoral fellowship from the Swedish Council for Forestry and Agricultural Research.

Received for publication 8 July 1993 and in revised form 20 May 1994.

References

Argraves, W. S., S. Suzuki, H. Arai, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 105:1183–1190.

Bangalore, I., A. J. Tanner, A. P. Laudane, and D. F. Stern. 1992. Antiserum raised against a synthetic phosphotyrosine-containing peptide selectively recognizes $p185^{neu/erb\text{-}2}$ and the epidermal growth factor receptor. *Proc. Natl. Acad. Sci. USA.* 89:11637–11641.

Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. Van Roy, M. M. Mareel, and W. Birchmeier. 1993. Loss of epithelial differentiation and gain of invasiveness correlate with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J. Cell Biol.* 120:757–766.

Benoit, R., P. Böhlen, N. Ling, A. Briskin, F. Esch, P. Brazeau, S.-y. Ying, and R. Guillemin. 1982. Presence of somatostatin-28-(1-12) in hypothalamus and pancreas. *Proc. Natl. Acad. Sci. USA.* 79:917–921.

Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487–525.

Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell.* 64:281–302.

Chen, W.-T., J.-M. Chen, S. J. Parsons, and J. T. Parsons. 1985. Local degradation of fibronectin at sites of expression of the transforming gene product $pp60^{src}$. *Nature (Lond.).* 316:156–158.

Chen, W.-T., J. Wang, T. Hasegawa, S. S. Yamada, and K. M. Yamada. 1986. Regulation of fibronectin of receptor distribution by transformation, exogenous fibronectin, and synthetic peptides. *J. Cell Biol.* 103:1649–1661.

Czernik, A. J., M.-A. Girault, A. C. Nairn, J. Chen, G. Snyder, J. Kebabian, and P. Greengard. 1991. Production of phosphorylation state-specific antibodies. *Methods Enzymol.* 201:264–283.

David-Pfeuty, T., and S. J. Singer. 1980. Altered distributions of the cytoskeletal proteins vinculin and α-actinin in cultured fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 77:6687–6691.

Davis, S., M. L. Lu, S. H. Lao, S. Lin, J. A. Butler, B. J. Druker, T. M. Roberts, Q. An, and L. B. Chen. 1991. Presence of an SH2 domain in the actin-binding protein tensin. *Science (Wash. DC).* 252:712–715.

Fitzgerald, L. A., B. Steiner, S. C. Rall Jr., S.-s. Lo, and D. R. Phillips. 1987. Protein sequence from endothelial glycoprotein IIIa derived from a cDNA clone. Identity with platelet GP IIIa and similarity to "integrin." *J. Biol. Chem.* 262:3936–3939.

Freed, E., J. Gailit, P. van der Geer, E. Ruoslahti, and T. Hunter. 1989. A novel integrin β subunit is associated with the vitronectin receptor α subunit (α_v) in a human osteosarcoma cell line and is a substrate for protein kinase C. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2955–2965.

Giancotti, F. G., P. M. Comoglio, and G. Tarone. 1986. A 135 000 molecular weight plasma membrane glycoprotein involved in fibronectin-mediated cell adhesion. *Exp. Cell Res.* 163:47–62.

Giancotti, F. G., and E. Ruoslahti. 1990. Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell.* 60:849–859.

Glenney, J. R., and L. Zokas. 1989. Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton. *J. Cell Biol.* 108:2401–2408.

Haimovich, B., B. J. Aneskievich, and D. Boettiger. 1991. Cellular partitioning of β_1 integrins and their phosphorylated forms is altered after transformation by Rous sarcoma virus or treatment with cytochalasin D. *Cell Reg.* 2:271–283.

Hamaguchi, M., N. Matsuyoshi, Y. Ohnishi, E. Gotoh, M. Takeichi, and Y. Nagai. 1993. $p60^{v\text{-}src}$ causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:307–314.

Hirst, R., A. Horwitz, C. Buck, and L. Rohrschneider. 1986. Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* 83:6470–6474.

Horvath, A. R., M. A. Elmore, and S. Kellie. 1990. Differential tyrosine-specific phosphorylation of integrin in Rous sarcoma virus transformed cells with differing phenotypes. *Oncogene.* 5:1349–1357.

Horwitz, A., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. *Nature (Lond.).* 320:531–533.

Hunter, T., and B. M. Sefton. 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA.* 77:1311–1315.

Hynes, R. O. 1990. Fibronectins. Springer Verlag New York Inc., New York. 546 pp.

Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11–25.

Kanner, S. B., A. B. Reynolds, H.-C. R. Wang, R. R. Vines, and J. T. Parsons. 1991. The SH2 and SH3 domains of pp60^{src} direct stable association with tyrosine phosphorylated proteins p130 and p110. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1689–1698.

Kitas, E., R. Knorr, A. Trzeciak, and W. Bannwarth. 1991. Alternative strategies for the solid-phase synthesis of O_4-phospho-L-tyrosine-containing peptides. *Helv. Chim. Acta.* 74:1314–1328.

Koch, A. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson. 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science (Wash. DC).* 252:668–674.

LaFlamme, S. E., S. A. Akiyama, and K. M. Yamada. 1992. Regulation of fibronectin receptor distribution. *J. Cell Biol.* 117:437–447.

Larsson, E., B. Lüning, and D. Heinegard. 1993. Synthesis and function of an *O*-phosphorylated peptide corresponding to the cell adhesion sequence of bone sialoprotein (BSP). *Acta Chem. Scand.* 47:565–569.

Maher, P. A., E. B. Pasquale, J. Y. J. Wang, and S. J. Singer. 1985. Phosphotyrosine-containing proteins are concentrated in focal adhesions and intercellular junctions in normal cells. *Proc. Natl. Acad. Sci. USA.* 82:6576–6580.

Marchisio, P. C., D. Cirillo, A. Teti, A. Zambonin-Zallone, and G. Tarone. 1987. Rous Sarcoma virus-transformed fibroblasts and cells of monocytic origin display a peculiar dot-like organization of cytoskeletal proteins involved in microfilament-membrane interactions. *Exp. Cell Res.* 169:202–214.

Matsuyoshi, N., M. Hamaguchi, S. Taniguchi, A. Nagafuchi, S. Tsukita, and M. Takeichi. 1992. Cadherin-mediated cell–cell adhesion is perturbed by v-*src* tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol.* 118:703–714.

Miura, O., N. Nakamura, J. N. Ihle, and N. Aoki. 1994. Erythropoietin-dependent association of phosphatidylinositol 3-kinase with tyrosine-phosphorylated erythropoietin receptor. *J. Biol. Chem.* 269:614–620.

Mueller, S. C., and W.-T. Chen. 1991. Cellular invasion into matrix beads. Localization of β_1 integrins and fibronectin to the invadiopodia. *J. Cell Science.* 99:213–225.

Otey, C. A., F. M. Pavalko, and K. Burridge. 1990. An interaction between α-actinin and the β_1 integrin subunit in vitro. *J. Cell Biol.* 111:721–729.

Otey, C. A., G. B. Vasquez, K. Burridge, and B. W. Erickson. 1993. Mapping of the α-actinin binding site within the β_1 integrin cytoplasmic domain. *J. Biol. Chem.* 268:21193–21197.

Pasquale, E. B., P. A. Maher, and S. J. Singer. 1986. Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 83:5507–5511.

Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* 40:191–198.

Reszka, A. A., Y. Hayashi, and A. F. Horwitz. 1992. Identification of amino acid sequences in the integrin β_1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* 117:1321–1330.

Ruoslahti, E. 1991. Integrins. *J. Clin. Invest.* 87:1–5.

Ruoslahti, E., and F. G. Giancotti. 1989. Integrins and tumor cell dissemination. *Cancer Cells.* 1:119–126.

Sarin, V., S. Kent, J. Tam, and R. Merrifield. 1981. Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem.* 177:147.

Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart. 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. *Cell.* 20:807–816.

Sefton, B. M., T. Hunter, E. H. Ball, and S. J. Singer. 1981. Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. *Cell.* 24:165–174.

Singer, I. I., S. Scott, D. W. Kawka, D. M. Kazazis, J. Gailit, and E. Ruoslahti. 1988. Cell surface distribution of fibronectin and vitronectin receptors depends on substrate composition and extracellular matrix accumulation. *J. Cell Biol.* 106:2171–2182.

Solowska, J., J.-L. Guan, E. E. Marcantonio, J. E. Trevithick, C. A. Buck, and R. O. Hynes. 1989. Expression of normal and mutant avian integrin subunits in rodent cells. *J. Cell Biol.* 109:853–861.

Tamkun, J. W., D. W. Simone, D. Fonda, R. S. Patel, C. Buck, A. F. Horwitz, and R. O. Hynes. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell.* 42:271–282.

Tapley, P., A. Horwitz, C. Buck, K. Duggan, and L. Rohrschneider. 1989. Integrins isolated from Rous sarcoma virus-transformed chicken embryo fibroblasts. *Oncogene.* 4:325–333.

Tarone, G., D. Cirillo, F. G. Giancotti, P. M. Comoglio, and P. M. Marchisio. 1985. Rous sarcoma virus-transformed fibroblasts adhere primarily at discrete protrusions of the ventral membrane called podosomes. *Exp. Cell Res.* 159:141–157.

Vogel, B. E., G. Tarone, F. G. Giancotti, J. Gailit, and E. Ruoslahti. 1990. A novel fibronectin receptor with an unexpected subunit composition ($\alpha_v\beta_1$). *J. Biol. Chem.* 265:5934–5937.

Volberg, T., Y. Zick, R. Dror, I. Sabanay, C. Gilon, A. Levitzki, and B. Geiger. 1992. The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1733–1742.

Wang, J. Y. J. 1991. Generation and use of anti-phosphotyrosine antibodies raised against bacterially expressed abl protein. *Methods Enzymol.* 201: 53–65.

Wu, H., A. B. Reynolds, S. B. Kanner, R. R. Vines, and T. Parsons. 1991. Identification and characterization of a novel cytoskeleton-associated pp60^{src} substrate. *Mol. Cell. Biol.* 11:5113–5124.

Yatohgo, T., M. Izumi, H. Kashiwagi, and M. Hayashi. 1988. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* 13:281–292.

Zhang, Z., A. O. Morla, K. Vuori, J. S. Bauer, R. L. Juliano, and E. Ruoslahti. 1993. The $\alpha_v\beta_1$ integrin functions as a fibronectin receptor but does not support fibronectin matrix assembly and cell migration on fibronectin. *J. Cell Biol.* 122:235–242.