Altered Localization and Cytoplasmic Domain-binding Properties of Tyrosine-phosphorylated β1 Integrin

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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 (PYβ1 peptide) that represents a portion of the cytoplasmic domain of the β1 integrin subunit and is phosphorylated on the tyrosine residue known to be the target of oncogenic tyrosine kinases. Antibodies prepared against the PYβ1 peptide, after removal of cross-reacting antibodies by absorption and affinity purification, recognized the PYβ1 peptide and the tyrosine-phosphorylated form of the intact β1 subunit, but did not bind the nonphosphorylated β1 peptide, the nonphosphorylated β1 subunit or other unrelated tyrosine-phosphorylated proteins. The anti-PYβ1 antibodies labeled the podosomes of Rous sarcoma virus-transformed fibroblasts, but did not detectably stain nontransformed fibroblasts. The localization of the tyrosine phosphorylated β1 subunit appeared distinct from that of the β subunit. Adhesion plaques were stained by the anti-β 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 PYβ1 peptide, but not to the nonphosphorylated β1 cytoplasmic peptide. Other SH2 domains did not bind to the PYβ1 peptide. These results show that the phosphorylated form of the β1 integrin subunit is detected in a different subcellular localization than the nonphosphorylated form and suggest that the phosphorylation on tyrosine of the β1 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 β1 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 β1 integrin subunit has been shown to interact with talin (Horton et al., 1986) and α-actinin (Otey et al., 1990, 1993) in vitro. Furthermore, the cytoplasmic tail is necessary and sufficient for β1 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 β1 subunit cytoplasmic domain (Hirst et al., 1986).

The transforming protein of Rous sarcoma virus (RSV),1 p60 onc, is a cytoplasmic tyrosine kinase with constitutive activity (Hunter and Sefton, 1980). Fibroblasts transformed by RSV express high levels of p60 onc, 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),

1. Abbreviations used in this paper: DMF, dimethylformamide; FMOC, 9-fluorenyl-methoxycarbonyl; 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, trifluoroacetic acid.
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Materials and Methods

Synthetic Peptides

The tyrosine-phosphorylated peptides KWDTGENFYP(K)SAVTT (PYβ1) and KWDTANNNPLY(P)KEATST(PYβ1), corresponding to parts of the cytoplasmic domain of the β1 subunit (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 (Matsuoyoshi 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 non-phosphorylated 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 (PYβ1), corresponding to a region of the cytoplasmic domain of the β1 subunit, to generate antibodies specific for the tyrosine-phosphorylated β1 integrin subunit. The residue that is phosphorylated in the peptide represents a consensus site for tyrosine-phosphorylation in the cytoplasmic domain of the β1 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-PYβ1 antibodies to show that the tyrosine-phosphorylated β1 subunit is concentrated in the podosomes of RSV-transformed NIH 3T3 cells, a localization distinct from both that of β1 integrins and phosphotyrosine in general. We discuss the possible function of tyrosine phosphorylated β1 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., 1983; Marchisio et al., 1987; Ruoslahti and Giancotti, 1989). We also present evidence that the tyrosine phosphorylated β1 subunit may specifically interact with phosphatidylinositol (PI)-3-kinase.

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 PYβ1 peptide in 300 μl PBS with 50 μM Na3VO4 was mixed with 12.5 μl of 1 mg/ml methytlated 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 antisera was made 1 mM Na3VO4 and precipitated with (NH4)2SO4 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 Na3VO4, dialyzed towards PBS-1 mM Na3VO4 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). Na3VO4 was added to the serum to inhibit the phosphotyrosine phosphatases that may be present and would cause the dephosphorylation of the tyrosine-phosphorylated peptides that were incubated (e.g., the PYβ1 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 Esherichia 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 PYβ1 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 Na3VO4. 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-PYβ1 antibodies from 40 ml antisera. Affinity-purified rabbit anti-phosphotyrosine (anti-PY) antibodies (Wang, 1991) and rabbit antisera against cytoplasmic peptides of the αβ integrin subunit were incubated with (NIH)2SO4 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 and precipitated with (NH4)2SO4 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 and precipitated with (NH4)2SO4 at 50% saturation; the precipitation and the subsequent steps were performed at 4°C. The precipitate was dissolved in half of the original volume of PBS and precipitated with (NIH)2SO4 at 50% saturation; the precipitation and the subsequent steps were performed at 4°C. The precipitate was dissolved in half of the original volume of PBS and precipitated with (NIH)2SO4 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 and precipitated with (NH4)2SO4 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 and precipitated with (NH4)2SO4 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 and precipitated with (NH4)2SO4 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 and precipitated with (NH4)2SO4 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 and precipitated with (NH4)2SO4 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 and precipitated with (NH4)2SO4 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 and precipitated with (NH4)2SO4 at 40% saturation; the precipitation and the subsequent steps were performed at 4°C.

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, Duluth, 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.

After removal of the last FMOC group the side chain protected peptide was released from the resin by trifluoroacetic acid (TFA), 1% in CH3CN, and was then desalted on Sephadex G15 in 0.1 M NH4HCO3 and lyophilized. The protected PYβ1 peptide gave a 31P NMR signal at -5.8 ppm. Attempts to remove the benzyl protecting groups through hydrogenolysis (Pt/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-buty1 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 31P NMR signal at -2.9 ppm. In a sample that had not been fully desalted a 31P NMR signal corresponding to the monobenzyl derivative of the peptide was observed at -4.2 ppm. The three positions of the 31P NMR signal were consistent with the appearance of the benzyl signals in the 1H NMR spectra. The tyrosine-phosphorylated β1 subunit peptide (PYβ1) was synthesized essentially as peptide PYβ1, giving in all steps similar 31P NMR signals. After the TFA deblocking procedure followed by desalting 77 mg PYβ1 peptide was obtained. Nonphosphorylated β1 integrin peptide KKKEKEMNNKWDGTENPFLYYSAVTVPWAYGK and β1 integrin peptide KFEERARKWDTANNPLYKEATSTFTNITYRGT were synthesized at the Protein Chemistry Facility at the La Jolla Cancer Research Foundation (La Jolla, CA).
Other Materials

CNP-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 PhosphoBlof\(^{TM}\) SH2 (src homology 2) domain reagents were from Oncogene Science (Uniondale, NY). The SH2 domains used are: the human p60\(^{src}\) SH2 domain, the bovine phospholipase C (PLC)\(_{\gamma}\) 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 \(\mu\)l of 10 \(\mu\)g/ml peptide solutions in PBS containing 0.5% glutaraldehyde, pH 7.4, or with 100 \(\mu\)l 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\(_2\)VO\(_4\), washed again with PBS, then incubated with 100 \(\mu\)l primary antibody in PBS with 0.05% Tween 20 and 1 mM Na\(_2\)VO\(_4\) for 4 h, washed three times with PBS-Tween, incubated with anti-rabbit IgG-horseradish peroxidase conjugate 1:1,000 in PBS-Tween-Na\(_2\)VO\(_4\) 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-phenyl-diamine and 0.01% H\(_2\)O\(_2\) in a buffer of 24.3 mM citric acid and 51.4 mM Na\(_2\)HPO\(_4\) 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 PY\(_{\beta_1}\) cytoplasmic peptide (A), the \(\beta_1\) cytoplasmic peptide (B), or phosphotyrosine (anti-PY) (C) in microtiter wells coated with PY\(_{\beta_1}\), \(\beta_1\), PY\(_{\beta_3}\), or \(\beta_3\) peptides at 10 \(\mu\)g/ml or with 10 \(\mu\)g/ml phosphotyrosine as a BSA conjugate (PYBSA). Antibodies against tyrosine-phosphorylated \(\beta_1\) (anti-PY\(_{\beta_1}\)) recognize only the PY\(_{\beta_1}\) peptide and not the nonphosphorylated \(\beta_1\) peptide or other tyrosine-phosphorylated substrates.

**Figure 2.** Specific inhibition of the binding of anti-PY\(_{\beta_1}\) antibodies by the PY\(_{\beta_1}\) peptide in ELISA. ELISA was performed with affinity-purified anti-PY\(_{\beta_1}\) subunit antibodies at 10 \(\mu\)g/ml in microtiter wells coated with 10 \(\mu\)g/ml of the PY\(_{\beta_1}\) peptide (A), or anti-phosphotyrosine antibodies at 1 \(\mu\)g/ml in wells coated with 10 \(\mu\)g/ml of phosphotyrosine as a BSA conjugate (PYBSA) (C). Buffer only (control), the indicated peptide at 100 \(\mu\)g/ml or 40 mM phenylphosphate were added in the primary antibody solution.
were coated onto microtiter wells diluted 1:50 in TBS which contained 1 mM CaCl₂ and 1 mM MgCl₂. ELISA was performed with affinity-purified anti-PYβ₁ (A) or anti-β (B) antibodies as described in Materials and Methods. The lysates were adjusted to the same protein concentration.

**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 Na₃VO₄ 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-0.1% BSA for 30 min, washed three times with PBS and finally developed as above.

**1²⁵I Surface Labeling of Cells**

RSV-transformed cells were treated for 15 min with 1 mM Na₃VO₄ and 2 mM H₂O₂ (Volberg et al., 1992) in serum-free DME, washed twice with cold PBS-100 μM Na₃VO₄, once with PBS, and then harvested and iodinated as described (Pytel et al., 1985), except that cells were lysed in 50 mM octylglucoside, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₃VO₄ in PBS, 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 Na₃VO₄ 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 Na₃VO₄ in PBS, washed twice with PBS and permeabilized with 0.5% NP-40, 1 mM Na₃VO₄, 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 Na₃VO₄ for 1 h, washed as before, incubated with anti-rabbit IgG-rhodamine 1:150 in PBS-3% BSA-1 mM Na₃VO₄ for 45 min and finally washed three times with PBS before observation.

**Phosphatase Treatment of Cells and PYβ₁ 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 Na₃VO₄.

### Results

**Specificity and Reactivity of Anti-tyrosine-phosphorylated β, Integrin Subunit Antibodies**

The crude antiserum from a rabbit immunized with the PYβ₁ peptide contained reactivities to both the PYβ₁ peptide and the nonphosphorylated β₁ cytoplasmic peptide as well as to phosphotyrosine. However, absorption of the antiserum on nonphosphorylated β₁ subunit peptide and on a mixture of tyrosine phosphorylated proteins, followed by affinity purification on a PYβ₁ peptide column, yielded antibodies specific for the PYβ₁ peptide. In ELISA experiments, these antibodies recognized only the tyrosine-phosphorylated β₁ peptide and not the nonphosphorylated β₁ peptide or other tyrosine-phosphorylated substrates, such as the related PYβ₃ peptide or phosphotyrosine coupled to BSA (Fig. 1A). In addition, dephosphorylation of the PYβ₁ peptide by alkaline phosphatase greatly reduced (by about 70%) the binding of the antibodies (not shown). In contrast, conventional anti-β₁ subunit cytoplasmic peptide antibodies (Giancotti and Ruoslahti, 1990) recognized β₁ peptides regardless of phosphorylation (Fig. 1B) and, similarly, an anti-β subunit cytoplasmic peptide antiserum recognized the PYβ₁ peptide (not shown). As expected, anti-phosphotyrosine antibodies (Wang, 1991) recognized the tyrosine-phosphorylated β₁ and β₁ peptides as well as phosphotyrosine coupled at BSA, but not the nonphosphorylated peptides nor BSA (Fig. 1C). The results with anti-phosphotyrosine antibodies also confirmed that the PYβ₁ and PYβ₃ peptides indeed contain phosphorylated tyrosine residues and that the phosphotyrosine-BSA coupling was effective.

The specificity of the anti-PYβ₁ antibodies was further demonstrated by the inhibition observed with the PYβ₁ peptide in ELISA experiments and by the lack of inhibition with the nonphosphorylated β₁ peptide (Fig. 2A). Moreover, phenylphosphate only marginally inhibited the anti-PYβ₁ antibodies (Fig. 2A), whereas it inhibited the binding of the anti-phosphotyrosine antibodies to phosphotyrosine-ml protease, 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-PYβ₁ (A) or anti-β₁ (B) antibodies as described in Materials and Methods. The lysates were coated onto microtiter wells diluted 1:50 in TBS which contained 1 mM CaCl₂ and 1 mM MgCl₂.
Figure 5. Immunofluorescent localization of the tyrosine phosphorylated β1 integrin subunit. Immunofluorescence was performed with 100 μg/ml of affinity-purified anti-PYβ1 antibodies, using RSV-transformed mouse NIH 3T3 fibroblasts plated on noncoated glass. No peptide (A), β1 (B), or PYβ1 (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 PYβ1 peptide (Fig. 2, B and C). The anti-phosphotyrosine antibodies were also inhibited by the PYβ1 peptide (Fig. 2, B and C), again confirming the presence of phosphotyrosine on this peptide.

Detection of Tyrosine-phosphorylated β1 Subunit in RSV-transformed Fibroblasts

Anti-PYβ1 antibodies immunoprecipitated the β1 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 β1 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 β1 subunit, as judged by immunoprecipitation from lysates of surface iodinated cells (not shown) and by ELISA on the cell lysates with anti-β1 (Fig. 4 B).

Distinct Localization of Tyrosine-phosphorylated β1 Integrin Subunit in the Podosomes of RSV-transformed Fibroblasts

We used the new anti-PYβ1 antibodies to determine the localization of the tyrosine phosphorylated β1 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-PYβ1 antibody staining was shown by inhibition experiments; the PYβ1 peptide specifically inhibited the staining of podosomes as well as the diffuse staining (Fig. 5 C, compare to A), whereas the nonphosphorylated β1 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 β₁ integrin subunit (PYβ₁), β₁ integrin subunit (β₁), and phosphotyrosine (PY) in normal (NIH3T3) and RSV-transformed (RSV-NIH-3T3) fibroblasts plated on non-coated glass. Immunofluorescence was performed with 100 μg/ml of affinity-purified anti-PYβ₁ antibodies, 10 μg/ml affinity-purified anti-β₁ antibodies, and 10 μg/ml affinity-purified anti-phosphotyrosine antibodies. Bar, 20 μm.

Table 1. Immunofluorescent Staining of RSV-transformed and Nontransformed Mouse NIH 3T3 Fibroblasts with Anti-tyrosine Phosphorylated β₁ Integrin Subunit, Anti-β₁ Integrin Subunit, Anti-phosphotyrosine, and Anti-p60src 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 β₁ subunit             | Podosomes diffuse staining                                       | Podosomes diffuse staining                            | Not detected                                                     | Not detected                                        |
| Anti-β₁ 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-p60src                                        | 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 β₁ integrin subunit cytoplasmic peptide (10 μg/ml), PYβ₁ (100 μg/ml) and phosphotyrosine (10 μg/ml) or with 10 μg/ml mouse anti-p60src antibodies as described in Materials and Methods.
transformed fibroblasts (Figs. 5, A, C, and D and Fig. 6 A), no anti-PYβ₁ staining above background was obtained in nontransformed fibroblasts (Fig. 6 B). Conventional anti-β₁ 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-PYβ₁, anti-β₁ and anti-phosphotyrosine antibodies were examined in cells plated on a fibronectin substrate. In RSV-transformed fibroblasts plated on fibronectin β₁ 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-PYβ₁ antibody staining is distinct from the localization of both β₁ subunit and phosphotyrosine immunoreactivities.

The localization of p60v-src was also examined for comparison (Table I). p60v-src was found both in podosomes and in adhesion plaques in the RSV-transformed NIH3T3 fibroblasts. Control experiments showed that the nonphosphorylated β₁ peptide greatly reduces anti-β₁ staining and phenylphosphate completely inhibits anti-phosphotyrosine antibody staining, as does the PYβ₁ peptide (not shown). As expected, the nonphosphorylated β₁ 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-β₁, anti-phosphotyrosine and anti-p60v-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-PYβ₁ antibody staining was not inhibited by phenylphosphate, the PYβ₁ epitope does indeed encompass a phosphate group, as demonstrated by the absence of anti-PYβ₁ 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-
Abolition of anti-PYβ₁ 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-PYβ₁ antibodies (A and B), 10 μg/ml of affinity-purified anti-phosphotyrosine antibodies (C and D) and anti-αv integrin antiserum (E and F) at a dilution of 1:100. Bar, 20 μm.

Discussion

We have successfully synthesized a phosphopeptide that mimics the tyrosine-phosphorylated cytoplasmic domain of the β₁ integrin subunit, prepared antibodies to it that specifically recognize the phosphorylated β₁ subunit, and shown with these antibodies that tyrosine-phosphorylated β₁ integrins are concentrated in different subcellular structures than nonphosphorylated β₁ integrins. Our results also show that an SH2 domain of PI-3-kinase binds to the phosphorylated β₁ 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 βErbB-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 PYβ₁ 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 31P NMR signal of the deblocked peptide was, however, later shown to have an incorrect chemical shift. We subsequently
The binding assay was performed with 5 μg/ml of the biotinylated PY/31, PY/33, or 83 peptides at 10 SH2 domains of the p85 subunit of PI-3-kinase, p60 v-sr~ or PLC3, in microtiter wells coated with PY/31, PY/33, or 83 peptides at 10 μg/ml (A) and with PYβ1 or PYβ1 dephosphorylated by treatment with alkaline phosphatase (B).

Figure 2. Binding of SH2 domains to the PYβ1 and PYβ3 peptides. The binding assay was performed with 5 μg/ml of the biotinylated SH2 domains of the p85 subunit of PI-3-kinase, p60 v-sr~ or PLC3 in microtiter wells coated with PYβ1, β1, PYβ3, or β peptides at 10 μg/ml (A) and with PYβ1 or PYβ1 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 β1 subunit cytoplasmic phosphopeptide recognize the phosphotyrosine residue in the context of the surrounding β1 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 peptide. The antibodies prepared against the PYβ1 subunit peptide recognized the intact tyrosine-phosphorylated β1 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 β1 subunit is phosphorylated in RSV-transformed fibroblasts. These experiments showed that the anti-PYβ1 antibodies were suitable for studying the subcellular localization of tyrosine-phosphorylated β1 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 β1 subunit was unknown (result not shown). With the anti-PYβ1 peptide antibodies, the phosphorylated β1 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 β1 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 β1 subunit represents only a minor fraction of the total β1 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-sr~ 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 β1 subunit, observed both on vitronectin and fibronectin substrates, is distinct from the distribution of the β1 subunit, 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 1). Thus, one consequence of β1 subunit phosphorylation appears to be substrate-independent localization in podosomes.

The β1 subunit primarily associates with the αo and α1 subunits in the RSV-transformed NIH 3T3 cells (not shown). However, because of the low quantity of the tyrosine-phosphorylated β1 subunit, we have not been able to identify its companion α subunit. The α 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 in the podosomes. This suggests that in the podosomes PYβ is present in complex with αo. The αoβ integrin is a fibronectin receptor (Vogel et al., 1990), but unlike αβ, it does not support the assembly of a fibronectin matrix (Zhang et al., 1993). Tyrosine-phosphorylated αoβ 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 β1 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, β1 subunit phosphorylation on tyrosine may contribute to the abnormal phenotype of RSV-transformed cells. Consistent with this possibility, β1 subunit tyrosine-phosphorylation was not de-
tected in chicken fibroblasts transformed by a virus encoding a mutant p60°-". Cells infected with this mutant virus retain surface fibronectin and have a relatively normal morphology (Horvath et al., 1990).

The tyrosine kinase p60°-"", 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 subunit is detected in podosomes, but not in adhesion plaques. It is not clear why the β subunit in the adhesion plaques is not phosphorylated by p60°-". A clear why the /~ subunit in the adhesion plaques is not a mutant p60°-". Cells infected with this mutant virus re- 

tected in podosomes, but not in adhesion plaques. It is not detected in chicken fibroblasts transformed by a virus encoding

However, the tyrosine-phosphorylated /~ subunit is de-localized in podosomes as well as in adhesion plaques in 

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