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Osteopontin Activation of c-*src* in Human Melanoma Cells Requires the Cytoplasmic Domain of the Integrin α_v-Subunit

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ABSTRACT

In human melanoma cells, expression of the $\alpha_v\beta_3$ integrin is correlated with the metastatic potential. The expression of osteopontin (OPN or OP), a protein ligand for the integrin $\alpha_v\beta_3$, also correlates with metastatic potential of some tumors. Analysis of signal transduction, stimulated by OPN/$\alpha_v\beta_3$ in human melanoma cells (M21), revealed activation of $pp^{60c\text{-}src}$ associated with the integrin. $pp^{60c\text{-}src}$ stimulation by OPN was dose dependent, and it was inhibited *in vitro* by a tyrosine kinase inhibitor, herbimycin-A. To determine the need for the cytoplasmic domain of the α_v-subunit, in the association of $pp^{60c\text{-}src}$ with $\alpha_v\beta_3$, a cell line expressing truncated α_v was studied. M21-L cells lacked α_v expression but stably transfected with complementary DNAs encoding α_v full length protein α_v1018 or α_v995 (lacking 23 carboxyl-terminal amino acids), and a fibroblast cell line (FG) expressing $\alpha_v\beta_5$ but not $\alpha_v\beta_3$, were used. Western analysis and immune complex kinase assays of anti-α_v immunoprecipitates demonstrated that M21-L/α_v995 cells did not exhibit $pp^{60c\text{-}src}$ association with α_v, whereas the α_v1018 complementary DNA transfected cells and FG cells had $pp^{60c\text{-}src}$ associated with the α_v integrins. Immunofluorescence analysis revealed $pp^{60c\text{-}src}$, $\alpha_v\beta_3$ integrin, and actin distribution along the plasma membrane of M21 cells. ^{35}S-labeling of cells and analysis of complexes immunoprecipitated by a monoclonal antibody against $\alpha_v\beta_3$ demonstrated association of actin with the immune complexes. We conclude that OPN stimulates $pp^{60c\text{-}src}$ kinase activity associated with the $\alpha_v\beta_3$ integrin and that the association requires the cytoplasmic tail of the α_v chain. (*Endocrinology* **137:** 2432–2440, 1996)

HUMAN MELANOMA is a highly metastatic skin cancer with a high mortality when not detected early. Melanoma cells express a wide variety of integrins (1) for extracellular matrix recognition. Integrin binding to matrix components such as vitronectin, fibronectin, or laminin influences cell morphology, growth, and differentiation (2). Some of the integrins reported in both human and murine melanomas are: $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (3), $\alpha_4\beta_1$ (4), $\alpha_6\beta_1$ (5), and $\alpha_v\beta_3$ (1, 6). During the process of tumor progression, there are marked changes in integrin expression. Specifically, the level of integrin $\alpha_v\beta_3$ expression has been directly correlated to neoplastic progression and tumorigenicity in melanoma cells (1, 7, 8).

Human melanoma (M21) and lung carcinoma cells (H2981) use two integrins containing α_v-subunits, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, in adhering to vitronectin (9), although only $\alpha_v\beta_3$ can be detected in focal contacts and colocalized with cytoskeletal proteins. A stable variant cell line, M21-L, lacking α_v, fails to express $\alpha_v\beta_3$ (6) and exhibits reduced rates of proliferation. M21-L cells, transfected with complementary DNA (cDNA) coding for α_v, exhibited a restored growth rate *in vivo* (8). These results demonstrated that integrins are important in metastatic processes and also in the control of cell proliferation. The effects of integrins on proliferation and cell migration are mediated by cell signals generated through their interactions with extracellular matrix proteins. Activation of the proto-oncogene $pp^{60c\text{-}src}$ has been implicated in the proliferation events triggered by growth-factor receptors (10) and recently has been demonstrated to be activated by association with pp^{125FAK} through the linker protein GRB_2 in fibroblasts adherent to fibronectin (11). Thus, we questioned whether $pp^{60c\text{-}src}$ was associated with $\alpha_v\beta_3$ and whether $\alpha_v\beta_3$-dependent activation of $pp^{60c\text{-}src}$ would be observed in M21 cells.

Many integrin receptors interact with extracellular matrix components via the tripeptide, arginine-glycine-aspartic acid, in the ligand. This sequence has been identified as a sequence that promotes cell attachment (12). Osteopontin (OPN), an arginine-glycine-aspartic acid-containing extracellular matrix protein, is one of the ligands for the integrin $\alpha_v\beta_3$. OPN expression has been observed in human cancer cells, and it promotes tumor cell migration and invasion predominantly through $\alpha_v\beta_3$ (13). Expression of antisense messenger RNA to OPN in high OPN-producing tumor cells reduces tumorigenicity (14, 15). It has been hypothesized that OPN might function as a chemoattractant and an attachment factor for tumor cells (16); specifically, it might play a role in targeting metastatic cells to bone. Also, OPN may be an autocrine factor in metastatic cells that suppresses the production of nitric oxide synthase, thereby inhibiting the production of nitric oxide, which is necessary for the cytotoxic oxidative burst of macrophages. Thus, OPN protects the tumor cells from being destroyed by macrophages (17).

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Integrin activation stimulates a number of intracellular signaling events including changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels (18, 19) and second messenger formation (20, 21). Signaling processes by integrins involve both inside-out and outside-in pathways (22, 23). Unlike growth factor receptors, integrin receptors are not tyrosine kinases and the exact biochemical mechanism of postreceptor signaling events remains elusive. Platelet-derived growth factor (PDGF)-stimulated phosphorylation of an $\alpha_v\beta_3$-associated phosphoprotein, 190 kDa, has been observed in NIH3T3 cells (24). Interaction of fibronectin with an integrin (25), or integrin clustering using an antiintegrin β_1-subunit antibody (26), triggers protein tyrosine phosphorylation. Also, integrin-induced phosphorylation of pp^{125FAK} was identified in human epidermoid carcinoma cells (KB cells) as a consequence of adhesion to fibronectin (27). We have previously shown that OPN treatment of chicken osteoclasts stimulates $pp^{60c\text{-}src}$ and phosphatidylinositol 3-hydroxyl kinase associated with the integrin $\alpha_v\beta_3$ (28). The increased tyrosine phosphorylation of pp^{125FAK} attracts the linker protein GRB2, which then allows $pp^{60c\text{-}src}$ and PtdIns 3-hydroxyl kinase to be modulated by cell adhesion to plasma fibronectin and transformation by $pp^{60v\text{-}src}$ in NIH3T3 cells (11, 29). These studies indicate that identification of the kinases associated with the integrin $\alpha_v\beta_3$ is critical to elucidating the mechanism of integrin-initiated signal transduction pathways.

We have used the human melanoma (M21) cell line to evaluate the role of the extracellular matrix protein, OPN, in $\alpha_v\beta_3$-mediated signaling events. We report here that OPN induces $pp^{60c\text{-}src}$ tyrosine kinase activity in human melanoma cells and that $pp^{60c\text{-}src}$ coimmunoprecipitates with the integrin $\alpha_v\beta_3$. Furthermore, the deletion of the α_v cytoplasmic domain resulted in the loss of $pp^{60c\text{-}src}$ association with integrin and failure of ligand activation of $pp^{60c\text{-}src}$ kinase. Our results demonstrate an important signaling event brought about by the interaction of the extracellular matrix protein, OPN, with the integrin $\alpha_v\beta_3$.

Materials and Methods

Materials

[^{32}P]-orthophosphate (9000 Ci/mmol), γ-^{32}P ATP, rainbow mol wt markers for proteins were obtained from Amersham (Arlington Heights, IL). [^{35}S]-methionine was obtained from ICN Bio-Medicals, Inc. (Costa Mesa, CA). Herbimycin-A was obtained from GIBCO-BRL (Gaithersburg, MD). Anti-β_3 antibody was obtained from Chemicon (Tamecula, CA). Protein A sepharose, mouse IgG, antiactin, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Polyvinyldifluoride membrane was obtained from Millipore Corp. (Bedford, MA). A protein assay reagent kit and all the reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad (Hercules, CA). pEZZ18 plasmid for the expression of human OPN cDNA and IgG sepharose were purchased from Pharmacia (Piscataway, NJ). Collagenase enzyme was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Rhodamine phalloidin was obtained from Molecular Probes, Inc. (Eugene, OR). Monoclonal antibody to $pp^{60v\text{-}src}$ was purchased from Oncogene (Uniondale, NY). Human OPN cDNA was kindly provided by Chiron Corp. (Emeryville, CA).

Cell culture

Human melanoma cell lines (M21, M21-L/1018 α_v and M21-L/995 α_v) and human carcinoma cell lines (FG) were previously reported (30, 31) and used in the present studies. All the cell lines were grown in Roswell Park Memorial Institute-1640 Media with 10% FBS and 50 μg/ml gentamicin.

Ligands

A plasmid encoding a fusion protein consisting of protein A and osteopontin joined by a peptide. NSGPVGPVGPVGW, sensitive to collagenase, was expressed in bacteria. The expressed protein was purified by IgG sepharose affinity chromatography. The isolated fusion protein was cleaved with collagenase, and pure OPN was isolated by a second purification through an IgG column. Purified OPN was quantitated and used for all the experiments.

Cell labeling and stimulation

Labeling of cells with [^{32}P]-orthophosphate was carried out as described previously (32) with some modifications. Briefly, cells were incubated with phosphate-free medium for 2 h. They were then washed three times with the same medium and incubated with carrier-free [^{32}P]-orthophosphate (500 μCi/ml) for 2 h. For [^{35}S]-methionine labeling, cells were washed three times with methionine-free medium and incubated in the same medium with 200 μCi/ml [^{35}S]-methionine for 16–18 h. Labeled cells were washed three times and incubated in the presence of vehicle or OPN (25 μg/ml except where otherwise mentioned) for 15 min at 37 C. Incubations at 37 C were stopped by rapidly aspirating the medium, washing the cells three times with ice-cold PBS containing 136 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, and immediately lysing them with ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 10 mM Chaps, 0.1 mM sodium orthovanadate, and 1 mM phenylmethyl sulfonyl fluoride (freshly prepared). Insoluble materials were removed from lysates by centrifugation (15,000 rpm, 15 min, 4 C). Protein concentrations were determined by using a Bio-Rad protein assay reagent kit. Equal amounts of protein lysates were used for immunoprecipitations. The lysates were precleared with protein A sepharose presoaked in a lysis buffer containing 0.1% BSA and with protein A sepharose preabsorbed with antimouse IgG. The precleared lysates were immunoprecipitated with either a monoclonal antibody to $pp^{60v\text{-}src}$ (oncogene) or with anti-α_v integrin antibodies, overnight at 4 C with shaking. The immune complexes were collected by addition of protein A sepharose (Sigma). The beads were collected by centrifugation and washed sequentially, each three times with the following buffers: lysis buffer, 0.2 M $LiCl_2$ with 20 mM Tris-HCl, pH 8.0, and finally with buffer containing 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$ and 0.1 mM Na_3VO_4. The immune complexes were eluted by boiling with electrophoresis sample buffer. The eluted samples were analyzed by SDS-PAGE (33). Gels were dried and autoradiographed using Kodak (Rochester, NY) X-OMAT film.

Immune complex kinase assay analysis

Cells were kept in serum-free media for 2 h and treated with vehicle or OPN (25 μg/ml)for 15 min at 37 C. Lysates were made with lysis buffer as mentioned above after washing the cells several times with ice-cold PBS. Equal amounts of protein lysates were immunoprecipitated with antiintegrin or anti-$pp^{60c\text{-}src}$ antibodies. The immune complexes collected by the addition of protein A sepharose were used for kinase assay. The sepharose beads, after washing several times with the buffers as mentioned above, were resuspended in 20 μl of kinase buffer (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$ and 0.1 mM Na_3V0_4) containing 10 μM [γ-^{32}P]ATP (10 μCi) either in the presence or absence of casein (1 mg/ml) as an exogenous substrate. The mixture was incubated at 25 C for 20 min, and the reaction was stopped by the addition of SDS-sample buffer (34, 35). The samples were boiled and subjected to SDS-PAGE and detected by autoradiography.

Herbimycin-A treatment (100 μg/ml) of the immune complexes of $\alpha_v\beta_3$ was performed for 20 min at room temperature. The herbimycin-A untreated immune complexes were also incubated at room temperature for 20 min with 1% dimethylsulfoxide (solvent for herbimycin-A). The immune complex kinase assays were then conducted in the presence of the exogenous substrate, casein. Recombinant $pp^{60c\text{-}src}$ protein (UBI) was used as a control in all the experiments.

Immunostaining

Cells were grown on coverslips and stained for indirect immunofluorescence as described (36). Briefly, cells were fixed with 3% paraformaldehyde and permeabilized with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM $CaCl_2$ containing 0.1% Triton X-100 for 1 min. The cells were washed and stained with antibodies to $pp^{60v\text{-}src}$ or LM609 (anti-$\alpha_v\beta_3$), washed, and counterstained with fluorescein isothiocyanate-conjugated goat antimouse IgG. Actin staining of the same cells stained for $pp^{60c\text{-}src}$ or $\alpha_v\beta_3$ was performed using rhodamine phalloidin (1:20 dilution) in PBS-EGTA. The cells were rinsed by several changes of PBS-EGTA and mounted on a slide in a mounting solution (Vector Laboratories, Inc., Burlingame, CA). Fluorescence was detected with a microscope fitted with epifluorescence (Zeiss Axioskop, Carl Zeiss, Inc., Oberkochen, Germany)

Results

OPN stimulation of $pp^{60c\text{-}src}$ phosphorylation in melanoma cells

Because autophosphorylation is a measure of *src* family kinase activation, M21 human melanoma cells were labeled with $^{32}[P_i]$-orthophosphate and treated with vehicle or OPN. After cell lysis, equal amounts of lysate proteins were immunoprecipitated with an anti-$pp^{60v\text{-}src}$ antibody. SDS-PAGE was performed, and autoradiograms were developed. As shown in Fig. 1, OPN stimulated $pp^{60c\text{-}src}$ phosphorylation (lane 2), as compared with vehicle-treated cells (lane 1). Immunoprecipitation performed with nonimmune serum failed to bring down a $pp^{60c\text{-}src}$ band (lane 3). The experiment shown in Fig. 1 was representative of three.

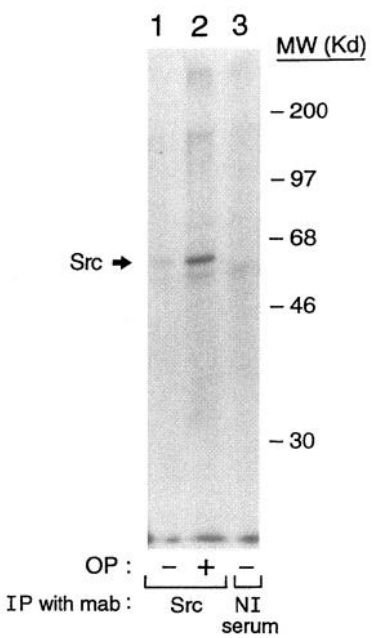

FIG. 1. OPN stimulated $pp^{60c\text{-}src}$ phosphorylation *in vivo*. After labeling with $[^{32}P]$-orthophosphate for 2 h, human melanoma cells (M21) were washed with phosphate and serum-free medium and then incubated with vehicle or OPN (25 μg/ml) for 15 min. Cell lysates were made as designed in *Methods*. Equal amounts of the lysate protein were immunoprecipitated with an anti-$pp^{60c\text{-}src}$ monoclonal antibody. SDS-PAGE and autoradiography of the *src* immunoprecipitates from vehicle- (lane 1) or OPN-treated (lane 2) cells demonstrated that OPN-stimulated $pp^{60c\text{-}src}$ phosphorylation. Lane 3, Immunoprecipitate obtained from untreated cell lysates by nonimmune serum.

Association of $pp^{60c\text{-}src}$ kinase with integrin $\alpha_v\beta_3$

To further investigate the basis of OPN stimulation of $pp^{60c\text{-}src}$ phosphorylation in melanoma cells and to analyze whether $pp^{60c\text{-}src}$ was associated with the integrin $\alpha_v\beta_3$, lysates were made from OPN-treated or vehicle-treated M21 cells. The lysates were immunoprecipitated using an antibody directed to the $\alpha_v\beta_3$ integrin complex (LM609) or an anti-$pp^{60c\text{-}src}$ antibody. The effect of OPN on the tyrosine kinase activity of $pp^{60c\text{-}src}$ was examined by the ability of $pp^{60c\text{-}src}$ associated with the integrin complex from vehicle-treated or OPN-treated cells to autophosphorylate itself and to phosphorylate an exogenous substrate, casein, *in vitro*. As shown in Fig. 2, a 60-kDa protein migrating exactly similar to $pp^{60c\text{-}src}$ was coimmunoprecipitated with the integrin $\alpha_v\beta_3$ (lanes 1 and 2), and OPN stimulated its autophosphorylation and the phosphorylation of the casein substrate (lane 2). OPN also stimulated the autophosphorylation and activity of $pp^{60c\text{-}src}$ kinase in anti-$pp^{60c\text{-}src}$ immunoprecipitates (lanes 3 and 4). Identification of the 60-kDa autophosphorylated protein as $pp^{60c\text{-}src}$ kinase was accomplished by cutting it from the gel and using V-8 protease to produce a peptide digest. SDS-PAGE of the digest of $\alpha_v\beta_3$-associated $pp^{60c\text{-}src}$ compared with the digest of phosphorylated $pp^{60c\text{-}src}$ positive control revealed an identical pattern of labeled peptides. The amount of $pp^{60c\text{-}src}$ immunoprecipitated by LM609 (lane 2) was less than the amount of phosphorylated $pp^{60c\text{-}src}$ seen in

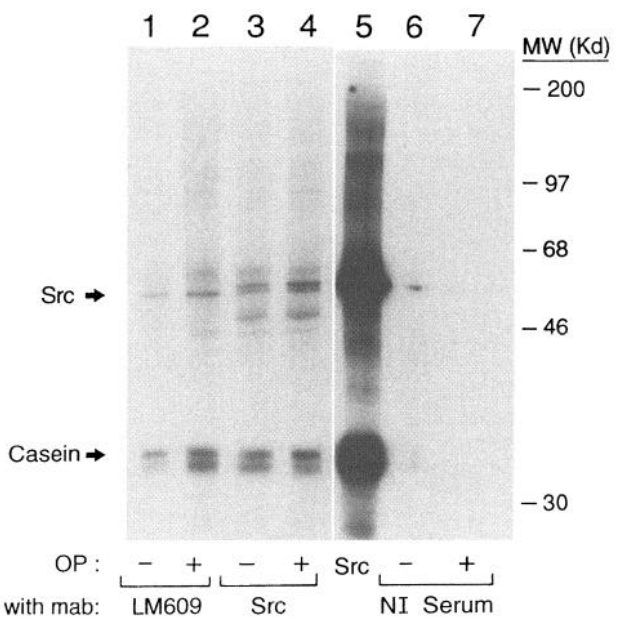

FIG. 2. Association of $pp^{60c\text{-}src}$ with the integrin $\alpha_v\beta_3$ and its activation by OPN treatment of M21 cells. M21 cells were treated with or without OPN (25 μg/ml for 15 min), and cell lysates were prepared. Equal amounts of lysate proteins were immunoprecipitated using anti-$\alpha_v\beta_3$ (LM609) or anti-$pp^{60v\text{-}src}$ monoclonal antibodies. The immune complexes were washed and assayed for protein kinase activity in the presence of γ-^{32}P-ATP and the exogenous substrate, casein, as described in *Methods*. Casein and $pp^{60c\text{-}src}$ are indicated by *arrows*. Lanes 1 and 2, anti-$\alpha_v\beta_3$; lanes 3 and 4, anti-$pp^{60v\text{-}src}$. Lane 5, UBI, which was used as a positive control. Lanes 6 and 7, Immunoprecipitates formed by nonimmune serum. They failed to demonstrate *c-src* or casein phosphorylation. This experiment was one of six. See text for the mean ± SE.

anti-pp$^{60c\text{-}src}$ immunoprecipitates (lane 4), but the OPN activated pp$^{60c\text{-}src}$ activity was roughly equal in the two types of immunoprecipitates. This suggested that a large portion of the OPN-activated c-*src* was $\alpha_v\beta_3$ associated. The integrin $\alpha_v\beta_3$-associated kinase activity was significantly increased by OPN treatment as shown in the representative experiment portrayed in Fig. 2. In six similar experiments, an increase in pp$^{60c\text{-}src}$ phosphorylation of 80% ± 27 ($P < 0.01$), and an increase in casein phosphorylation of 25.8 ± 9.7 ($P < 0.05$) was observed.

The effect of OPN on pp$^{60c\text{-}src}$ phosphorylation was dose dependent. As shown in Fig. 3, the increased phosphorylation of pp$^{60c\text{-}src}$ is dependent upon the concentration of soluble OPN added to cells. A dose of 2 μg/ml of OPN increased pp$^{60c\text{-}src}$ autophosphorylation. The response to OPN had not saturated at the highest dose of OPN used. Twenty-five μg/ml of OPN was more than half as effective as 100 μg/ml, and therefore it was used for the rest of the studies. The dose responsiveness of M21 melanoma cells to OPN was similar to that of avian osteoclasts (28). The physiological concentrations of OPN are unknown and difficult to ascertain because OPN is an autocrine substance and because the concentration in particular local environments is what is important. It circulates at concentrations circa 2 ng/ml.

Truncation of the α_v chain reduces pp$^{60c\text{-}src}$ association

To examine the role of the cytoplasmic domain of α_v in the association of the $\alpha_v\beta_3$ integrin with pp$^{60c\text{-}src}$, cell lines expressing a mutant α_v were used. The M21-L cell line, which does not express endogenous α_v protein or messenger RNA, but maintains abundant β_3-subunit proteins (6), was transfected with cDNAs, encoding one of two α_v-subunit proteins, α_v1018 (wild-type) or α_v995 (a truncation mutant missing 23 amino acid residues) at the carboxyl terminus of α_v (30). Lysates made from M21-L cells transfected with α_v1018 or α_v995 were immunoprecipitated using an antibody against α_v (LM142). LM142 is equally effective in immunoprecipitating a_v1018 or a_v995. Immune complex kinase assays were performed using the immunoprecipitates formed by LM142 as enzyme sources. The results revealed that OPN stimulated pp$^{60c\text{-}src}$ kinase activity in M21-L/α_v1018 cells (Fig. 4, lane 2) as compared with vehicle-treated cells (lane 1). The immunoprecipitates from the α_v995 cell lysates revealed the absence of pp$^{60c\text{-}src}$ kinase autophosphorylation. Phosphorylation of the exogenous casein substrate was observed both in vehicle-treated (lane 3) and OPN-treated (lane 4) α_v995 cells, but OPN did not stimulate casein phosphorylation. The phosphorylation of casein observed in α_v995 transfected cells was due to kinases besides c-*src* in the immunoprecipitates. The phosphoprotein detected, and potentially auto-phosphorylated in these experiments (Fig. 4, lanes 3 and 4), had approximate electrophoretic mobilities of 120 kDa, but its identity is unknown. In cell lysates from a human pancreatic carcinoma cell line, FG, which does not express $\alpha_v\beta_3$ but uses the integrin $\alpha_v\beta_5$ as a vitronectin receptor (37), the anti-α_v immunoprecipitates revealed that pp$^{60c\text{-}src}$ was associated with α_v (lanes 5 and 6). OPN had inconsistent effects on pp$^{60c\text{-}src}$ and kinase activity in FG cells, perhaps because basal pp$^{60c\text{-}src}$ activity associated with α_v was higher in FG cells than M21 cells.

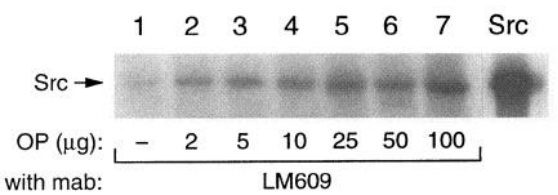

FIG. 3. Dose-dependent effect of OPN on pp$^{60c\text{-}src}$ kinase activity. M21 cells were treated with increasing concentrations of recombinant human OPN (2–100 μg/ml) (lanes 2–7) for 15 min at 37 C. Cell lysates were immunoprecipitated with anti-$\alpha_v\beta_3$ (LM609). The immune complexes were assayed *in vitro* for pp$^{60c\text{-}src}$ autophosphorylation as described in *Methods*. A dose-dependent increase in the phosphorylation of pp$^{60c\text{-}src}$ kinase is observed. pp$^{60c\text{-}src}$ is indicated by *arrow*. These results are representative of two additional experiments.

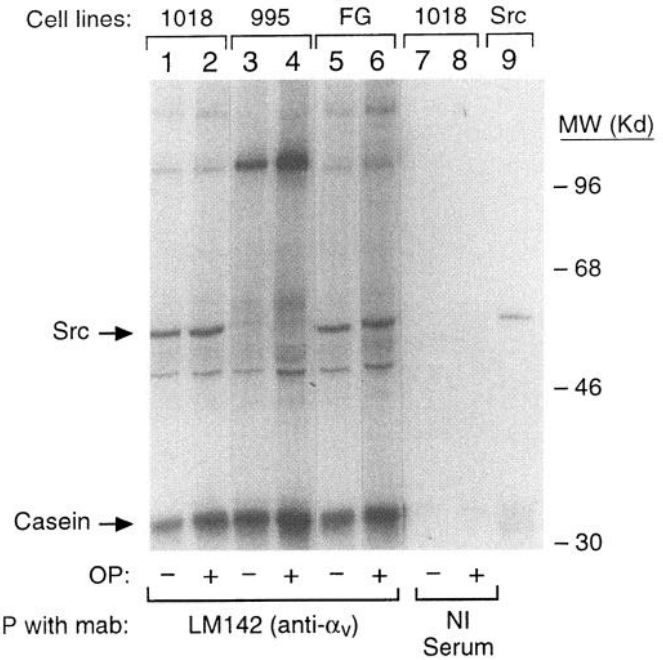

FIG. 4. Presence of pp$^{60c\text{-}src}$ kinase activity in anti-α_v immunoprecipitates. Requirement for the cytoplasmic domain of α_v. Cell lysates from M21, M21L/α_v1018, and M21L/α_v995 cells were prepared after treatment with vehicle (-) or OPN (+) (25 μg/ml). Equal amounts of lysate proteins were used for immunoprecipitation with anti-α_v (LM142) antibody. The immune complexes were assayed *in vitro* for protein kinase activity using casein as the exogenous substrate. OPN-stimulated pp$^{60c\text{-}src}$ kinase activity in M21 cell immunoprecipitates formed by the anti-α_v antibody (lane 2) compared with immunoprecipitates from vehicle-treated cell. Immunoprecipitates from M21L/α_v1018 cells revealed pp$^{60c\text{-}src}$ kinase activity associated with the α_v chain (lanes 3 and 4). Deletion of the cytoplasmic domain of α_v in α_v995 cells did not show pp$^{60c\text{-}src}$ phosphorylation in anti-α_v immunoprecipitates. PP$^{60c\text{-}src}$ is seen in anti-α_v immunoprecipitates of FG-cells, but OPN did not stimulate kinase activity (lanes 5 and 6). Lanes 7 and 8, Immunoprecipitates of cell lysates (α_v1018) with nonimmune serum. Lane 9, Recombinant UBI. The positions of pp$^{60c\text{-}src}$ and casein are indicated by *arrows*.

The profiles of pp$^{60c\text{-}src}$ kinase activity in anti-α_v immunoprecipitates were compared with Western analysis performed with an anti-pp$^{60c\text{-}src}$ antibody (Fig. 5). In agreement with immune complex kinase assay results, Western analysis revealed association of pp$^{60c\text{-}src}$ with the α_v chain of M21 cells (lanes 1 and 2) and FG cell (lanes 5 and 6). The

changes observed in OPN-stimulated M21 cells in immune complex kinase assays (Fig. 2, lanes 1 and 2) were quantitatively and qualitatively similar to the Western analysis as shown in Fig. 5 (lanes 1 and 2). α_v995 transfected M21-L cells did not show $pp^{60c\text{-}src}$ association with the α_v chain (lanes 3 and 4).

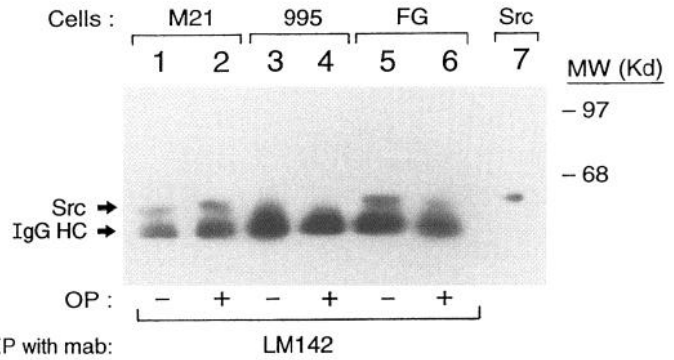

FIG. 5. Detection of $pp^{60c\text{-}src}$ association with the α_v by immunoblotting. M21 (lanes 1 and 2), M21/α_v995 (lanes 3 and 4), and FG (lanes 5 and 6) cells were treated with vehicle (-) or OPN, and cell lysates were prepared. Immunoprecipitation were performed in the antibody against the α_v chain and immune complexes were subjected to SDS-PAGE. Proteins were transferred to a polyvinyldifluoride membrane. $pp^{60c\text{-}src}$ was detected by immunoblotting with monoclonal antibody directed to $pp^{60c\text{-}src}$. Protein bands were visualized by chemiluminescence using the ECL-kit. IgG heavy chain, and $pp^{60c\text{-}src}$ are marked by *arrows*.

Immunofluorescent localization of integrin, $pp^{60c\text{-}src}$, and actin in M21 cells

The distribution of $pp^{60c\text{-}src}$ (Fig. 6B) and integrin $\alpha_v\beta_3$ (Fig. 6E) were examined in M21 cells plated on coverslips and grown in the presence of serum-containing media. The same coverslips stained for $pp^{60c\text{-}src}$ or integrin $\alpha_v\beta_3$ were also stained for actin as shown in Fig. 6, C and F, respectively. Much of the integrin $\alpha_v\beta_3$ or $pp^{60c\text{-}src}$ immunoreactivity colocalized with the actin at the cell periphery.

Coimmunoprecipitation of actin with integrin $\alpha_v\beta_3$

To further investigate the apparent colocalization of c-*src* and $\alpha_v\beta_3$ with the actin cytoskeleton, we determined whether immunoprecipitation of the integrin $\alpha_v\beta_3$ coimmunoprecipitated actin. Lysates made from [^{35}S]-methionine-labeled cells were immunoprecipitated with LM609 or an anti-β_3 antibody. As expected, numerous proteins were found in association with the integrin. The results revealed immunoprecipitation of an integrin complex containing α_v and β_3 chains and coimmunoprecipitation of actin (Fig. 7, lanes 1 and 2). Coimmunoprecipitation of actin with the integrin $\alpha_v\beta_3$ was

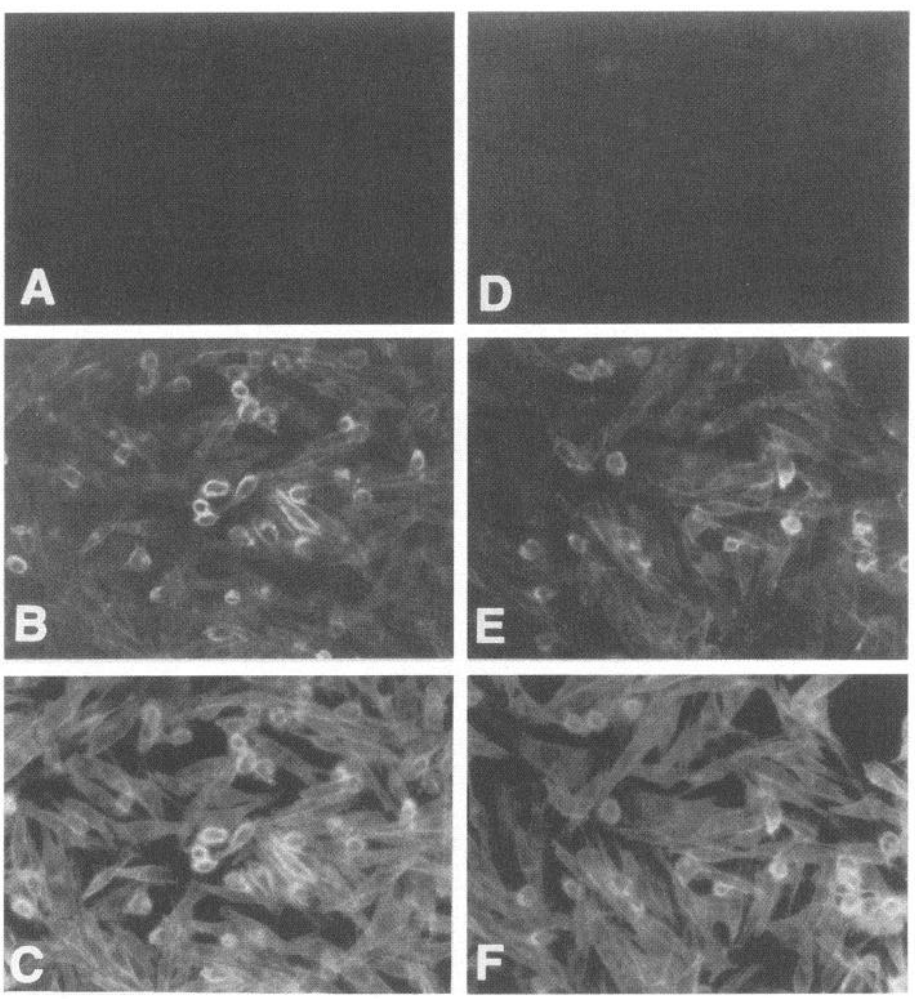

FIG. 6. Localization of integrin, $\alpha_v\beta_3$, $pp^{60c\text{-}src}$, and actin in M21 cells. M21 cells were grown on cover slips in RPMI/1640 media with 10% FBS. Cells were fixed and stained with monoclonal antibodies for $pp^{60c\text{-}src}$ (B) and integrin $\alpha_v\beta_3$ (E). The same sets of cells stained for $pp^{60c\text{-}src}$ or integrin $\alpha_v\beta_3$ were stained for actin and are shown in C and F, respectively. The distribution of actin showed a peripheral staining closer to the plasma membrane where $pp^{60c\text{-}src}$ (B) and integrin $\alpha_v\beta_3$ (E) are localized. A and D, Cells stained with nonimmune serum which shows negative staining.

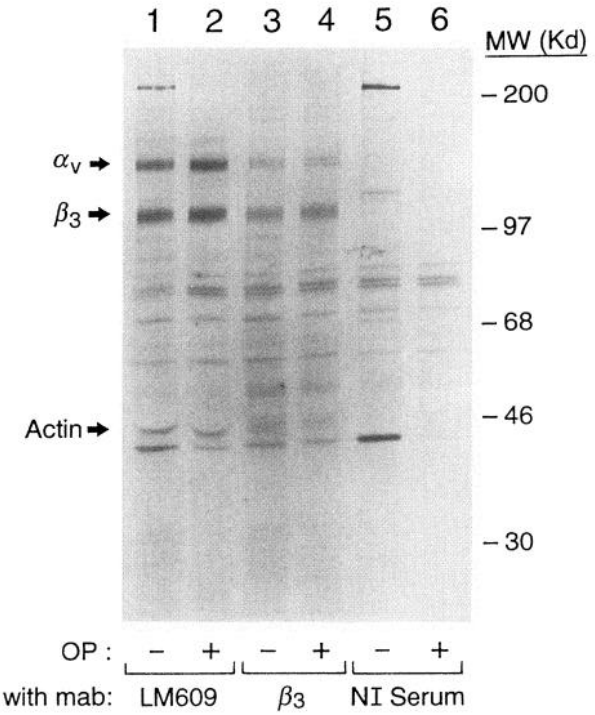

FIG. 7. Coimmunoprecipitation of actin with integrin $\alpha_v\beta_3$. M21 cells were metabolically labeled with [^{35}S]-methionine, and cell lysates were made from vehicle-treated (-) or OPN-treated (+) cells. Immunoprecipitations with anti-$\alpha_v\beta_3$ (lanes 1 and 2) or anti-β_3 antibody (lanes 3 and 4) or with nonimmune serum (lanes 5 and 6) are shown. Immune complexes were analyzed by SDS-PAGE and autoradiography. Antiintegrin $\alpha_v\beta_3$ immunoprecipitates revealed coimmunoprecipitation of actin with the integrin $\alpha_v\beta_3$. The actin band was not seen in anti-β_3 immunoprecipitates. Positions of the integrin subunits and actin bands are indicated by *arrows*.

reconfirmed by Western analysis of $\alpha_v\beta_3$ immunoprecipitated with antiactin antibody (data not shown). Immunoprecipitation of the lysates with antibody against the β_3 chain brought down the β_3 chain but less the α_v chain compared with immunoprecipitation of the integrin complexes with LM609. The ratio of the intensity of the α_v and β_3 chains bands (as shown by *arrows*) was relatively equal when immunoprecipitated with anti-$\alpha_v\beta_3$ (LM609). Actin was not detected in the anti-β_3 immunoprecipitates (Fig. 7, lanes 3 and 4), although many other proteins in the precipitated complexes were similar to those observed with LM609.

Immunolocalization of actin and pp$^{60c\text{-}src}$ in vivo α_v/995 cells

To examine whether deletion of β-turn sequences of α_v cytoplasmic domain alters colocalization of actin and pp$^{60c\text{-}src}$, M21 melanoma cells (Fig. 8, A and B) and M21-L/α_v 995 cells (Fig. 8, C and D) were double stained with rhodamine phalloidin for actin and anti-*src* antibody. The same sets of cells stained for actin and *src* are shown in Fig. 8. There was significant morphological differences between M21 cells (Fig. 8, A and B) and M21-L/α_v 995 cells (Fig. 8, C and D), but the colocalization of actin [A and C] and *src* [B and D] was unchanged. Colocalization was observed by yellow color formed by fusion of green pseudocolor images of pp$^{60c\text{-}src}$ with the red actin staining.

Discussion

In the present study, we have demonstrated that recombinant human OPN, an extracellular matrix protein, stimulated pp$^{60c\text{-}src}$ kinase activity in M21 melanoma cells. These results are in agreement with studies demonstrating that soluble vitronectin-containing ligands triggered phosphorylation of proteins on (19, 38, 39) tyrosine in bovine pulmonary artery (37). It is known that one of the receptors for OPN is the $\alpha_v\beta_3$ integrin (19, 38, 39). A previous study has reported OPN stimulated phosphorylation and activity of integrin-associated pp$^{60c\text{-}src}$, as well as phosphorylation of pp^{125FAK} and PLCγ in avian osteoclasts (28). Binding of extracellular ligands to integrin receptors generates a cascade of biochemical events including changes in intracellular pH (40, 41); intracellular Ca^{2+} (42–44); transient increase in phosphorylation of proteins (25, 26, 28, 45–47), (reviewed in Refs. 48 and 49); activation of protein kinases such as protein kinase C (50) and mitogen-activated protein kinase (51); and activation of gene expression (reviewed in Ref. 22).

The pp$^{60c\text{-}src}$ family kinases become transiently activated when the receptor for PDGF is activated by ligand binding (52, 53). Unlike growth factor receptors, integrin receptors are not kinases. Cell surface receptors have been shown to associate with members of the pp$^{60c\text{-}src}$ family such as *lyn*, *hck*, and *fgr* (54), the *syk* kinase (55), and the *fes* kinase (56). Tyrosine phosphorylation of pp^{125FAK} has been observed in several cell types upon integrin clustering or adhesion to fibronectin (27, 57, 58). Activation of pp^{125FAK} was observed in NIH3T3 fibroblast cells by transformation with pp$^{60c\text{-}src}$ (29). Adhesion of murine NIH3T3 fibroblasts to fibronectin promotes pp$^{60c\text{-}src}$ and focal adhesion association and formation of an integrin-activated signaling complex (11). Phosphorylation of pp^{125FAK} by pp$^{60c\text{-}src}$ (or other family kinases) is an important step in the formation of an active signal complex (59). These and our results indicate that the nonreceptor tyrosine kinase pp$^{60c\text{-}src}$ phosphorylation is an early if not the initial response of integrin-mediated signal transduction and that activation of kinases elicited by integrin-mediated signaling is similar to growth-factor-mediated responses. Members of the pp$^{60c\text{-}src}$ family of kinases have been shown to associate with the autophosphorylated PDGF or colony stimulating factor-1 or interleukin-2 receptors (52, 60–62) through SH2 domains. However, the scenario is more complex because the integrin receptors do not have the sequences for the interaction of nonreceptor tyrosine kinases. Thus, their mechanism of integrin association and activation is unknown.

The α_v integrin has been suggested to play a role in the regulation of adhesive properties and invasiveness of carcinoma and melanoma cells (9, 63, 64). The α_v integrin is a component of several α_v-containing integrins and shares structural motifs with other integrin α-subunits. A β-turn motif is found in several integrin α-subunits. Truncation of the β-turn motif in the cytoplasmic tail of α_v (α_v995) reduced ligand binding properties of the integrin (30). Deletion of the

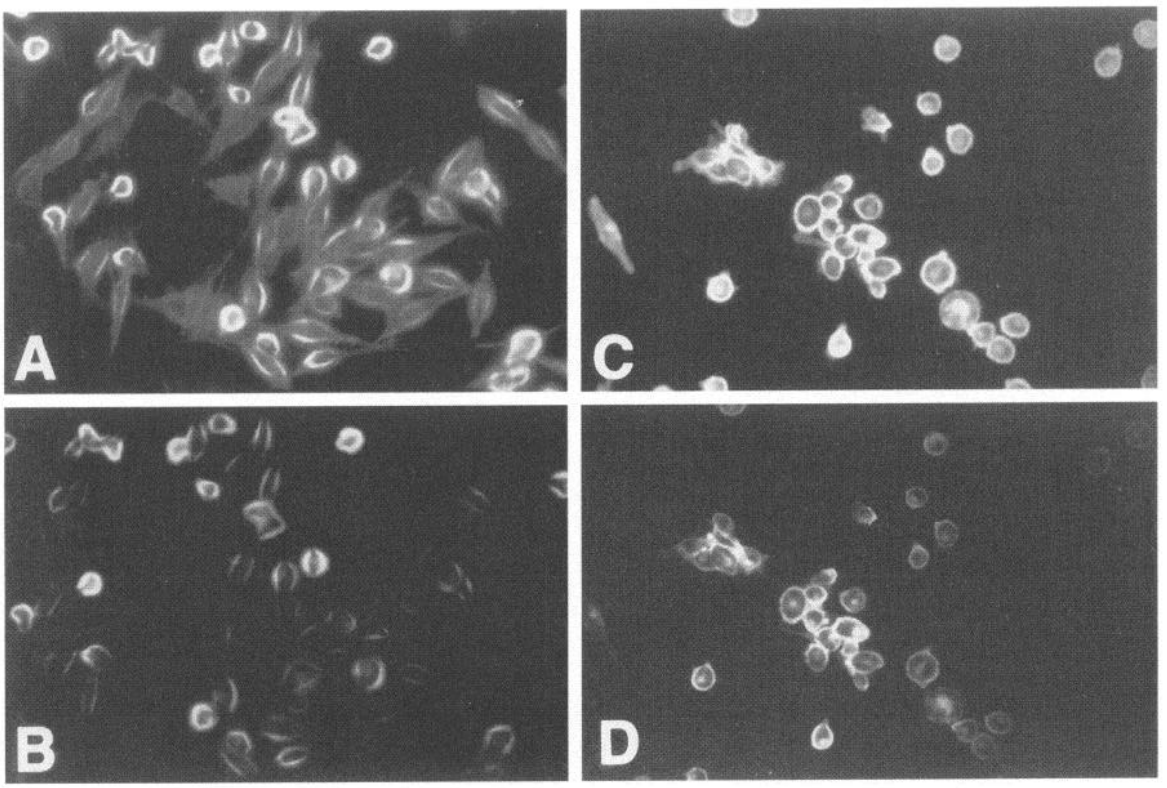

FIG. 8. Immunolocalization of actin and $pp^{60c\text{-}src}$ in M21 and M21-L/α_v995. M21 (A and B) and M21-L/α_v995 (C and D) cells were grown on cover slips in RPMI 1640 media with 10% FBS. Immunostaining for actin (A and C) and $pp^{60c\text{-}src}$ (B and D) was done on the same cells as shown. There was significant colocalization of actin and $pp^{60c\text{-}src}$ in both cell types despite the morphological differences observed in the M21-L α_v995 cells compared with the M21 cells.

cytoplasmic tail of α_5 from the $\alpha_5\beta_1$ fibronectin receptor resulted in the loss of the ligand-binding function (65). Our studies with α_v995-truncation mutant transfected cells demonstrated that $pp^{60c\text{-}src}$ was no longer associated with $\alpha_V\beta_3$. However, association of $pp^{60c\text{-}src}$ with $\alpha_v\beta_3$ was observed in M21 cells as well as in human carcinoma cells (FG), which express $\alpha_v\beta_5$ as a cell adhesion receptor. These analyses are in agreement with demonstrations by Chan *et al.* (66) that the cytoplasmic domain of the α_v chain has an important role in post-ligand-binding events. Our observations with M21 and M21L/α_v995 cells indicate that the α_v cytoplasmic tail is required in mediating the association of the cytoskeletal/$pp^{60c\text{-}src}$ complex with the integrin. Coimmunoprecipitation of actin and $pp^{60c\text{-}src}$ with the integrin $\alpha_v\beta_3$ demonstrates the association of the cytoskeletal/$pp^{60c\text{-}src}$ complex with the integrin $\alpha_v\beta_3$.

Demonstration of colocalization of actin/$pp^{60c\text{-}src}$ and the integrin along the periphery of melanoma cells provides additional evidence for the above observations. In platelets, association of $pp^{60c\text{-}src}$ and GpIIb/IIIa with the cytoskeleton and tyrosine phosphorylation are related phenomena (67). GpIIb/IIIa complexes were associated with actin bundles in aggregated platelets (68, 69). $pp^{60c\text{-}src}$ could be an integral part of the so-called "integrin-rich cytoskeleton" as postulated by Kouns *et al.* (70). Immunofluorescent images of focal adhesions showed colocalization of integrins with the termini of actin bundles and actin-binding proteins (71). The cytoplasmic tails of integrins have been shown to interact with talin, α-actinin, tensin, and other unidentified proteins to link the integrins to the actin cytoskeletal network (72, 73). The protein(s) of the cytoskeletal structure to which $pp^{60c\text{-}src}$ directly binds is not yet known. Also, a more direct mode of attachment at focal adhesions is suggested by the observation that two different forms of the integrin with actin tightly bound have been isolated (74, 75). In conclusion, our present study suggests that the cytoplasmic domain of the α_v chain is essential for the association of the $pp^{60c\text{-}src}$/cytoskeleton complex. Furthermore, the $\alpha_v\beta_3$ ligand activates $pp^{60c\text{-}src}$ kinase associated with $\alpha_v\beta_3$ in the complex.

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References

1. **Albelda SM, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M, Buck CA** 1990 Integrin distribution in malignant melanoma: association of the β_3 subunit with tumor progression. Cancer Res 50:6757–6764
2. **Ruoslahti E** 1991 Integrins. J Clin Invest 87:1–5
3. **Kramer R, Marks N** 1989 Identification of integrin collagen receptors on human melanoma cells. J Biol Chem 264:4684–4688
4. **Mould PP, Wheldon LA, Komoriya A, Wayner EA, Yamada KM, Humphries MJ** 1990 Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin $\alpha_v\beta_3$. J Biol Chem 265:4020–4024

5. **Ramos DM, Berston ED, Kramer RH** 1990 Analysis of integrin receptor for laminin and type IV collagen on metastatic B16 melanoma cells. Cancer Res 50:728–734
6. **Cheresh DA, Spiro RC** 1987 Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen and von Willebrand factor. J Biol Chem 262:17703–17711
7. **Cheresh DA** 1991 Structure, function and biological properties of integrin $\alpha_v\beta_3$ on human melanoma cells. Cancer Metastasis Rev 10:3–10
8. **Habermann FB, Mueller BMC, Romerdahl CA, Cheresh DA** 1992 Involvement of integrin α_v gene expression in human melanoma tumorigenicity. J Clin Invest 89:2018–2022
9. **Wayner EA, Orlando RA, Cheresh DA** 1991 Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ contribute to cell attachment to vitronectin but differentially distribute on the cell surface. J Cell Biol 113:919–929
10. **Alonso G, Loegi M, Mazurenko N, Courtneidge SA** 1995 Sequence requirements for binding of src family tyrosine kinases to activated growth factor receptors. J Biol Chem 270:9840–9848
11. **Schlaepfer DD, Hanks SK, Hunter T, van der Geer P** 1994 Integrin-mediated signal transduction linked to Ras pathway by GRB binding to focal adhesion kinase. Nature 372:786–791
12. **Pierschbacher MD, Ruoslahti E** 1987 Influence of stereochemistry of the sequence Arg-Gly- Asp-Xaa on binding specificity in cell adhesion. J Biol Chem 262:17294–17298
13. **Senger DR** 1995 Osteopontin at the tumor/host interface. Functional regulation by thrombin-cleavage and consequences for cell adhesion. Ann NY Acad Sci 760:83–100
14. **Gardner HA R, Berse B, Senger DA** 1994 Specific reduction in osteopontin synthesis by antisense RNA inhibits the tumorigenicity of transformed rat1 fibroblasts. Oncogene 9:2321–2326
15. **Behrend EI, Craig AM, Wilson SM, Denhardt DT, Chambers AF** 1995 Expression of antisense osteopontin RNA in metastatic mouse fibroblasts is associated with reduced malignancy. Osteopontin: Role in cell signalling and adhesion. Ann NY Acad Sci 760:299–301
16. **Chambers AF, Hota C, Prince CW** 1993 Adhesion of metastatic, ras-transformed NIH3T3 cells to osteopontin, fibronectin and laminin. Cancer Res 53:701–706
17. **Denhardt DT, Chambers AF** 1994 Overcoming obstacles to metastasis - defence against host defenses: osteopontin as a shield against attack by cytotoxic host cells. J Cell Biochem 56:48–51
18. **Schwartz MA** 1993 Spreading of human endothelial cells on fibronectin or triggers elevation intracellular free calcium. J Cell Biol 120:1003–1010
19. **Miyauchi A, Alvarez J, Greenfield EM, Teti A, Grano M, Colucci S, Zambonin-Zallone A, Ross FP, Teitelbaum SL, Cheresh DA, Hruska KA** 1991 Recognition of osteopontin and related peptides by an $\alpha_v\beta_3$ integrin stimulates immediate cell signals in osteoclasts. J Biol Chem 266:20369–20374
20. **Schuch U, Lohse MJ, Schachner M** 1989 Neural cell adhesion molecules influence second messenger system. Neuron 3:13–20
21. **Frei T, von Bohlen un Halbach F, Wille W, Schachner M** 1992 Neural cell adhesion molecules influence second messenger system. J Cell Biol 117:177–194
22. **Juliano RL, Haskill S** 1993 Signal transduction from the extracellular matrix. J Cell Biol 120:577–585
23. **Williams MJ, Hughes PE, O'Toole TE, Ginsberg MH** 1994 The inner world of cell adhesion integrin cytoplasmic domains. Trends Cell Biol 4:109–112
24. **Bartfeld NS, Pasquale EB, Geltosky JE, Languino LR** 1993 The $\alpha_v\beta_3$ integrin associates with a 190-kDa protein that is phosphorylated on tyrosine in response to platelet-derived growth factor. J Biol Chem 268:17270–17276
25. **Guan JL, Trevithick J, Hynes RO** 1991 Fibronectin/integrin interaction induces tyrosine phosphorylation of 120 kD protein. Cell Regul 2:951–964
26. **Kornberg LJ, Earp HS, Turner CE, Procop C, Juliano RL** 1991 Signal transduction by integrins: Increased protein tyrosine phosphorylation caused by clustering of β_1 clustering. Proc Natl Acad Sci USA 88:8392–8396
27. **Kornberg L, Earp HS, Parsons J, Schaller M, Juliano RL** 1992 Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion associated tyrosine kinase. J Biol Chem 267:23439–23442
28. **Hruska KA, Rolnick F, Huskey M, Alvarez U, Cheresh D** 1995 Engagement of the osteoclast integrin $\alpha_v\beta_3$ by osteopontin stimulates phosphoinositol 3-hydroxyl kinase activity. Endocrinology 136:2984–2992
29. **Guan JL, Shalloway D** 1992 Regulation of focal adhesion associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature 358:690–692
30. **Filardo EJ, Cheresh DA** 1994 The β turn in the cytoplasmic tail of the integrin α_v subunit influences conformation and ligand binding of $\alpha_v\beta_3$. J Biol Chem 269:4641–4647
31. **Leavesley DI, Ferguson GD, Wayner EA, Cheresh DA** 1992 Requirement of the integrin beta 3 subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. J Cell Biol 117:1101–1109
32. **Meenakshi T, Ross FP, Martin J, Teitelbaum SL** 1993 1,25-dihydroxyvitamin D_3 and mC synergistically phosphorylate talin. J Cell Biochem 53:1–11
33. **Laemmeli VP** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–685
34. **Uehara Y, Fukazawa H, Murakami Y, Mizuno S** 1989a Irreversible inhibition of v-*src* tyrosine kinase activity by herbimycin A and its abrogation by sulfhydryl compounds. Biochem Biophys Res Commun 163:803–809
35. **Uehara Y, Murakami Y, Sugimoto Y, Mizuno S** 1989b Mechanism of reversion of Rous Sarcoma virus transformation by herbimycin A reduction of total phosphorylation levels due to reduced kinase activity and increased turn over of $pp60^{v\text{-}src}$. Cancer Res 49:780–785
36. **Wu H, Kanner SB, Reynolds AB, Vines RR, Parsons JT** 1991 Identification and characterization of a novel cytoskeleton- associated pp60c-src substrate. Mol Cell Biol 11:5113–5124
37. **Bhattacharya S, Fu C, Bhattacharya J, Greenberg S** 1995 Soluble ligands of the $\alpha_v\beta_3$ integrin mediate enhanced tyrosine phosphorylation of multiple proteins in adherent bovine pulmonary artery endothelial cells. J Biol Chem 270:16781–16787
38. **Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM, Giachelli CM** 1995 The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. J Clin Invest 95:713–724
39. **Ross FP, Chappel J, Alvarez JI, Sander D, Butler WT, Farach-Carson MC, Mintz KA, Robey P G, Teitelbaum SL, Cheresh DA** 1993 Interactions between the bone matrix proteins osteopontin and the osteoclast integrin $\alpha_v\beta_3$ potentiate bone resorption. J Biol Chem 268:9901–9907
40. **Ingber DE, Prusty D, Frangioni JV, Cragoe EJ, Lechene C, Schwartz MA** 1990 Control of intracellular pH and growth by fibronectin in capillary endothelial cells. J Cell Biol 110:1803–1811
41. **Schwartz MA, Lechene C, Ingber DE** 1991 Insoluble fibronectin activates the Na/H antiporter by clustering and immobilizing integrin $\alpha_v\beta_3$, independent of cell shape. Proc Natl Acad Sci USA 88:7849–7853
42. **Schwartz MA, Denninghoff K** 1994 α_v integrin mediate the rise in intracellular calcium in endothelial cells on fibronectin even though they play a minor role in adhesion. J Biol Chem 269:11133–11137
43. **Zimolo Z, Wesolowski G, Tanaka H, Hyman JL, Hoyer JR, Rodan GA** 1994 Soluble $\alpha_v\beta_3$ integrin ligands raise Ca_{2+} in rat osteoclasts and mouse derived osteoclast-like cells. Am J Physiol C376–C381
44. **Pelletier AJ, Bodary SC, Levinson AD** 1992 Signal transduction by the platelet integrin $\alpha_{IIb}\beta_3$: induction of calcium oscillations required for protein tyrosine phosphorylation and ligand induced spreading of stably transfected cells. Mol Biol Cell 3:989–998
45. **Golden A, Brugge JS, Shattil SJ** 1990 Role of platelet membrane glycoprotein IIb-IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. J Cell Biol 111:3117–3127
46. **Kapron-Bras C, Fitz-Gibbon L, Jeevaratnam P, Wilkins J, Dedhar S** 1993 Stimulation of tyrosine phosphorylation and accumulation of GTP-bound $p21^{ras}$ upon antibody-mediated $\alpha_2\beta_1$ integrin activation in T-lymphoblastic cells. J Biol Chem 268:20701–20704
47. **Kanner SB, Grosmaire LS, Ledbetter JA, Damle NK** 1993 β_2 integrin LFA-1 signalling through phospholipase C-g^1 activation. Proc Natl Acad Sci USA 90:7099–7103
48. **Schwartz MA, Ingber DE** 1994 Integrating with integrins. Mol Biol Cell 5:389–393
49. **Sastry SK, Horowitz AF** 1993 Integrin cytoplasmic domains: me-

diators of cytoskeletal linkages and extra and intracellular initiated transmembrane signalling. Curr Opin Cell Biol 5:819–831

50. **Vuori K, Ruoslahti E** 1993 Activation of protein kinase C precedes $\alpha_5\beta_1$ integrin-mediated cell spreading on fibronectin. J Biol Chem 268:21459–21462
51. **Chen Q, Kinch MS, Lin TH, Burridge K, Juliano RL** 1994 Integrin-mediated cell adhesion activates mitogen activated protein kinases. J Biol Chem 269:26602–26605
52. **Gould K, Hunter T** 1988 PDGF- induces multisite phosphorylation of $pp60^{c\text{-}src}$ and increases its kinase activity. Mol Cell Biol 8:3345–3356
53. **Krypta RM, Goldberg Y, Ulug ET, Courtneidge SA** 1990 Association between the PDGF receptor and members of the src family of tyrosine kinase. Cell 62:481–492
54. **Stefanova I, Corcoran ML, Horak EM, Wahl LM, Bolen JB, Horak ID** 1993 Lipopolysaccharide induces activation of CD-14 associated protein tyrosine kinase $p53/p56^{lyn}$. J Biol Chem 268:20725–20728
55. **Taniguchi T, Kobayashi T, Kondo J, Takahashi K, Nakamura H, Suzuki J, Nagai K, Yamada T, Nakamura S, Yamamura H** 1991 Molecular cloning of a porcine gene *syk* that encodes a 72 kDa protein tyrosine kinase showing high susceptibility to proteolysis. J Biol Chem 266:15790–15796
56. **Greer P, Maltby V, Rossant J, Bernstein A, Pawson T** 1990 Myeloid expression of the human *c-fps/fes* proto-oncogene in transgenic mice. Mol Cell Biol 10:2521–2527
57. **Burridge K, Turner CE, Romer LH** 1992 Tyrosine phosphorylation of paxillin and $pp125^{fak}$ accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. J Cell Biol 119:893–903
58. **Lipfert L, Haimovich B, Schaller MD, Cobb BS, Parsons JT, Brugge JM** 1992 Integrin-dependent phosphorylation and activation of the protein tyrosine kinase $pp125^{Fak}$ in platelets. J Cell Biol 119:893–903
59. **Calalb MB, Polte TR, Hanks SK** 1995 Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity. A role for src family kinases. Mol Cell Biol 15:954–963
60. **Twamley-Stein GM, Pepperkok R, Ansorge W, Courtneidge SA** 1993 The src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH3T3 cells. Proc Natl Acad Sci USA 90:7696–7700
61. **Courtneidge SA, Dhand R, Pilat D, Twamley GM, Waterfield MD, Roussel M** 1993 Activation of src family kinases by colony stimulating factor-1 and their association with its receptor. EMBO J 12: 943–950
62. **Hatakeyama M, Kono T, Koboyashi N, Kawahara A, Levin SD, Perlmutter RM, Taniguchi T** 1991 Interaction of the IL-2 receptor with the src-family kinase $p56^{lck}$. Identification of novel intermolecular association. Science 252:1523–1528
63. **Seftor REB, Seftor EA, Gehlsen KR, Stelter-Stevenson WG, Brown PO, Ruoslahti E, Hendrix MJC** 1992 Role of $\alpha_v\beta_3$ integrin in human melanoma cell invasion. Proc Natl Acad Sci USA 89:1557–1561
64. **Cheresh DA, Smith JW, Cooper HM, Quaranta V** 1989 A novel vitronectin receptor integrin ($\alpha_v\beta_x$) is responsible for distinct adhesive properties of carcinoma cells. Cell 57:59–69
65. **Bauer JS, Varner J, Schreiner E, Kornberg L, Nicholas R, Juliano RL** 1993 Functional role of the cytoplasmic domain of the integrin α_5 subunit. J Cell Biol 122:209–221
66. **Chan BMC, Kassner PD, Schiro JA, Byers HR, Kupper TS, Hemler ME** 1992 Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. Cell 68:1051–1060
67. **Oda A, Druker BJ, Smith M, Salzman EW** 1992 Association of $pp60^{c\text{-}src}$ with Triton X-100 insoluble residue in human blood platelet aggregation and actin polymerization. J Biol Chem 267:20075–20087
68. **Fox JEB** 1987 In: Verstraete M, Vermylen J, Lijnen R, Arnout J (eds) Thrombosis and Haemostasis. International Society of Thrombosis and Homeostasis and Leuven University Press, Leuven, Belgium, pp 175–225
69. **Phillips DR, Jennings LK, Edwards HH** 1980 Identification of the membrane protein mediating the interaction of human platelets. J Cell Biol 86:77–86
70. **Kouns WC, Fox CF, Lamoreaux WJ, Coons LB, Jennings LK** 1991 The effect of glycoprotein IIb-IIIa receptor occupancy on the cytoskeleton of resting and activated platelets. J Biol Chem 266:13891–13900
71. **Heidemann SR** 1993 A new twist on integrins and the cytoskeleton. Science 260:1080–1081
72. **Burridge K, Fath K, Kelly T, Nuckolls G, Turner C** 1988 Transmembrane junctions between the extracellular and the cytoskeleton. Annu Rev Cell Biol 4:487–525
73. **Otey CA, Pavalko FM, Burridge K** 1990 An interaction between α-actinin and the -β_1 integrin subunit *in vitro*. J Cell Biol 111:721–729
74. **Painter RG, Prodouz KN, Gaarde W** 1985 Isolation of a subpopulation of glycoprotein IIb/IIIa from platelet membranes that is bound to membrane actin. J Cell Biol 100:652–657
75. **Molony L, Kelly T, Burridge K** 1987a Does integrin bind actin directly? J Cell Biol 105:177a