

## Integrin $\alpha 2\beta 1$ Promotes Activation of Protein Phosphatase 2A and Dephosphorylation of Akt and Glycogen Synthase Kinase 3 $\beta$

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**Serine/threonine kinase Akt is a downstream effector protein of phosphatidylinositol-3-kinase (PI-3K). Many integrins can function as positive modulators of the PI-3K/Akt pathway. Integrin  $\alpha 2\beta 1$  is a collagen receptor that has been shown to induce specific signals distinct from those activated by other integrins. Here, we found that, in contrast what was found for cells adherent to fibronectin,  $\alpha 2\beta 1$ -mediated cell adhesion to collagen leads to dephosphorylation of Akt and glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) and concomitantly to the induction of protein serine/threonine phosphatase 2A (PP2A) activity. PP2A activation can be inhibited by mutation in the  $\alpha 2$  cytoplasmic domain and by a function-blocking anti- $\alpha 2$  antibody. Akt can be coprecipitated with PP2A, and coexpression of Akt with PP2Ac (catalytic subunit) inhibits Akt kinase activity. Integrin  $\alpha 2\beta 1$ -related activation of PP2A is dependent on Cdc42. These results indicate that cell adhesion to collagen modulates Akt activity via the  $\alpha 2\beta 1$ -induced activation of PP2A.**

The integrins are a large family of heterodimeric transmembrane receptors composed of  $\alpha$  and  $\beta$  subunits (22). In addition to mediating cell-matrix interactions, integrins have been shown to activate intracellular signaling pathways which, in collaboration with growth factor-induced signals, regulate cellular functions (46). Some integrin signaling cascades are activated via the  $\beta$  subunit cytoplasmic domain, and they are therefore triggered by several integrin heterodimers. These signals include the activation of protein tyrosine kinases of the Src and focal adhesion kinase (FAK) families (9, 47). More-recent studies have revealed signaling events that are activated specifically by an  $\alpha$  subunit (19). Integrins may associate with other membrane proteins, such as caveolin-1, and a subset of integrins can activate extracellular signal-related kinase, one of the mitogen-activated protein kinases, via Fyn and Shc (53, 54). Some integrins interact with other membrane proteins to regulate distinct signaling cascades. For example laminin receptor  $\alpha 3\beta 1$  associates with tetraspanin proteins and activates phosphatidylinositol-3-kinase (PI-3K) and PI-4K (4). We have shown that  $\alpha 2\beta 1$  integrin specifically activates the p38 pathway via a mechanism involving the  $\alpha 2$  cytoplasmic tail and Cdc42 (25). The p38 signaling pathway seems to regulate the expression of type I collagen and collagenase-3 (25, 42), and it is required for cell migration on collagen (29).

The PI-3K/Akt pathway is activated by a wide range of extracellular stimuli, including the integrins (12), and it has been linked to cell survival (13). Recently it was demonstrated that the different variants of the cytoplasmic domain in the  $\beta 1$  subunit can equally activate Akt (14, 16) and that the binding of  $\alpha 5\beta 1$  to fibronectin activates Akt, unlike the binding of

$\alpha 2\beta 1$  to monomeric collagen (15). Thus the activation of Akt may be dependent on the integrin  $\alpha$  subunit.

Reversible phosphorylation of proteins is a major mechanism for the control of cellular signaling pathways and maintenance of homeostasis (21). Although numerous kinases have been implicated in integrin signaling, the function and possible regulation of the corresponding phosphatases are largely unknown. Adhesion of cultured fibroblasts to extracellular matrix proteins has been shown to induce recruitment and activation of SHP-2, a nontransmembrane protein tyrosine phosphatase (39, 51). SHP-2 seems to play an active role in integrin-mediated signaling events, such as cell adhesion and migration (36, 62). Very little is known about the role of protein serine/threonine phosphatases in integrin signaling. Recent data have indicated a positive role for protein serine/threonine phosphatase 2A (PP2A) in integrin inside-out signaling. Inhibition of PP2A activity induces a selective loss of  $\beta 1$  integrins from focal adhesion sites (38) and inhibits cell adhesion (11); in addition PP2A has been shown to colocalize with  $\beta 1$  integrin at adhesion sites (38). However, the role of serine/threonine phosphatases in modulating integrin outside-in signals remains to be studied. Many studies have demonstrated the importance of PP2A in regulating a variety of cellular functions (52). Therefore it is likely that PP2A activity is tightly controlled in vivo.

Cell adhesion to three-dimensional (3D) fibrillar collagen, unlike adhesion to monomeric two-dimensional collagen, inhibits cell proliferation in different cell types (15, 20, 30) and induces specific integrin-mediated signals, which regulate gene expression (25, 42, 44). Here, a novel  $\alpha 2\beta 1$ -mediated signaling mechanism is introduced. Using human primary fibroblasts and human osteosarcoma (Saos-2) cell clones expressing either the wild-type  $\alpha 2$  subunit or a signaling-deficient  $\alpha 2/\alpha 1$  chimera, we have analyzed the ability of  $\alpha 2\beta 1$  integrin to regulate signals that have been linked with cell proliferation and survival. We and others have shown that  $\alpha 2\beta 1$  integrin is not

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involved in the regulation of the extracellular signal-related kinase mitogen-activated protein kinase pathway in response to collagen (25, 42, 53). However, here we show that cell adhesion to 3D collagen attenuates Akt and glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) phosphorylation by a mechanism involving  $\alpha 2\beta 1$ -induced activation of PP2A.

#### MATERIALS AND METHODS

**Plasmids, adenoviruses, and antibodies.** The  $\alpha 2$  integrin and the chimerical  $\alpha 2/\alpha 1$  integrin expression constructs have been described previously (25, 43). In  $\alpha 2/\alpha 1$  the intracellular domain of  $\alpha 2$  was replaced with one from  $\alpha 1$  integrin. Cdc42<sup>Asn17</sup>, Cdc42QL, Rac1<sup>Asn17</sup>, and RhoA<sup>Asn19</sup> were provided by J. C. Léal (Consejo Superior de Investigaciones Científicas, Madrid, Spain). pCMVHA-Akt and pCMVHA (hemagglutinin [HA] vector) were provided by K. Vuori (Burnham Institute), the HA-PP2Ac expression construct was provided by D. Brautigan (University of Virginia) (8), and pCMV was from Invitrogen. The adenovirus vector for the expression of membrane-targeted, active Akt (myrAkt) was obtained from K. Walsh (Tufts University School of Medicine) and was constructed as described previously (17, 18). The antibodies against Akt, the HA probe, and Cdc42 were purchased from Santa Cruz Biotechnology. Anti-PP2A was a gift from D. Brautigan. The phospho-specific antibodies were from New England Biolabs. The anti- $\alpha 2$  (MCA743) and anti- $\alpha 1$  (MCA1133) integrin antibodies were from Serotec.

**Collagen gels.** Collagen gels were prepared from bovine dermal collagen, which contains 95% type I collagen and 5% type III collagen (Cellon). Eight volumes of Cellon were mixed with 1 volume of 10-fold-concentrated Dulbecco's modified Eagle medium (DMEM) and 1 volume of 0.05 M NaOH in HEPES buffer (0.2 M) and kept on ice. Cells were trypsinized, resuspended in 1/10 gel volume of DMEM supplemented with inhibitors or anti-integrin antibodies, mixed into neutralized Cellon solution, and transferred into cell culture plates. The collagen was allowed to polymerize for 1 h at 37°C, after which 1 gel volume of DMEM was added, the gels were detached from the sides of the cell culture plates, and incubation was continued for various times. Cells from various Saos-2 cell clones were cultured on plastic and harvested directly from the plate or kept in suspension (1 ml of DMEM) for 1 h. The release of cells from collagen gel was performed as described previously (25).

**Immunoblot analysis.** Cells were released from collagen, washed once with ice-cold phosphate-buffered saline, and lysed in Laemmli sample buffer. The samples were sonicated, fractionated by sodium dodecyl sulfate–10 or 12% polyacrylamide gel electrophoresis, and transferred to a Hybond ECL membrane (Amersham Corp.). Western blotting was performed as described previously (25) with New England Biolabs antibodies at the dilution of 1:1,000 and other antibodies at the dilution of 1:250. Specific binding of antibodies was detected with peroxidase-conjugated secondary antibodies and visualized by an enhanced chemiluminescence detection system (Amersham).

**Transfections and kinase assay.** For the Akt kinase assay, subconfluent cells plated on 10-cm-diameter dishes were transfected using 18  $\mu$ l of Fugene 6 transfection reagent (Boehringer Mannheim) and 6  $\mu$ g of either empty vector, HA vector, or PP2Ac expression vector together with 3  $\mu$ g of pCMVHA-Akt. At 36 h after transfections, the cells were treated with collagen gel for 1.5 h, and a kinase assay was performed as described previously (18). Briefly, equal amounts of protein in cell lysis buffer (1% NP-40; 10% glycerol; 137 mM NaCl; 20 mM Tris-HCl [pH 7.4]; 20 mM NaF; 1 mM phenylmethylsulfonyl fluoride [PMSF]; leupeptin, antipain, and pepstatin [2  $\mu$ g/ml each]) from each sample were pre-incubated with protein G-Sepharose for 30 min at 4°C. After centrifugation, an anti-HA antibody was added together with protein G-Sepharose and 2 mg of bovine serum albumin/ml. Immunoprecipitation was performed at 4°C for 2 h. The immunoprecipitates were washed twice with cell lysis buffer, twice with H<sub>2</sub>O, and twice with kinase buffer (20 mM HEPES [pH 7.2], 10 mM MgCl<sub>2</sub>, 20 mM MnCl<sub>2</sub>), and they were incubated in 50  $\mu$ l of kinase buffer containing 2  $\mu$ g of myelin basic protein (Boehringer Mannheim) and [ $\gamma$ -<sup>32</sup>P]ATP (5  $\mu$ M, 10  $\mu$ Ci; Amersham) at room temperature for 30 min. Kinase reactions were terminated by adding sodium dodecyl sulfate sample solution. The reactions were run on 15% polyacrylamide gels and subjected to autoradiography. Okadaic acid (1 nM) was included at every stage to inhibit any *in vitro* phosphatase activity from influencing the kinase activity after the lysis of the cells. The *in vitro* kinase assays for PI-3K (3, 58) were performed in the presence of [ $\gamma$ -<sup>32</sup>P]ATP and PI (Avanti Polar Lipids). The formed [<sup>32</sup>P]PI was measured using thin-layer chromatography and autoradiography.

**Phosphatase assay.** Subconfluent human osteosarcoma cells and human primary fibroblasts were treated with collagen for various times in the presence or absence of antibodies (function-blocking anti- $\alpha 2$ , nonfunctional anti- $\alpha 1$  immu-

noglobulin G [IgG]; 5  $\mu$ g/ml) or inhibitors (bisindolylmaleimide [BIM], 10  $\mu$ M; SB203580, 20  $\mu$ M; Calbiochem) or were lysed directly on the plate. Alternatively, cells plated on 10-cm-diameter dishes were transfected using 20  $\mu$ l of Fugene 6 transfection reagent (Boehringer Mannheim) and 10  $\mu$ g of expression vector pCEV either alone or with Cdc42<sup>Asn17</sup>, Cdc42QL, Rac1<sup>Asn17</sup>, or RhoA<sup>Asn19</sup>. Thirty-six hours after transfections, the cells were placed inside a collagen gel for 2 h. Cells were released from collagen as described previously (25) and lysed in phosphatase lysis buffer (20 mM HEPES [pH 7.4]; 10% glycerol; 0.1% NP-40; 1 mM EGTA; 30 mM  $\beta$ -mercaptoethanol; 1 mM PMSF; leupeptin, antipain, and pepstatin [2  $\mu$ g/ml each]). The protein phosphatase assay system (Life Technologies) was used to determine phosphatase activity according to manufacturer's instructions. Briefly, <sup>32</sup>P-labeled phosphorylase (a substrate for both PP1 and PP2) was allowed to react with 2 to 20  $\mu$ g of protein from cell lysate for 10 min in 30°C, after which the proteins were precipitated with 20% trichloroacetic acid and the radioactivity released was measured by scintillation counting. Protein content was determined by the Bradford method, and phosphatase activity relative to protein content was calculated.

**Immunoprecipitation.** For immunoprecipitation, the cells were transfected with Fugene 6 and 10  $\mu$ g of the wt-PP2Ac-HA expression construct as described above (provided by D. Brautigan, University of Virginia) (8). Eight hours after transfection the cells were infected with myrAkt adenovirus at a multiplicity of infection of 50 and incubated for 16 h. Serum-free DMEM was added, and the incubation was continued for 24 h. The cells were treated with 3D collagen for 1 h under serum-free conditions, and the cells were lysed in solubilization buffer (10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 1% Triton X-100, 30 mM sodium pyrophosphate, 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF) (1). Immunoprecipitation was performed as described for the Akt kinase assay, with either a specific antibody or control IgG from the same species, except that the washes were performed six times with solubilization buffer. Western blot detection was performed with antibodies against Akt and the HA probe and antiserum against the PP2A catalytic subunit.

#### RESULTS

**Adhesion of  $\alpha 2\beta 1$  integrin to collagen promotes dephosphorylation of Akt.** A previous study suggested that integrin  $\alpha 5\beta 1$ -mediated cell adhesion to fibronectin induces Akt phosphorylation, while adhesion to monomeric collagen has no effect (32). Since monomeric two-dimensional and fibrillar three-dimensional (3D) collagen may have distinct properties in triggering integrin signaling (25, 42), immunoblotting was performed to determine whether cell adhesion to 3D collagen influences the Akt kinase. We examined the phosphorylation of Ser473 on Akt with a phospho-specific antibody, since this residue is considered to indicate active kinase (6). Serum-starved human primary fibroblasts were harvested in the monolayer or trypsinized and replated on fibronectin-coated plates or seeded inside collagen (for 2 h), and Akt phosphorylation was studied by immunoblotting. Cells adhering on fibronectin showed increased Akt phosphorylation (67%  $\pm$  11% [mean  $\pm$  standard error of the mean {SEM}]; four separate experiments) compared to cells cultured on plastic, while phosphorylation of Ser473 was reduced by 48%  $\pm$  3% (mean  $\pm$  SEM; four separate experiments) in cells cultured inside 3D fibrillar collagen (Fig. 1A). We also analyzed the effect of collagen-mediated reduction of Akt phosphorylation on GSK3 $\beta$ , a well-characterized Akt substrate that is inactivated by the active Akt kinase via phosphorylation at Ser9 (12). In only 1 h, 3D collagen induced a rapid decrease of phosphorylation of Akt, and after 3 h nearly all Akt was in a nonphosphorylated state (Fig. 1B). Inactivation of Akt resulted in decreased phosphorylation of GSK3 $\beta$  (Fig. 1B). In seven separate experiments the phosphorylation of Akt and GSK3 $\beta$  in cells cultured inside collagen was reduced by 40%  $\pm$  16% and 47%  $\pm$  17% (means  $\pm$  SEMs), respectively. To evaluate

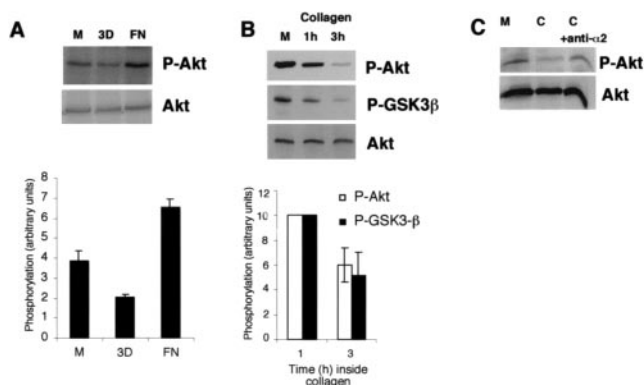


FIG. 1. Differential regulation of Akt phosphorylation by extracellular matrix molecules. (A) Fibroblasts were deprived of serum for 16 h and then harvested from the cell culture dish (M, monolayer), seeded inside 3D collagen gel, or plated on fibronectin (FN) for 2 h. P-Akt, phosphorylated Akt. (B) Primary fibroblasts were deprived of serum for 16 h and then harvested from the cell culture dish (M) or seeded inside 3D collagen gel for 1 and 3 h. P-GSK3 $\beta$ , phosphorylated GSK3 $\beta$ . (C) Primary fibroblasts were deprived of serum for 16 h and then harvested from the cell culture dish (M) or seeded inside collagen gel (C) or treated with an anti- $\alpha$ 2 function-blocking MAb (C + anti- $\alpha$ 2) and placed inside collagen for 1.5 h. Western blot analyses with an antibody that recognizes Akt when it is phosphorylated at Ser473 or with an antibody that recognizes GSK3 $\beta$  when it is phosphorylated are shown. Immunoblotting with an antibody that recognizes all forms of Akt was performed for reference. Densitometric quantitation of phosphorylated Akt and GSK3 $\beta$  levels relative to loading is also shown (A and B).

the role of  $\alpha$ 2 $\beta$ 1 in collagen-induced Akt inactivation, we treated human fibroblasts with an anti- $\alpha$ 2 integrin function-blocking monoclonal antibody (MAb). Importantly, this antibody has previously been shown to block  $\alpha$ 2 $\beta$ 1-initiated signals inside collagen (42). The antibody blocked collagen-induced Akt dephosphorylation (Fig. 1C). The results indicate that  $\alpha$ 2 $\beta$ 1 integrin function is involved in the observed reduction of Akt phosphorylation.

To confirm that the effect of collagen on Akt activation is mediated by  $\alpha$ 2 $\beta$ 1 integrin, we used osteosarcoma cells (Saos) expressing  $\alpha$ 1 $\beta$ 1 and lacking endogenous  $\alpha$ 2 $\beta$ 1 integrin. The cells were transfected to have stable expression of wild-type  $\alpha$ 2 (Saos- $\alpha$ 2) or  $\alpha$ 2 with the  $\alpha$ 1 cytoplasmic domain (Saos- $\alpha$ 2/ $\alpha$ 1) (Fig. 2A) (25). The mutant  $\alpha$ 2/ $\alpha$ 1 $\beta$ 1 integrin mediates cell adhesion to collagen as efficiently as the wild-type receptor but lacks  $\alpha$ 2 signaling functions (25). In agreement with a previous study (27), detachment and maintenance of the cells in suspension resulted in complete Akt inactivation in 1 h (Fig. 2B, lane S). After 2 h inside collagen, phosphorylation of Ser473 on Akt was clearly reduced in Saos- $\alpha$ 2 cells compared to that in Saos- $\alpha$ 2/ $\alpha$ 1 cells, indicating the importance of the  $\alpha$ 2 cytoplasmic domain in Akt inactivation (Fig. 2B). This was further confirmed using different stable cell clones. Akt phosphorylation was monitored over a course of some hours, and, according to the densitometric analysis, after 6 h no phosphorylated Akt was detected in Saos- $\alpha$ 2 cells, while Akt remained phosphorylated at Ser473 in Saos- $\alpha$ 2/ $\alpha$ 1 cells (Fig. 2C). In correlation with that of Akt, the phosphorylation of GSK3 $\beta$  decreased inside collagen in Saos- $\alpha$ 2 cells while phosphorylation at Ser9 was maintained in cells expressing the mutant  $\alpha$ 2/ $\alpha$ 1 integrin

chimera (Fig. 2D). Densitometric analysis performed in six separate experiments showed that the amount of phosphorylated GSK3 $\beta$  in  $\alpha$ 2-expressing cells was 40%  $\pm$  22% (mean  $\pm$  SEM) lower after 1 h, and 36%  $\pm$  8% (mean  $\pm$  SEM) lower after 4 h, inside collagen than the amount in Saos- $\alpha$ 2/ $\alpha$ 1 cells. These results indicate that functional  $\alpha$ 2 $\beta$ 1 integrin is essential for the collagen-induced rapid reduction of Akt and GSK3 $\beta$  phosphorylation, while integrin  $\alpha$ 1 $\beta$ 1 does not seem to contribute to the negative regulation of Akt in the cells studied. The active role of the cytoplasmic domain of  $\alpha$ 2 in the regulation of Akt was further confirmed using Saos cells expressing an  $\alpha$ 2/ $\alpha$ 5 integrin chimera (5). When these cells were exposed to 3D collagen, the levels of phosphorylated Akt were the same as those in  $\alpha$ 2/ $\alpha$ 1 cells and higher than those in Saos- $\alpha$ 2 cells (Fig. 2E).

**Integrin  $\alpha$ 2 $\beta$ 1 regulates Akt via PP2A.** Akt activity can be decreased by either inhibition of upstream activators or dephosphorylation of Thr308 and Ser473 (37). To determine which mechanism is involved, activation of PI-3K was tested by a direct kinase assay performed with Saos- $\alpha$ 2 and Saos- $\alpha$ 2/ $\alpha$ 1 cells cultured inside collagen. No constant differences between Saos- $\alpha$ 2 and Saos- $\alpha$ 2/ $\alpha$ 1 cell clones were detected (mean of two Saos- $\alpha$ 2 and two Saos- $\alpha$ 2/ $\alpha$ 1 clones: 80 and 73 arbitrary units, respectively). Furthermore, inhibition of PI-3K with LY-294002 had no further effect on the low levels of phosphorylated Akt measured in Saos- $\alpha$ 2 cells after 1.5 h inside collagen (not shown). We therefore concluded that  $\alpha$ 2 $\beta$ 1 integrin regulates Akt phosphorylation downstream of PI-3K. Integrin  $\alpha$ 2 $\beta$ 1-mediated reduction of Akt and GSK3 $\beta$  phosphorylation could, indeed, be prevented by protein serine/threonine phosphatase inhibitor calyculin A. This was observed in both the human primary fibroblasts and Saos- $\alpha$ 2 cells cultured inside collagen (Fig. 3). The above experiments strongly indicated that  $\alpha$ 2 $\beta$ 1 regulates Akt dephosphorylation rather than its activation via PI-3K and phosphoinositide-dependent kinase 1 but did not resolve the exact mechanism of Akt deactivation.

Using a specific assay for serine/threonine phosphatases, we asked whether cell adhesion to 3D collagen could induce serine/threonine phosphatase activity and, if so, whether the differences would be large enough to be detected against the background of all cellular phosphatase activity. We observed that human primary fibroblasts seeded inside collagen have two- to fivefold (range in three separate experiments, each with three parallel measurements)-higher phosphatase activity than cells cultured in a monolayer (Fig. 4A). Interestingly, the collagen-induced phosphatase activity could be almost completely blocked with a function-blocking antibody against anti- $\alpha$ 2 integrin (Fig. 4A), while a control antibody (nonfunctional anti- $\alpha$ 1 antibody) had no effect (not shown). The phosphatase assay measures PP2A activity but is not specific for it. However, PP2A can be distinguished from PP1 by its over-100-fold-higher susceptibility to okadaic acid (10). The integrin-induced phosphatase activity was inhibited by 52% with 0.1 nM okadaic acid (a very low inhibitor concentration, which does not influence PP1 [50% inhibitory concentration, 15 nM]), reducing the phosphatase activity to that measured in cells treated with collagen in the presence of an anti-integrin antibody (Fig. 4B). The data indicate that  $\alpha$ 2 $\beta$ 1 integrin induces PP2A-type phosphatase activity in response to collagen.

This was further supported by the fact that serine/threonine

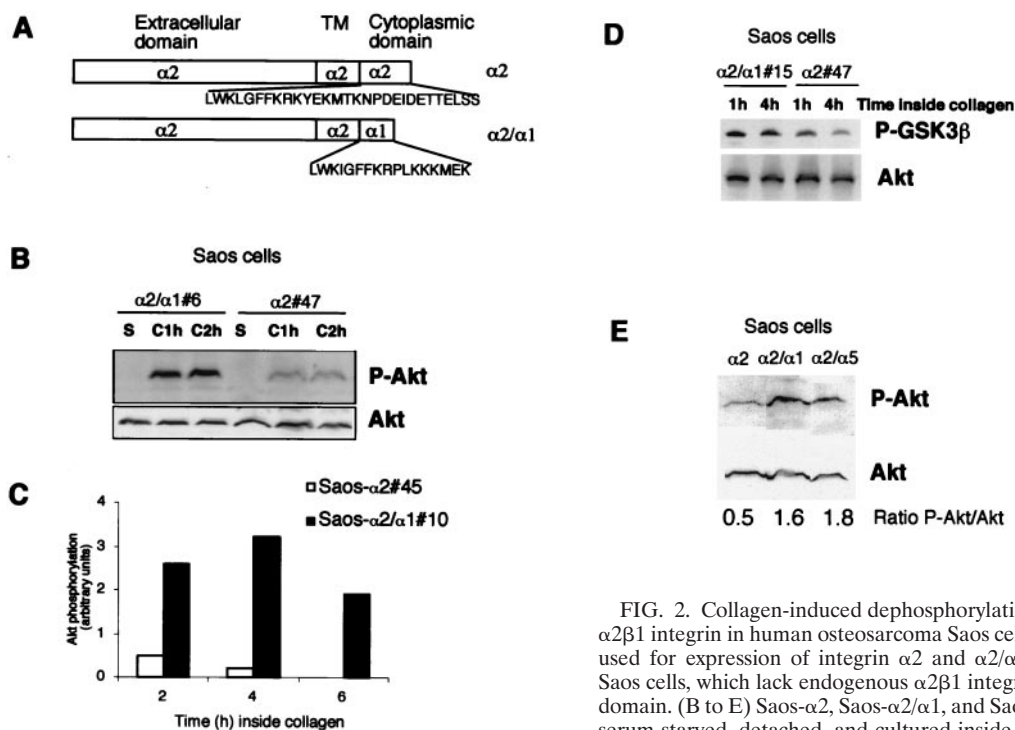


FIG. 2. Collagen-induced dephosphorylation of Akt is regulated by  $\alpha 2\beta 1$  integrin in human osteosarcoma Saos cells. (A) cDNA constructs used for expression of integrin  $\alpha 2$  and  $\alpha 2/\alpha 1$  (chimera) subunits in Saos cells, which lack endogenous  $\alpha 2\beta 1$  integrin. TM, transmembrane domain. (B to E) Saos- $\alpha 2$ , Saos- $\alpha 2/\alpha 1$ , and Saos- $\alpha 2/\alpha 5$  cell clones were serum starved, detached, and cultured inside 3D collagen gel (C, collagen) for the indicated times or left in suspension (S) for 1 h. For panel E cells were cultured inside collagen for 2 h. Western blot analysis with antibodies that recognize Akt (B and E) and GSK3 $\beta$  (D) when they are phosphorylated (P-Akt and P-GSK3 $\beta$ , respectively) is shown. (B, D, and E) Immunoblotting with an antibody that recognizes all forms of Akt was performed for reference. (C) A separate experiment with longer incubation times inside collagen was performed with different Saos cell clones. Densitometric quantitation of phosphorylated Akt levels at different time points relative to loading is shown. If the experiment was performed with several cell clones, the clone numbers are indicated (e.g., #6).

phosphatase activity was significantly higher (3.3- to 4.5-fold) in Saos- $\alpha 2$  clones than in Saos- $\alpha 2/\alpha 1$  clones (Fig. 5A), further confirming the role of  $\alpha 2\beta 1$  in PP2A regulation and the generality of this observation in different cell types. Introduction of  $\alpha 2$  or  $\alpha 2/\alpha 1$  cDNAs into Saos cells did not alone influence serine/threonine phosphatases, since both  $\alpha 2$ - and  $\alpha 2/\alpha 1$ -expressing cells showed equal phosphatase activities when cultured on fibronectin and since the phosphatase activities in Saos- $\alpha 2/\alpha 1$  and Saos vector control cells were the same (Fig. 5C). In accordance with the results of the measurement done in fibroblasts, in Saos cells the  $\alpha 2\beta 1$ -induced phosphatase activity was predominantly PP2A, since 0.2 nM okadaic acid inhibited the activity by 60% (Fig. 5B). The activation of PP2A by  $\alpha 2\beta 1$  integrin represents a novel integrin-mediated signaling mechanism, where an integrin  $\alpha$  subunit specifically activates a serine/threonine phosphatase.

**Akt forms a complex with PP2A.** Recently, PP2A has been shown to form a complex with a number of kinases (1, 42, 57, 58). In most cases, PP2A inactivates the kinases. Here, we examined whether Akt associates physically with PP2A. Human primary fibroblasts were transfected with the endogenous catalytic subunit of PP2A (PP2Ac) containing a HA tag or with an empty HA tag vector only. The anti-HA immunoprecipitates were Western blotted with an anti-Akt antibody, and the Akt protein was found in HA-PP2Ac-transfected samples (Fig. 6A). The control samples had some Akt but much less than the samples from HA-PP2Ac-transfected cells (Fig. 6A). The matrix (fibronectin or collagen) had no effect on the coprecipitation (Fig. 6A). In another set of experiments human primary fibroblasts were infected with adeno-myrAkt to increase the level of active kinase in the cells. PP2Ac was detected in Akt immunoprecipitates. Neither Akt nor PP2A could be detected in precipitates prepared with control IgG (not shown). We also verified the interaction between Akt and PP2Ac in fibroblasts transfected with a vector encoding HA-tagged PP2Ac and infected with adeno-myrAkt (not shown). To confirm the specificity of the antibodies used, Western blotting was performed on whole-cell extracts from collagen-stimulated, nontransfected human primary fibroblasts. Anti-PP2A and anti-Akt antibodies detected a single specific band, and anti-HA showed no unspecific binding to cellular proteins (Fig. 6B).

By transient cotransfections using PP2Ac together with tagged Akt, we assessed whether PP2A is directly responsible for the dephosphorylation of Akt. Cells were lysed, and Akt was immunoprecipitated in the presence of okadaic acid (1 nM) to prevent PP2A action during and after cell lysis. Over-

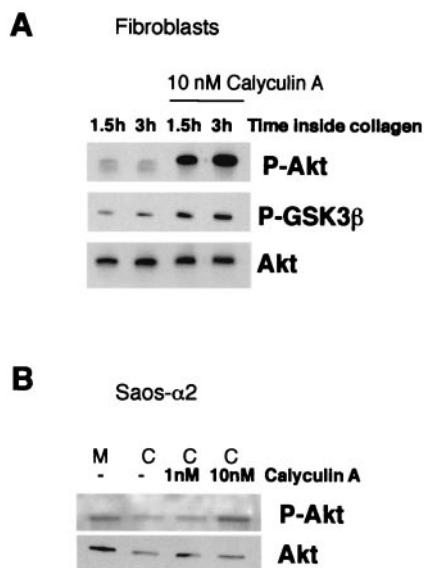


FIG. 3. Regulation of Akt phosphorylation involves serine/threonine phosphatase activity. (A) Western blot analysis with an antibody that recognizes Akt or GSK3 $\beta$  when they are phosphorylated (P-Akt and P-GSK3 $\beta$ , respectively). Fibroblasts were deprived of serum for 16 h and seeded inside 3D collagen gel in the presence or absence of serine/threonine phosphatase inhibitor calyculin A (10 nM). (B) Western blot analysis with an antibody that recognizes phosphorylated Akt. Saos- $\alpha$ 2 cells were deprived of serum for 16 h and then harvested from the cell culture dish (M, monolayer) and seeded inside 3D collagen (C) gel for 2 h. Cells were cultured in the presence or absence of serine/threonine phosphatase inhibitor calyculin A (1 and 10 nM). Immunoblotting with an antibody that recognizes all forms of Akt was performed for reference (A and B).

expression of PP2Ac reduced Akt activity in an in vitro kinase assay of serum-starved Saos- $\alpha$ 2/ $\alpha$ 1 cells that were stimulated with collagen for 1 h (Fig. 7). In three separate experiments the reduction in Akt activity was  $36\% \pm 23\%$ . Thus, overexpression of PP2A was able to reduce Akt phosphorylation in cells where active endogenous Akt could be observed due to the inability of the mutant integrin to activate PP2A. The data suggest a tight interrelationship between PP2A activity and Akt dephosphorylation.

**PP2A activation by  $\alpha$ 2 $\beta$ 1 integrin is independent of p38 and dependent on Cdc42.** The mechanism of PP2A activation by  $\alpha$ 2 $\beta$ 1 integrin was studied. We have previously shown that integrin  $\alpha$ 2 $\beta$ 1 stimulates p38 kinase via small GTPase Cdc42 (25). This led us to study the possible role of Cdc42 in  $\alpha$ 2 $\beta$ 1-mediated activation of PP2A. Saos- $\alpha$ 2 cells were transiently transfected with a dominant-negative Cdc42 mutant (Cdc42DN). Transfection efficiency was determined and seemed to vary between 25 and 50% (not shown). Cdc42DN effectively inhibited integrin  $\alpha$ 2 $\beta$ 1-induced activation of PP2A. In five separate experiments PP2A activity was  $53\% \pm 13\%$  lower in Cdc42DN-transfected cells than in vector control cells. The inhibitory effect of Cdc42DN was found to be statistically significant (Student *t* test;  $P < 0.016$ ). Figure 8A shows a representative experiment. We also performed control assays to show that the transfections had no effect on the amount of PP2A in cells (Fig. 8A). Dominant-negative RhoA and Rac1 mutants had no effect on PP2A activity in collagen-stimulated

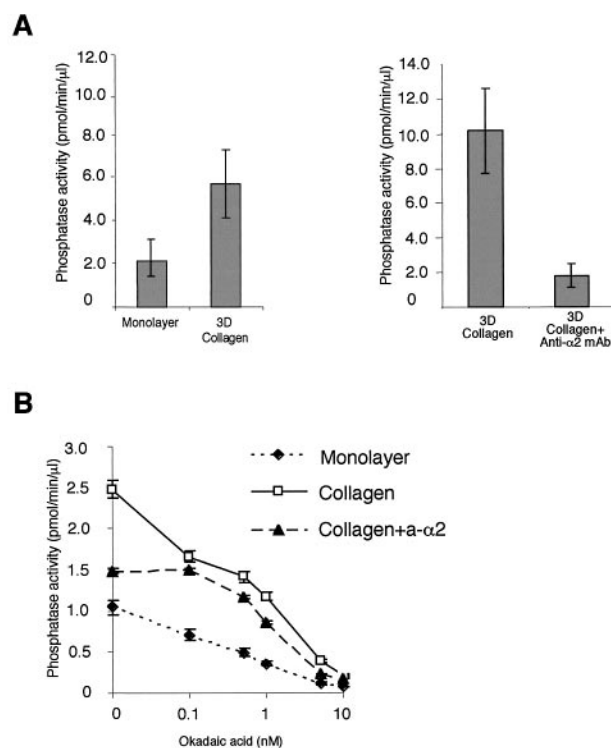


FIG. 4. Serine/threonine phosphatase PP2A is activated inside 3D collagen gel. Fibroblasts were serum starved and then harvested from the cell culture dish (monolayer) or seeded inside collagen gel for 1.5 h. Serum-starved fibroblasts were detached and placed inside collagen in the presence or absence of either an anti- $\alpha$ 2 function-blocking MAb (collagen + anti- $\alpha$ 2; 2.5  $\mu$ g/ml) or a nonfunctional anti- $\alpha$ 1 MAb (2.5  $\mu$ g/ml; not shown). Thereafter, cells were lysed and equal amounts of protein were assayed for PP1-PP2A activity using  $^{32}$ P-labeled glycogen phosphorylase as a substrate. Phosphatase activity of cells seeded inside collagen versus that of cells cultured in a monolayer and the effect of anti- $\alpha$ 2 MAb are shown (A). To distinguish between PP1 and PP2A type activity, the effect of okadaic acid (causes half-maximal inhibition of PP2A at 0.1 nM and has no effect on PP1 below 5 nM) was tested by an in vitro phosphatase reaction (B). Phosphatase activity relative to cell lysate protein content (mean  $\pm$  SEM;  $n = 3$ ) is shown.

Saos- $\alpha$ 2 cells (Fig. 8A). In contrast, the active Cdc42 mutant increased PP2A activity to some extent (not shown).

The roles of other previously identified  $\alpha$ 2 $\beta$ 1 downstream effectors, p38 kinase (25) and protein kinase C- $\zeta$  (PKC- $\zeta$ ) (61), were also studied. Inhibition of p38 kinase with SB203580 did not influence PP2A activity, while PKC inhibitor BIM I, capable of inhibiting the PKC- $\zeta$  isoform at the concentration used, showed some effect (Fig. 8B). However, this inhibition was not found to be statistically significant, and the high BIM I concentration may also inhibit other kinases. The inability of p38 inhibition to influence PP2A activity indicates that  $\alpha$ 2 $\beta$ 1 must regulate at least two distinct signaling functions, namely, the activation of PP2A and p38.

## DISCUSSION

PP2A comprises a family of protein serine/threonine phosphatases. They can interact with a substantial number of proteins and contribute to the regulation of numerous signaling pathways (26). Active PP2A can inhibit the cell cycle, predom-

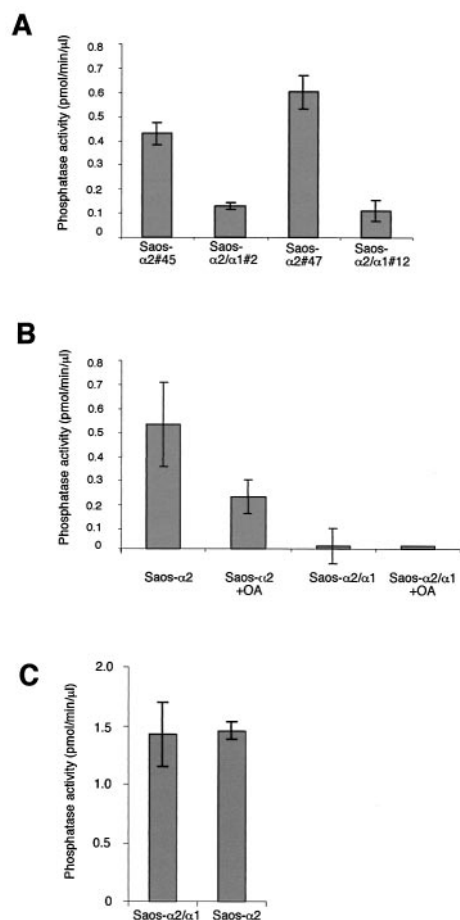


FIG. 5. An integrin  $\alpha 2$  cytoplasmic tail is needed for activation of a serine/threonine phosphatase PP2A. Saos- $\alpha 2$  and Saos- $\alpha 2/\alpha 1$  cells were serum starved, detached, and cultured inside collagen for 1.5 h (A and B) or cultured on fibronectin (C). Measurement of PP1-PP2A phosphatase activity was done from cell lysates using  $^{32}$ P-labeled glycogen phosphorylase as a substrate. Two different Saos- $\alpha 2$  and Saos- $\alpha 2/\alpha 1$  single-cell clones were tested. The effect of 0.1 nM okadaic acid (OA) (inhibits half maximally PP2A but has no effect on PP1) was tested in the in vitro phosphatase reaction (B). Phosphatase activity relative to cell lysate protein content is shown. If the experiment was performed with several cell clones, the clone numbers are indicated (e.g., #2).

inantly at the G<sub>2</sub>/M checkpoint (33), induce apoptosis (13, 48), and act as a tumor suppressor (45). In this study we demonstrate that  $\alpha 2\beta 1$  integrin modulates Akt and GSK3 $\beta$  phosphorylation in response to cell adhesion to 3D collagen by activating PP2A. In general integrin-mediated cell adhesion is known to trigger initial tyrosine phosphorylation events, such as the activation of FAK and Src family kinases, that result in the activation of downstream signaling pathways, a number of which involve the activity of various serine/threonine kinases (50). However, relatively little is known about how specific protein tyrosine and serine/threonine phosphatases function to regulate integrin-mediated signals. The suggestion that the activation of PP2A in response to collagen is specifically mediated by  $\alpha 2\beta 1$  integrin is based on the following facts: (i) PP2A activation could be inhibited by a function-blocking antibody to  $\alpha 2$  integrin and (ii) human osteosarcoma cells lacking

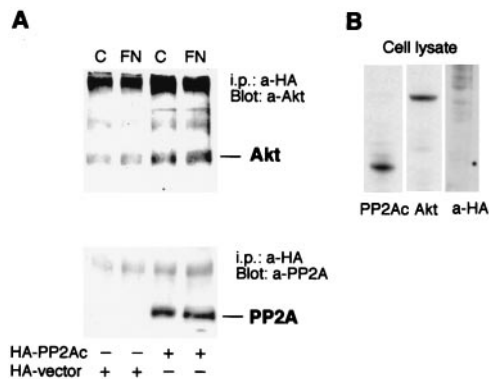


FIG. 6. PP2A interacts with Akt in transfected fibroblasts in vivo. (A) Human primary fibroblasts were transfected with Fugene 6 reagent and 6  $\mu$ g of plasmid encoding the HA-tagged PP2A catalytic subunit (HA-PP2Ac) or with empty plasmid (HA vector). After 1 h inside collagen or on fibronectin, the cells were harvested, lysed, and immunoprecipitated (i.p.) with anti-HA antibody. The immunoprecipitates were analyzed by Western blotting with antibodies against Akt or the PP2A catalytic subunit. (B) Immunoblots from whole-cell lysates (nontransfected) using the same antibodies, indicating that the antibodies against Akt and PP2A recognize the corresponding wild-type proteins and that the anti-HA antibody shows no unspecific binding to cellular proteins.

$\alpha 2\beta 1$  integrin or expressing a signaling deficient, chimeric  $\alpha 2/\alpha 1$  subunit were shown to be unable to activate PP2A in response to collagen even though these cells adhere to collagen similarly to the  $\alpha 2\beta 1$ -expressing cells (25). Thus, our results demonstrate the association of integrin function with a protein serine/threonine phosphatase. In the previous reports, a link between integrins and protein tyrosine phosphatase SHP-2 has been recently established and the phosphatase function has been shown to be a positive regulator of integrin function (23,

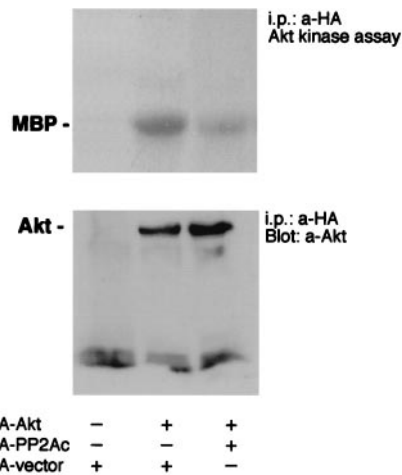


FIG. 7. Overexpression of PP2A inactivates Akt. The effect of the HA-PP2A catalytic subunit or HA vector on the kinase activity of cotransfected Akt was tested. Akt was immunoprecipitated from transfected (36 h posttransfection) and serum-starved Saos- $\alpha 2/\alpha 1$  cells after 1.5 h of treatment inside collagen. Akt activity was determined by the ability to phosphorylate myelin basic protein (MBP). A control blot shows that the overexpression of the PP2A catalytic subunit has no effect on the amount of Akt protein in cells.

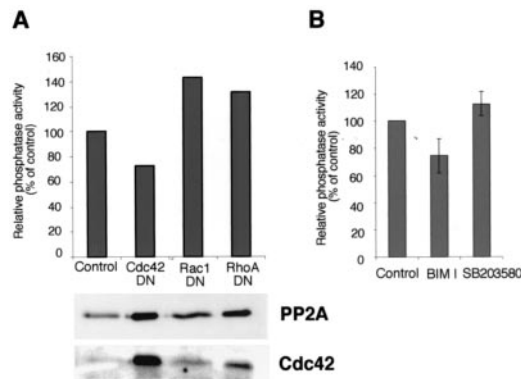


FIG. 8. Activation of PP2A by  $\alpha 2\beta 1$  integrin is dependent on Cdc42. (A) The effect of the transfected dominant-negative mutant forms of Cdc42 (Cdc42DN), Rac1 (Rac1DN), and RhoA (RhoADN) on the activation of PP2A in Saos- $\alpha 2$  cells. Phosphatase activity relative to cell lysate protein content is shown. Immunoblotting with antibodies against PP2A and Cdc42 was performed to show that Cdc42 was overexpressed in transfected cells and that it had no effect on the amount of PP2A protein in cells. (B) Serum-starved fibroblasts were detached and left untreated or treated with inhibitor BIM I (10  $\mu$ M) or SB203580 (20  $\mu$ M) and placed inside collagen for 1 h. Phosphatase activity relative to cell lysate protein content is shown (mean  $\pm$  SEM). BIM I, four separate experiments; SB203580, two separate experiments ( $n = 3$  for each).

39). Another example of the interrelationship between phosphatases and integrins comes from studies demonstrating that the expression of dual-specificity phosphatase PTEN is essential in maintaining the anchorage dependence of growth via negative regulation of FAK and downstream kinases (49).

The *in vitro* activity of PP2A can be regulated by phosphorylation (7) and methylation (31, 59). Cells also have specific PP2A inhibitor proteins (34, 35). The cellular pathways regulating PP2A activity are incompletely known, and accordingly the exact mechanism of PP2A activation by  $\alpha 2\beta 1$  integrin remains to be shown. Active Cdc42 seems indispensable in the stimulation of PP2A by  $\alpha 2\beta 1$  and collagen, since expression of dominant-negative Cdc42 blocked  $\alpha 2\beta 1$ -mediated PP2A activation. Cdc42 may regulate the formation of a PP2A-containing adhesion and signaling complex. Indeed, a previous report indicates that PP2A can accumulate at focal contact sites due to its interaction with paxillin (24). Furthermore, our results suggest that the cytoplasmic domain of  $\alpha 2$  integrin may also participate in the activation of PP2A.

Cdc42 may be one of the prime effectors of  $\alpha 2\beta 1$  signaling, since it is also required for  $\alpha 2\beta 1$ -mediated activation of the p38  $\alpha$  isoform (25). p38 can activate PP2A (55), but here the inhibition of the p38 pathway had no effect on PP2A activation by  $\alpha 2\beta 1$  integrin. Additionally, a PKC inhibitor did not affect PP2A activation. PKC- $\zeta$  has been described as one of the signaling proteins activated by  $\alpha 2\beta 1$ -mediated binding to collagen (60). Ceramides can concomitantly activate PKC- $\zeta$  and PP2A, suggesting that these pathways might be connected to each other (40).

Akt activity can, in general, be inhibited by either inhibition of upstream activators or by direct dephosphorylation (37). The fact that here  $\alpha 2\beta 1$  integrin inhibited Akt without concomitant attenuation of PI-3K led us to consider a possible link

between the observed PP2A induction and Akt inactivation. PP2A has been shown to inactivate Akt *in vitro*, while other serine/threonine phosphatases, such as PP1, are unable to use phosphorylated Akt as a substrate (2). Calyculin A is able to prevent PP2A-dependent dephosphorylation of Akt (37), and here we show that it prevented  $\alpha 2\beta 1$ -related Akt inactivation. Furthermore, the cotransfection experiments using the PP2A catalytic subunit with Akt demonstrated a direct relationship between PP2A and Akt kinase activity.

Recent studies indicated that PP2A associates with kinases and thereby negatively regulates their function (1, 41, 56, 57). Here we suggest that activated PP2A is the mechanism behind the modulation of Akt activity by  $\alpha 2\beta 1$  integrin. Furthermore, the dephosphorylation of GSK3 $\beta$ , one of the downstream targets of Akt, correlated with Akt deactivation. Dephosphorylation of GSK3 $\beta$  leads to its activation (28). Besides the Akt/GSK-3 $\beta$  pathway, PP2A has the potency to affect several other signaling pathways, and therefore the activation of PP2A may participate in many  $\alpha 2\beta 1$ -mediated cellular events, including inhibition of the cell cycle (30).

To conclude, we have introduced a novel  $\alpha 2\beta 1$ -related signaling pathway, namely, activation of PP2A. Protein phosphatases have wider substrate specificity than protein kinases, and therefore they may affect several signaling pathways. In general, the regulation of cellular phosphatase activity is less studied than that of kinases, and there are only a few reports indicating their regulation by a specific integrin. Integrin  $\alpha 2\beta 1$ -mediated PP2A activation provides a versatile mechanism to regulate numerous signaling mechanisms and cellular functions.

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