

Research article

## The linkage between $\beta 1$ integrin and the actin cytoskeleton is differentially regulated by tyrosine and serine/threonine phosphorylation of $\beta 1$ integrin in normal and cancerous human breast cells

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### Abstract

**Background:** Structural requirements for the  $\beta 1$  integrin functions in cell adhesion, spreading and signaling have been well documented mainly for fibroblasts. In this study, we examined the reason for the reduced surface expression of  $\beta 1$  integrin in human breast cancer MCF-7 cells compared to normal human breast epithelial (HBE) cells, both of which adhered to collagen type IV.

**Results:** The  $\beta 1$  integrin immunoprecipitates from either HBE or MCF-7 cells involved  $\alpha$ -actinin while actin coprecipitated with  $\beta 1$  integrin from HBE cells but not from MCF-7 cells. Immunoblotting using the anti-phosphotyrosine (PY) antibody indicated the phosphorylation of  $\beta 1$  integrin at least at tyrosine in both cells. Dephosphorylation of  $\beta 1$  integrin from HBE cells by protein tyrosine phosphatase (PTP), but not by protein serine/threonine phosphatase (PP), caused dissociation of actin from  $\beta 1$  integrin, although dephosphorylation of it from MCF-7 cells by either PTP or PP caused association of the two proteins. In MCF-7 cells  $\beta 1$  integrin coprecipitated doublet of proteins having the  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (CaMK) II activity that was susceptible to KN-62, a specific inhibitor of CaMKII.

**Conclusion:** The results suggest that  $\beta 1$  integrin is tyrosine phosphorylated and links with actin via  $\alpha$ -actinin in HBE cells but prevented from linking with actin in MCF-7 cells by phosphorylation at both tyrosine and serine/threonine of  $\beta 1$  integrin which forms a complex with  $\alpha$ -actinin and CaMKII. Thus the linkage formation of  $\beta 1$  integrin with actin may be differentially regulated by its tyrosine and serine/threonine phosphorylation in normal HBE cells and breast cancer MCF-7 cells.

### Background

The integrin family of surface receptors play a critical role in many cellular processes that include cell adhesion, cell spreading, and growth signaling [1–6]. Integrins interact extracellularly with the substratum such as collagens at specific sites called focal adhesions and intracellularly with many actin-binding proteins such as

$\alpha$ -actinin, talin, and vinculin, thereby linking these proteins with the actin cytoskeleton. Links between cell surface receptor  $\beta$  integrins and the actin cytoskeleton are thought to be established in more than one way. Integrin binds to talin [7], which also binds to vinculin [8–10], which in turn binds to  $\alpha$ -actinin [11,12], which then binds to actin. This constitutes a three-protein link between in-

tegrin and actin. In addition, talin can also bind actin directly [13,14], so that talin may form a direct one-protein link between integrin and actin. Similarly, the actin-binding protein  $\alpha$ -actinin binds to the  $\beta$ 1 integrin subunit [15]. While many isoforms of  $\beta$ 1 integrin have been reported [16,17], a short cytoplasmic domain of 47 amino acids of  $\beta$ 1 integrin isoform A contains the three putative domains with the highest affinity for  $\alpha$ -actinin [18]. In addition, several amino acids and motifs within the cytoplasmic domain of the  $\beta$  subunit, which are potential phosphorylation sites, have been implicated in the integrin function. The  $\beta$ 1 subunit contains a Val-Thr-Thr sequence and these two threonines appear to be important for adhesion of fibroblast cells [19], and adhesion and invasion of lymphoid cells [20]. However, the relevance of phosphorylation of these amino acids within the cytoplasmic domain of  $\beta$ 1 integrin in its function remains largely unclear.

Human breast cancer MCF-7 cells have many cellular properties characteristic to malignantly transformed cells; such as an enhanced growth ability in soft agar and poor cell adhesion to the substratum compared to their normal counterpart HBE cells [21]. Poor cell adhesion of MCF-7 cells to the substratum is thought to be mainly caused by a low level of  $\beta$ 1 integrin expression on the cell surface [21]. This observation gave a clue to further understanding the relevance of  $\beta$ 1 integrin function to cell adhesion, cell spreading, and anchorage-independent growth in cancerous cells. In this study, we examined why only a small population of  $\beta$ 1 integrin present in MCF-7 cells is expressed on the cell surface compared to HBE cells, both of which adhere to collagen type IV. Immunoprecipitation and Western blot analysis revealed that intracellular linkage between  $\beta$ 1 integrin and actin formed in HBE cells was lost in MCF-7 cells. The relevance of phosphorylation of  $\beta$ 1 integrin to its linkage forming ability with actin was also examined in this study using PTP and PP.

## Results

### **The absence of coprecipitation of actin with $\beta$ 1 integrin in MCF-7 cells**

To determine whether expression of  $\beta$ 1 integrin on the cell surface requires its intracellular interaction with the actin cytoskeleton,  $\beta$ 1 integrin was immunoprecipitated with the anti- $\beta$ 1 integrin antibody or control IgG from HBE or MCF-7 cells which adhered to collagen IV. Western blot analysis revealed that actin coprecipitated with  $\beta$ 1 integrin but not with the IgG precipitates from quiescent HBE cells (Fig. 1A). In contrast, actin did not coprecipitate with  $\beta$ 1 integrin or the IgG precipitates from adherent MCF-7 cells (Fig. 1A). To determine whether the failure to coprecipitate actin with  $\beta$ 1 integrin was due to the absence of actin expression, whole cell lysates were

prepared from HBE or MCF-7 cells, both of which were quiescent and adherent to collagen IV. Immunoblot analysis indicated that total amounts of actin and  $\beta$ 1 integrin were comparable between HBE and MCF-7 cells (Fig. 1B).

### **$\alpha$ -Actinin-mediated interaction between $\beta$ 1 integrin and actin**

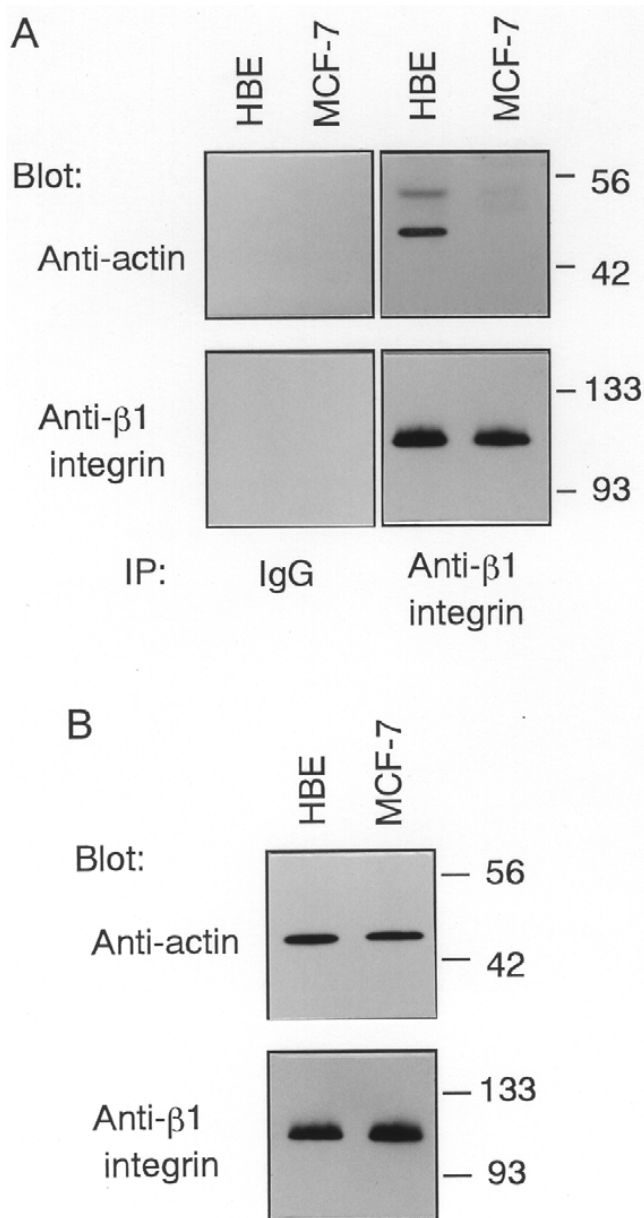
Interaction between  $\beta$ 1 integrin and the actin cytoskeleton is mediated by the actin-binding proteins which are involved in the focal adhesion complex where integrin  $\beta$ 1 through its cytoplasmic domain links with the actin cytoskeleton. We next determined which actin-binding proteins mediate this linkage between  $\beta$ 1 integrin and actin. Immunoblot analysis of the  $\beta$ 1 integrin or control IgG precipitates for  $\alpha$ -actinin, talin and vinculin, revealed that only  $\alpha$ -actinin among three proteins coprecipitated with  $\beta$ 1 integrin but not with the IgG precipitates from quiescent HBE cells (Fig. 2A). Similar to HBE cells,  $\alpha$ -actinin, but not talin or vinculin, coprecipitated with  $\beta$ 1 integrin but not with the IgG precipitates from quiescent MCF-7 cells (Fig. 2A). Western blotting for the whole cell lysates revealed that total amounts of  $\alpha$ -actinin and vinculin in HBE and MCF-7 cells were comparable and that the amount of talin was slightly smaller in MCF-7 cells than that in HBE cells (Fig. 2B). The results indicated that  $\alpha$ -actinin coprecipitated with  $\beta$ 1 integrin in both HBE and MCF-7 cells.

### **Expression of the wild-type integrin $\beta$ 1A in both HBE and MCF-7 cells**

The cytoplasmic domain of  $\beta$ 1 integrin is short and has the putative binding domains for  $\alpha$ -actinin [18]. To determine the difference in the cytoplasmic domain of  $\beta$ 1 integrin between HBE and MCF-7 cells, total RNA was extracted from the cells and the cDNA that covers the full-length of the cytoplasmic domain of  $\beta$ 1 integrin was amplified. Reverse transcription-polymerase chain reaction (RT-PCR) products from HBE or MCF-7 cells were identical in length with 462-bp on agarose gels (Fig. 3A). Sequence analysis of the cDNA fragments of HBE and MCF-7 cells revealed that both fragments were identical to the wild-type cytoplasmic domain of  $\beta$ 1 integrin isoform A (Fig. 3B), indicating that there is no difference in the cytoplasmic domain of  $\beta$ 1 integrin between HBE and MCF-7 cells.

### **The phosphorylation state of $\beta$ 1 integrin**

The importance of  $\beta$ 1 integrin phosphorylation to its functions which include cell adhesion and invasion has been suggested by many investigations [19,20,22,23]. To determine this possibility, we examined the phosphorylation state of  $\beta$ 1 integrin in HBE and MCF-7 cells. Metabolic labeling of cells with [ $^{32}$ P]orthophosphoric acid indicated that  $\beta$ 1 integrin (110 kDa), and not  $\alpha$ -actinin



**Figure 1**  
**Coprecipitation of actin with  $\beta 1$  integrin in HBE cells and not in MCF-7 cells.** (A) Integrin  $\beta 1$  was immunoprecipitated (IP) with the anti- $\beta 1$  integrin antibody or control IgG from HBE or MCF-7 cells which were quiescent and adherent to collagen IV. The immunoprecipitates were immunoblotted with anti-actin or anti-integrin  $\beta 1$  monoclonal antibody. Molecular size markers are indicated at right in kDa. (B) Whole cell lysates were prepared from HBE or MCF-7 cells which were quiescent and adherent to collagen IV, and equal amounts (5  $\mu$ g protein) of the lysates were resolved by SDS-PAGE and blotted. The blots were probed with anti-actin or anti-integrin  $\beta 1$  monoclonal antibody. Molecular size markers are indicated at right in kDa.

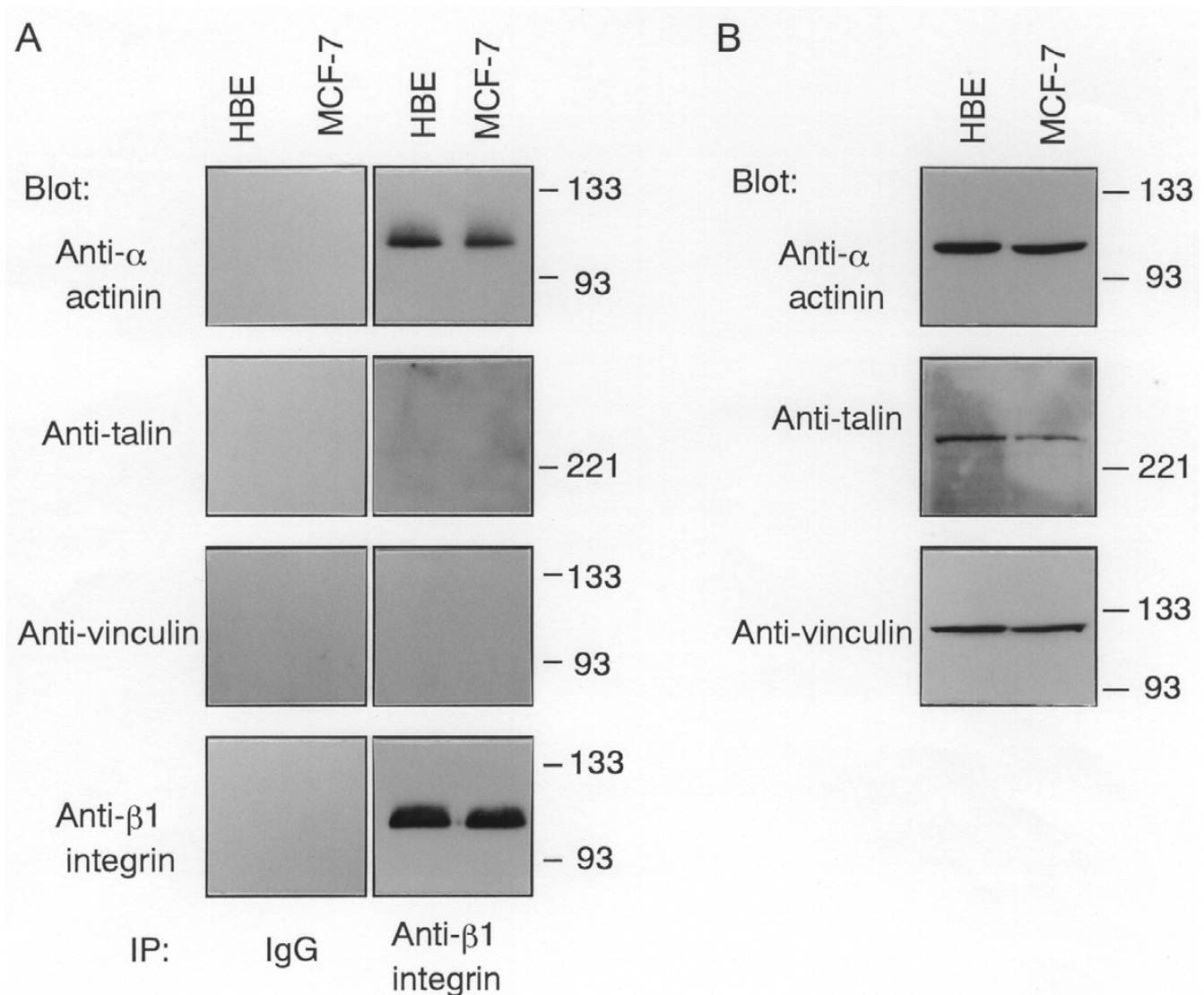
(100 kDa), was phosphorylated in either HBE and MCF-7 cells (Fig. 4A). When the  $\beta 1$  integrin immunoprecipitates were probed with anti-phosphotyrosine (PY) antibody,  $\beta 1$  integrin, and not  $\alpha$ -actinin, reacted with the antibody in both HBE and MCF-7 cells (Fig. 4B). This indicates that  $\beta 1$  integrin is phosphorylated at least at tyrosine in MCF-7 cells as well as in HBE cells.

#### **Effects of dephosphorylation of $\beta 1$ integrin on its link with actin**

To determine the role of phosphorylation of  $\beta 1$  integrin in the link formation between  $\beta 1$  integrin and actin, the  $\beta 1$  integrin immunoprecipitates were dephosphorylated by PTP or PP. Treatment of the  $\beta 1$  integrin immunoprecipitates from HBE cells with PTP resulted in tyrosine dephosphorylation of  $\beta 1$  integrin as assessed by the anti-PY antibody and concomitant dissociation of actin from it (Fig. 5A). In contrast, tyrosine dephosphorylation of  $\beta 1$  integrin from MCF-7 cells by PTP did not affect the absence of coprecipitation of actin with  $\beta 1$  integrin but successive incubation of the PTP-treated  $\beta 1$  integrin with the supernatant of the  $\beta 1$  immunoprecipitates that contained unbound actin or with exogenous human platelet actin caused coprecipitation of actin with  $\beta 1$  integrin (Fig. 5A). The result indicated that  $\beta 1$  integrin in either cells was tyrosine phosphorylated. On the other hand, dephosphorylation of the  $\beta 1$  integrin immunoprecipitates from either cells by PP2A<sub>1</sub> did not result in any alteration in coprecipitation of two proteins or the reactivity of  $\beta 1$  integrin with the anti-PY antibody (Fig. 5B). Actin remained coprecipitated with  $\beta 1$  integrin from HBE cells, while it remained dissociated from  $\beta 1$  integrin from MCF-7 cells. However, incubation of the PP2A<sub>1</sub>-treated  $\beta 1$  integrin from MCF-7 cells with the supernatant of the  $\beta 1$  immunoprecipitates or with exogenous human platelet actin caused coprecipitation of actin with  $\beta 1$  integrin (Fig. 5B). The results indicated that  $\beta 1$  integrin in HBE cells is not serine/threonine phosphorylated but in MCF-7 cells.

#### **CaMKII binding to $\beta 1$ integrin in MCF-7 cells**

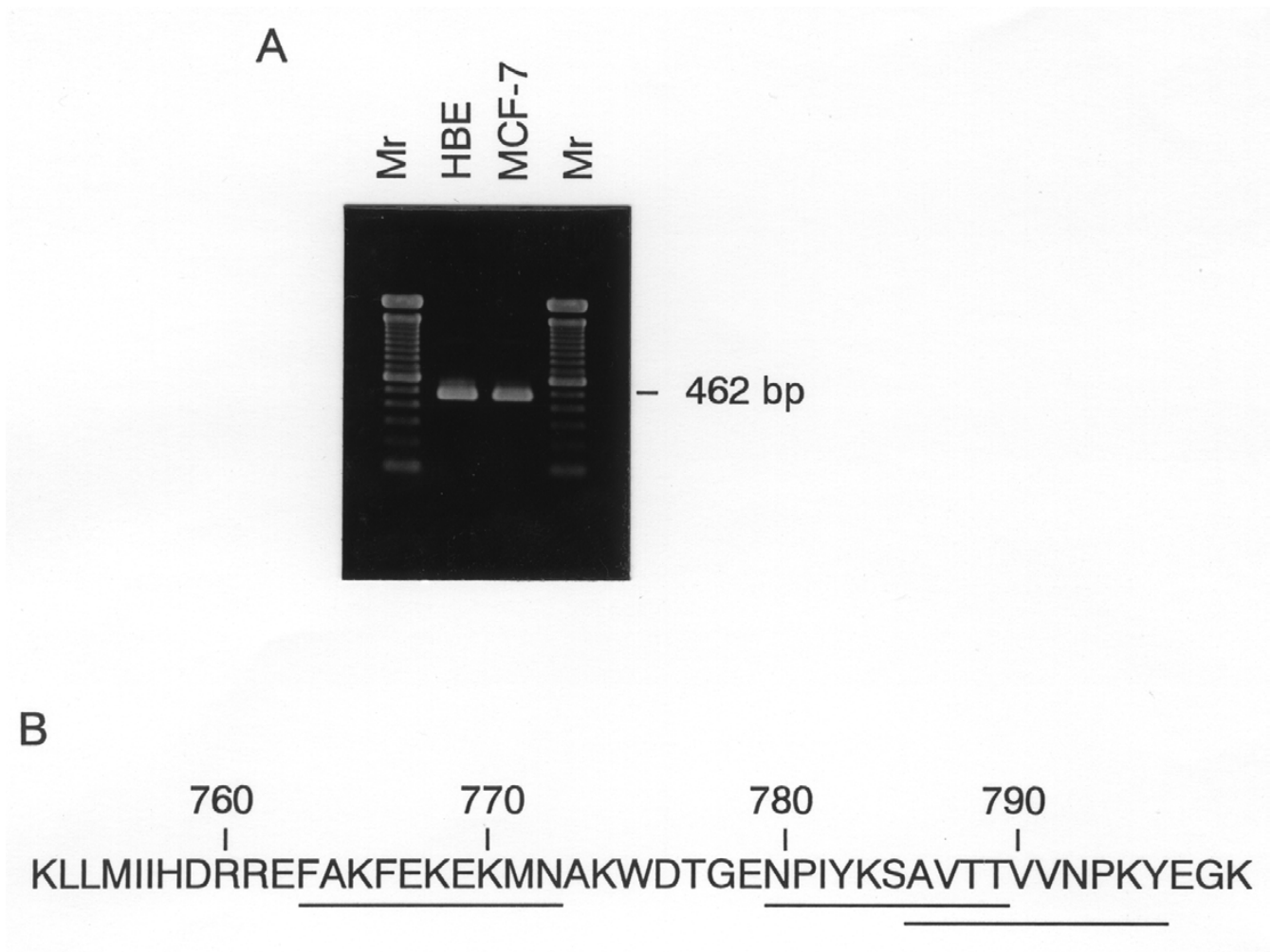
Loss of linkage between the cell-cell adhesion molecule E-cadherin and the actin cytoskeleton, that is mediated by a three-protein link of  $\beta$ -catenin,  $\alpha$ -catenin and  $\alpha$ -actinin, is induced by association of the EGF receptor having the protein tyrosine kinase activity with  $\beta$ -catenin that leads to tyrosine phosphorylation of  $\beta$ -catenin and concomitant dissociation of  $\alpha$ -catenin from it [24,25]. By analogy with this phenomenon, the  $\beta 1$  integrin immunoprecipitates were probed with the antibodies to several serine/threonine protein kinases to determine whether serine/threonine protein kinase associates with  $\beta 1$  integrin. Among these, the anti-CaMKII antibody, which reacts broadly with all CaMKII isoforms, reacted with doublet of proteins having molecular mass of around 56

**Figure 2**

**Coprecipitation of  $\alpha$ -actinin, but not talin or vinculin, with  $\beta$ 1 integrin in HBE and MCF-7 cells.** (A) Integrin  $\beta$ 1 was immunoprecipitated (IP) from HBE and MCF-7 cells using the anti- $\beta$ 1 integrin antibody or control IgG. The immunoprecipitates were probed with anti- $\alpha$ -actinin, anti-talin, or anti-vinculin antibody. Molecular size markers are indicated at right in kDa. (B) Equal amounts (5  $\mu$ g protein) of whole cell lysates from quiescent HBE and MCF-7 cells were probed with anti- $\alpha$ -actinin, anti-talin, or anti-vinculin antibody. Molecular size markers are indicated at right in kDa.

kDa in the  $\beta$ 1 integrin immunoprecipitates but not in the control IgG precipitates from MCF-7 cells (Fig. 6A). These proteins which were reactable with the anti-CaMKII antibody were not detected in the  $\beta$ 1 integrin or control IgG precipitates from HBE cells (Fig. 6A). To confirm the CaMKII kinase activity of the doublet of proteins, *in vitro* kinase assay was carried out using a specific substrate peptide Autocamtide II, in the presence of calmodulin and inhibitor peptides of cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). Whereas the  $\beta$ 1 integrin immunoprecipitates from HBE

cells exhibited a very low level of the kinase activity that was slightly above that of the precipitates without anti- $\beta$ 1 integrin antibody, the relative kinase activity of the immunoprecipitates from MCF-7 cells was 17-fold (Student's t-test,  $p < 0.01$ ) above that of the  $\beta$ 1 integrin immunoprecipitates from HBE cells (Fig. 6B). When 10  $\mu$ M KN-62, a specific inhibitor of CaMKII [26–28], was present, the kinase activity of the  $\beta$ 1 integrin immunoprecipitates from HBE cells was 7-fold above ( $p < 0.05$ ) that in the absence of KN-62 (Fig. 6B). Contrary to this,

**Figure 3**

**Amino acid sequence of the cytoplasmic domain of  $\beta 1$  integrin in HBE or MCF-7 cells.** (A) Agarose gel electrophoresis of RT-PCR products. Total RNA was extracted from HBE or MCF-7 cells which were quiescent and adherent to collagen IV, and cDNA encoding the cytoplasmic domain of  $\beta 1$  integrin was amplified by RT-PCR. The cDNA products were resolved in 1.6% (w/v) agarose gel electrophoresis with molecular size markers (Mr) and stained with etidium bromide. Molecular size of the cDNA products are indicated at right in bp. (B) The cDNA fragments of 462-bp were extracted from agarose gels and sequence analysis was carried out. Amino acid sequences of the three peptides with the highest affinity for  $\alpha$ -actinin are underlined (adapted from [18]).

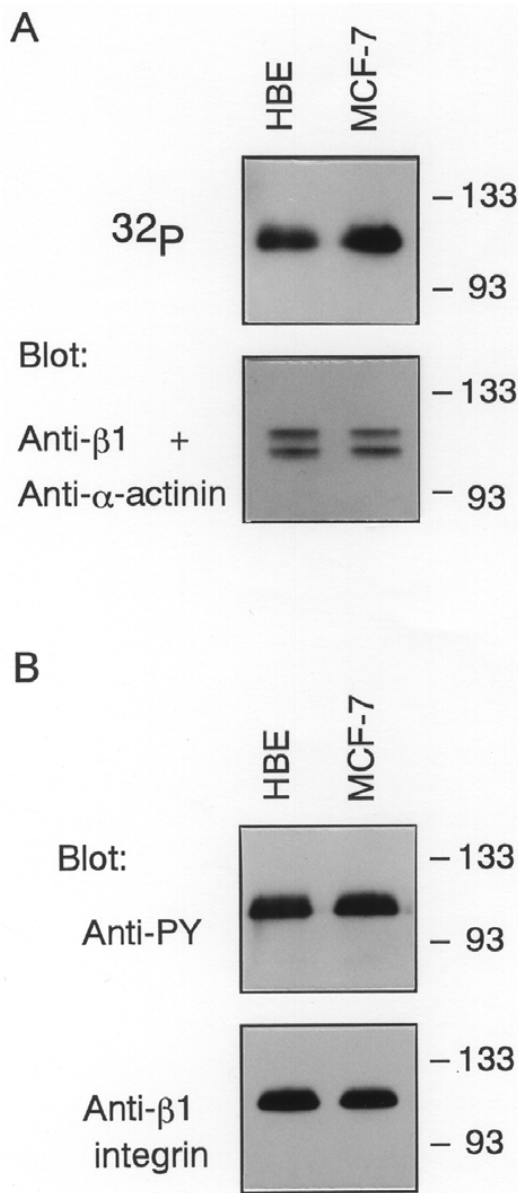
10  $\mu$ M KN-62 significantly inhibited the kinase activity of the  $\beta 1$  integrin immunoprecipitates from MCF-7 cells by 92% ( $p < 0.01$ ) (Fig. 6B).

### Discussion

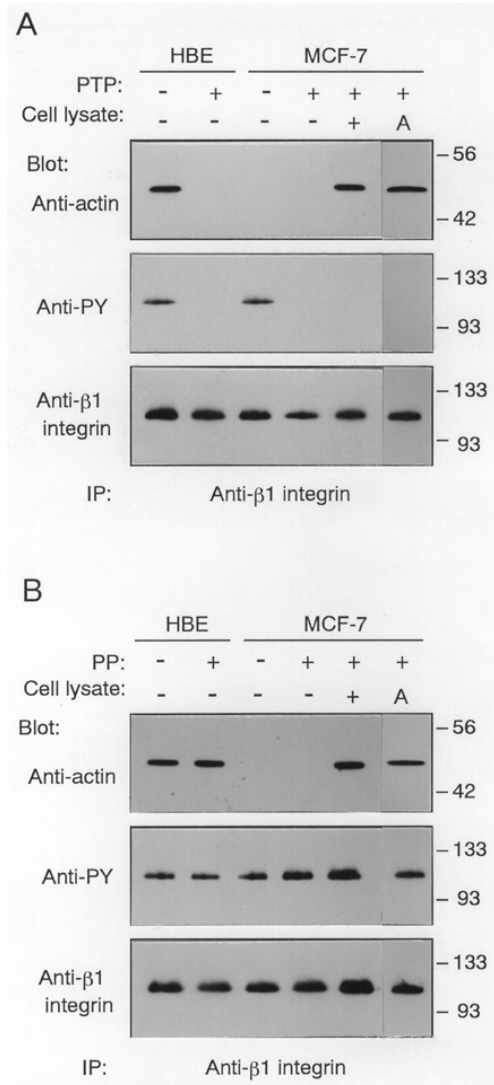
Immunoprecipitation and Western blotting revealed that actin coprecipitated with  $\beta 1$  integrin from quiescent HBE cells but not from MCF-7 cells. Coprecipitation of actin with  $\beta 1$  integrin but not with the control IgG precipitates from HBE cells indicates that association of  $\beta 1$  integrin with actin is not non-specific. Since actin expression in MCF-7 cells adhering to collagen IV was

comparable with that in HBE cells, the result suggests that loss of linkage between  $\beta 1$  integrin and the actin cytoskeleton in MCF-7 cells may not be due to the absence of actin expression.

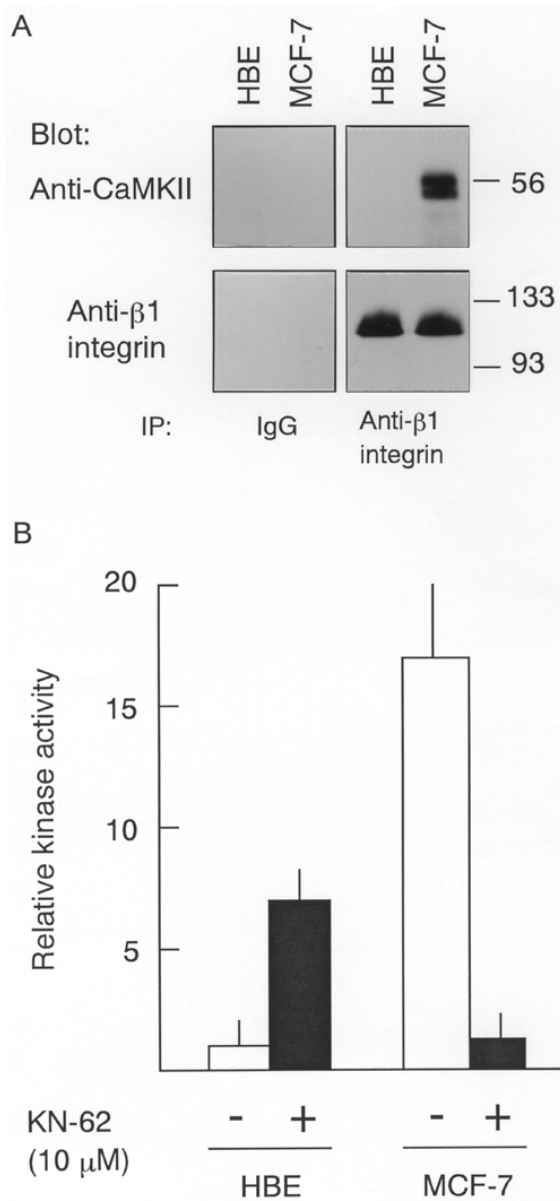
The  $\beta 1$  integrin subunit interacts by its cytoplasmic domain with many actin-binding proteins, such as  $\alpha$ -actinin, talin, and vinculin, thereby forming a link between  $\beta 1$  integrin and the actin cytoskeleton [7–12]. Immunoprecipitation followed by Western blotting revealed that only  $\alpha$ -actinin, but not talin or vinculin, coprecipitated with  $\beta 1$  integrin but not with the IgG precipitates from



**Figure 4**  
**The phosphorylation state of β1 integrin.** (A) HBE or MCF-7 cells which were quiescent and adherent to collagen IV were metabolically labeled with [<sup>32</sup>P]orthophosphoric acid and β1 integrin was immunoprecipitated from the cells. After separation on 8% SDS-PAGE, gels were dried and autoradiographed. Unlabeled samples were electrophoresed and transferred onto membranes. The blots were probed with antibodies to β1 integrin and α-actinin. Molecular size markers are indicated at right in kDa. (B) Tyrosine phosphorylation of β1 integrin in HBE and MCF-7 cells. Integrin β1 was immunoprecipitated from HBE or MCF-7 cells and the immunoprecipitates were resolved by 8% SDS-PAGE before blotting. The blots were then probed with anti-PY or anti-integrin β1 monoclonal antibody. Molecular size markers are indicated at right in kDa.



**Figure 5**  
**Effects of dephosphorylation of β1 integrin on its coprecipitation with actin.** (A) The β1 integrin immunoprecipitates from quiescent HBE or MCF-7 cells were treated with (+) or without (-) PTP, then immunoblotted with anti-actin, anti-β1 integrin, or anti-PY antibody. The PTP-treated β1 integrin immunoprecipitates from MCF-7 cells were incubated for 30 min with (+) or without (-) the supernatant of the β1 immunoprecipitates containing unbound actin (Cell lysate), or with 5 μg/ml exogenous human platelet actin (A). Molecular size markers are indicated at right in kDa. (B) The β1 integrin immunoprecipitates from quiescent HBE or MCF-7 cells were treated with (+) or without (-) PP2A<sub>1</sub>, then the PP2A<sub>1</sub>-treated β1 integrin from MCF-7 cells were incubated for 30 min with (+) or without (-) the supernatant of the β1 immunoprecipitates (Cell lysate), or with 5 μg/ml exogenous human platelet actin (A). The immunoblots were probed with anti-actin, anti-β1 integrin, or anti-PY antibody. Molecular size markers are indicated at right in kDa.



**Figure 6**  
**CaMKII binding to  $\beta$ 1 integrin in MCF-7 cells.** (A) The  $\beta$ 1 integrin immunoprecipitates (IP) with the anti- $\beta$ 1 integrin antibody or control IgG from HBE or MCF-7 cells were probed with antibody to all CaMKII isoforms or anti- $\beta$ 1 integrin antibody. Molecular size markers are indicated at right in kDa. (B) The  $\beta$ 1 integrin immunoprecipitates from HBE or MCF-7 cells were incubated with the substrate peptide Autocamtide II, calmodulin, inhibitor peptides of PKA and PKC, and [ $^{32}$ P]ATP with (closed columns) or without (open columns) 10  $\mu$ M KN-62 at 30°C for 10 min. After subtraction of the background, the activity was normalized to the amount of  $\beta$ 1 integrin and expressed relative to that of the  $\beta$ 1 integrin immunoprecipitates from HBE cells without KN-62. Results represent the mean  $\pm$  S.D. (bars) of duplicate assays of three independent experiments.

both HBE and MCF-7 cells. This indicates the specific association between  $\alpha$ -actinin and  $\beta$ 1 integrin in both HBE and MCF-7 cells. Whereas comparable amounts of  $\alpha$ -actinin and vinculin are present in HBE and MCF-7 cells and slightly reduced level of talin is seen in MCF-7 cells, the result suggests that  $\alpha$ -actinin, but not talin or vinculin, may predominantly mediate a one-protein link between  $\beta$ 1 integrin and actin in both HBE and MCF-7 cells and that the loss of linkage between the two proteins in MCF-7 cells may be due to the absence of a link between  $\beta$ 1 integrin-associated  $\alpha$ -actinin and actin.

In spite of the fact that  $\alpha$ -actinin may directly bind with actin [15], structural requirements for the functions of the cytoplasmic domain of  $\beta$ 1 or  $\beta$ 3 integrin in cell adhesion and cell spreading are strongly suggested by many investigations [19,20,22,23]. In addition, there are several isoforms within the cytoplasmic domain of  $\beta$ 1 integrin [16,17]. These observations prompted us to examine which isoform of  $\beta$ 1 integrin is expressed in MCF-7 cells. RT-PCR, using a set of primers corresponding to the sequences covering the cytoplasmic domain of  $\beta$ 1 integrin isoform A, demonstrated that the same cDNA fragments in length were amplified from HBE and MCF-7 cells. Sequence analysis indicated that the cDNA fragments had the identical sequence and corresponded to sequences encoding the cytoplasmic domain of the wild-type  $\beta$ 1A integrin. This suggests that both HBE and MCF-7 cells express the same wild-type  $\beta$ 1 integrin and that the loss of linkage between  $\beta$ 1 integrin and actin may not be due to the structural difference in the cytoplasmic domain of  $\beta$ 1 integrin in MCF-7 cells from that in HBE cells.

The relevance of phosphorylation of the  $\beta$  subunit of integrin at threonine [19,20] or tyrosine residue [23] to the integrin functions that include cell adhesion and invasion has been documented. Therefore we next examined the phosphorylation state of  $\beta$ 1 integrin and its relation to the linkage formation with actin. Metabolic labeling of cells with [ $^{32}$ P]orthophosphoric acid and Western blotting using the anti-PY antibody indicated that  $\beta$ 1 integrin, but not  $\alpha$ -actinin, in either HBE or MCF-7 cells was phosphorylated at least on a tyrosine residue and that there was no apparent difference in the phosphorylation state of  $\beta$ 1 integrin between two cells. Therefore, the susceptibility of the linkage forming ability of  $\beta$ 1 integrin with actin to PTP or PP was examined. Effects of the PTP treatment of the  $\beta$ 1 immunoprecipitates from HBE and MCF-7 cells were contradictory; that is, coprecipitation of actin with  $\beta$ 1 integrin from HBE cells was lost, while that of two proteins from MCF-7 cells was induced after incubation of the PTP-treated immunoprecipitates with the supernatant of the immunoprecipitates or with exogenous human platelet actin. As the reactivity of anti-PY antibody with  $\beta$ 1 integrin from either HBE or

MCF-7 cells was lost by PTP and exogenous actin coprecipitated with PTP-treated  $\beta 1$  integrin from MCF-7 cells, alterations in coprecipitation of actin with  $\beta 1$  integrin may be due to tyrosine dephosphorylation of  $\beta 1$  integrin and not other proteins involved in the immunoprecipitates or the supernatant of the immunoprecipitates. In addition, the result suggests that tyrosine phosphorylation is necessary for  $\beta 1$  integrin to link with actin in HBE cells but inhibitory for it in MCF-7 cells. Contrary to this, treatment of the  $\beta 1$  integrin immunoprecipitates with PP2A<sub>1</sub> did not result in any alteration in coprecipitation of actin with  $\beta 1$  integrin from HBE cells but caused coprecipitation of the two proteins from MCF-7 cells. As PP2A<sub>1</sub> did not affect tyrosine phosphorylation of  $\beta 1$  integrin from either HBE or MCF-7 cells and exogenous actin became coprecipitated with PP2A<sub>1</sub>-treated  $\beta 1$  integrin from MCF-7 cells as endogenous actin in the supernatant, the results suggested that PP2A<sub>1</sub>-induced coprecipitation of  $\beta 1$  integrin from MCF-7 cells with actin may be due to serine/threonine dephosphorylation of  $\beta 1$  integrin rather than other cellular proteins and that the ability of  $\beta 1$  integrin to link with actin in MCF-7 cells may be prevented by its serine/threonine phosphorylation. While it appears that phosphorylation at either tyrosine or serine/threonine is necessary for  $\beta 1$  integrin to link with actin and phosphorylation at both tyrosine and serine/threonine prevents  $\beta 1$  integrin from linking with actin, the linkage formation of  $\beta 1$  integrin with the actin cytoskeleton may be differentially regulated in MCF-7 cells as compared to HBE cells by tyrosine or serine/threonine phosphorylation of  $\beta 1$  integrin.

Finally we examined whether some protein kinases associate with  $\beta 1$  integrin in MCF-7 cells, using antibodies to several serine/threonine protein kinases. Among these, the anti-CaMKII antibody reacted with doublet of proteins with molecular mass of around 56 kDa which coprecipitated with  $\beta 1$  integrin from MCF-7 cells but not from HBE cells. As the antibody to CaMKII did not react with any protein in the control IgG precipitates from MCF-7 cells, association of the doublet of proteins with  $\beta 1$  integrin may be specific. The results demonstrating that the  $\beta 1$  integrin immunoprecipitates from MCF-7 cells had the kinase activity for a specific substrate peptide, Autocamtide II, under the conditions where calmodulin and inhibitor peptides of PKA and PKC were present and that this kinase activity was completely inhibited by 10  $\mu$ M KN-62, a specific inhibitor of CaMKII [26–28], suggest that the doublet of proteins which associate with  $\beta 1$  integrin in MCF-7 cells may be CaMKII isoforms. Even though the reason for an elevated level of the kinase activity of the  $\beta 1$  immunoprecipitates from HBE cells by KN-62 is unknown at present, it is possible to assume that some unidentified protein kinases which are responsive to KN-62 may associate with  $\beta 1$  integrin in

HBE cells. Coprecipitation of CaMKII with  $\beta 1$  integrin in MCF-7 cells does not imply their direct association. As direct association between CaMKII and  $\alpha$ -actinin has been reported by yeast two-hybrid and biochemical assays [29], CaMKII in MCF-7 cells may bind to  $\alpha$ -actinin, which may also bind to  $\beta 1$  integrin, forming a ternary complex with  $\alpha$ -actinin and  $\beta 1$  integrin. Whether serine/threonine phosphorylation of  $\beta 1$  integrin in MCF-7 cells is regulated by the  $\beta 1$  integrin-associated CaMKII, or whether loss of intracellular linkage between  $\beta 1$  integrin and the actin cytoskeleton causes the reduced cell surface expression of  $\beta 1$  integrin, are areas for further investigation.

### Conclusion

Intracellular linkage of  $\beta 1$  integrin with the actin cytoskeleton *via*  $\alpha$ -actinin was formed in HBE cells which adhered to type IV collagen. However, this linkage was lost in adherent MCF-7 cells. The present results suggest that tyrosine phosphorylation may be required for  $\beta 1$  integrin to link with actin in HBE cells, but phosphorylation of  $\beta 1$  integrin at both tyrosine and serine/threonine residues in MCF-7 cells may cause dissociation of actin from it. Thus the linkage formation of  $\beta 1$  integrin with the actin cytoskeleton may be differentially regulated by its tyrosine and serine/threonine phosphorylation in normal HBE cells and breast cancer MCF-7 cells.

### Materials and methods

#### Cell culture and replating on collagen type IV

HBE cells obtained from Clonetics were maintained in serum-free defined medium MCDB 170 [30] supplemented with 10 ng/ml EGF, 1.4  $\mu$ M hydrocortisone, 5  $\mu$ g/ml insulin, 0.1 mM ethanalamine, 0.1 mM phosphoethanolamine, and 25  $\mu$ M prostaglandin E2 [24] at 37° C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. MCF-7 cells were maintained in 5% fetal bovine serum (FBS)-containing RPMI 1640 medium. Subconfluent culture of HBE or MCF-7 cells were harvested with 0.25% (w/v) trypsin-2.65 mM EDTA solution and the cells were replated on 60-mm collagen IV-coated dishes (Becton-Dickinson). HBE or MCF-7 cells were then growth arrested by incubation in an EGF-deprived medium for 3 days or in 0.1% FBS-containing medium for 2 days, respectively.

#### Immunoprecipitation and Western blot analysis

Cells adherent to collagen type IV were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate) [25] and sonicated briefly using an ultrasonic cell disruptor (Misonix Inc.). The cell lysates were incubated with the anti-integrin  $\beta 1$  monoclonal antibody (Life Technologies) or control IgG followed by



Protein A-Sepharose (Amersham Pharmacia). The immunoprecipitates were washed three times with PB buffer (20 mM phosphate buffer, pH 8.6, 0.5% Nonidet P-40, and 0.1% SDS) and boiled for 2 min in lysis buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol). Whole cell lysates were prepared by homogenizing cells in 1% SDS in 20 mM Tris-HCl, pH 7.4, 1 mM PMSF and 1 mM sodium orthovanadate, and then boiling for 2 min, before sonicating briefly. The protein content was determined using a protein reagent kit (Pierce) with bovine serum albumin (BSA) as the standards. The immunoprecipitates or whole cell lysates were dissolved in SDS-polyacrylamide gel electrophoresis (PAGE) and after electrophoresis the samples were transferred onto membranes (Millipore). The blots were probed with anti-actin (ICN), anti- $\beta$ 1 integrin, anti- $\alpha$ -actinin (Upstate Biotechnology), anti-talin (Chemicon), anti-vinculin (Sigma), anti-phosphotyrosine (PY) (Upstate Biotechnology), or anti-CaMKII antibody (Santa Cruz Biotechnology). After incubation of the membrane with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia), the reactivity was visualized using an ECL kit (Amersham Pharmacia) and X-ray films (Fuji-film).

#### RT-PCR and direct sequencing

Total RNA was extracted from unstimulated cells which adhered to collagen IV, using TRIzol reagent and then digested with DNase I (Life Technologies). A one-step RT-PCR was carried out using an RT-PCR kit (CLONTECH) at the annealing temperature of 55°C. The primer set for the cytoplasmic domain of  $\beta$ 1A integrin was 5'-TCCTATTTTAACATTACCAA-3' and 5'-ACTGTGACTATGGAAATTGC-3' [16]. The PCR products were electrophoresed on 1.6% (w/v) agarose gels and the cDNA was extracted from the gel bands. The DNA sequence was confirmed for both forward and reverse strands of cDNA fragments using the set of PCR primers on an automated DNA sequencer (ABI PRISM 3100, Applied Biosystems).

#### Metabolic labeling of cells

Adherent cells to collagen IV-coated dish were labeled with 100  $\mu$ Ci/ml of [ $^{32}$ P]orthophosphoric acid (Amersham Pharmacia) in phosphate-free medium for the last 24 h during incubation to arrest the cells. Immunoprecipitation of  $\beta$ 1 integrin and SDS-PAGE of the immunoprecipitates were carried out as described above. After electrophoresis, gels were dried under vacuum and autoradiographed using X-ray films at -70°C for a week.

#### Phosphatase treatment

Immunoprecipitation of  $\beta$ 1 integrin was carried out as described above and the supernatant obtained by centrifugation of the immunoprecipitates with the Protein

A-Sepharose beads was retained at room temperature. The  $\beta$ 1 integrin immunoprecipitates were washed twice with PB buffer and once with phosphatase buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, and 0.1 mM EDTA). Then the immunoprecipitates were treated with or without 1.0 unit of PTP (LAR, Calbiochem) [31–33] for 10 min or 0.1 units of serine/threonine-specific PP (PP2A<sub>1</sub>, Calbiochem) [34–36] for 15 min in 100  $\mu$ l of phosphatase buffer at 30°C. In some experiments, the phosphatase-treated immunoprecipitates were washed with RIPA buffer and incubated with the retained supernatant of the  $\beta$ 1 immunoprecipitates that contained unbound actin or with 5  $\mu$ g/ml human platelet actin (Cytoskeleton, Inc.) in RIPA buffer containing 0.2 mM CaCl<sub>2</sub> for 30 min at room temperature.

#### CaMKII assay

Immunoprecipitation of  $\beta$ 1 integrin was carried out as described above, and the  $\beta$ 1 integrin immunoprecipitates were washed twice with PB buffer and once with 20 mM phosphate-buffered saline, pH 7.2. *In vitro* kinase assay for  $\beta$ 1 integrin-associated CaMKII was carried out at 30°C for 10 min using [ $\gamma$ - $^{32}$ P]ATP (NEN Life Science Products) and a CaMKII assay kit (Upstate Biotechnology) that included specific substrate peptide Autocamtide II, calmodulin, and inhibitor peptides of PKA and PKC. In some experiments, the kinase assay was done in the presence of KN-62 (1-[N,O-Bis(5-isoquinolinesulfonyl)]-N-methyl-L-tyrosyl-4-phenylpiperazine) (Sigma), a specific inhibitor of CaMKII [26–28] at the indicated concentrations. The background kinase assay was carried out using the precipitates with the Protein A-Sepharose beads but without the antibody to  $\beta$ 1 integrin. Radioactivity incorporated into the substrate peptide was determined with a liquid scintillation counter. After subtraction of the background, the activity was normalized to the amount of  $\beta$ 1 integrin that was quantified by measurement of the band intensity of  $\beta$ 1 integrin on SDS-PAGE gels using an image analyzer equipped with a digital camera (EDAS 290, Kodak).

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