

## Calcium/calmodulin-dependent protein kinase II controls $\alpha_5\beta_1$ integrin-mediated inside-out signaling

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### SUMMARY

**Fibronectin binding on  $\alpha_5\beta_1$  integrin is strictly dependent on intracellular calcium. Using an in vitro assay, we previously found that either calcineurin inhibitors or a blocking calcineurin monoclonal antibody added to cell lysates completely abolished the fibronectin/integrin interaction, which suggested that the activity of calcineurin, a calcium/calmodulin-dependent phosphatase, was required to counteract some kinase activity and maintain the high affinity state of  $\alpha_5\beta_1$ . In this paper, we show that blocking of the calcium/calmodulin kinase II (CaMKII) activity with the specific inhibitor KN-62 or with its pseudosubstrate Autocamtide-2 preserved the high affinity state of the integrin even under experimental conditions that inhibit calcineurin. Conversely, the addition**

**of purified CaMKII to the cell lysate inhibited  $\alpha_5\beta_1$  binding to fibronectin in vitro. Consistent with these results, cell adhesion on fibronectin was stimulated by KN-62. Moreover, Scatchard analysis of fibronectin binding on CHO cells revealed that KN-62 decreased the  $K_d$  value from 0.3 to 0.05  $\mu\text{M}$ . Finally the expression of exogenous constitutively active CaMKII resulted in a dramatic defect in cell adhesion with no significant modification in  $\alpha_5\beta_1$  cell surface expression. In summary our results demonstrate that CaMKII controls the affinity state of the integrin  $\alpha_5\beta_1$  in vitro and in living cells.**

Key words: Integrin, Calcium/calmodulin kinase II, Calcineurin, Fibronectin, Adhesion

### INTRODUCTION

The integrins, the most prominent of the extracellular matrix adhesion receptors, are a large family of heterodimeric transmembrane proteins with different  $\alpha$  and  $\beta$  subunits. To date 16  $\alpha$  and 8  $\beta$  subunits have been characterized from more than 20 receptors (Hynes, 1992). Subsequent to extracellular ligand binding, most integrins cluster within the plasma membrane into subcellular structures called focal adhesions. These structures provide a molecular link between the extracellular matrix and cytoskeleton. Cytoskeletal proteins such as talin,  $\alpha$ -actinin and vinculin have been detected by immunocytochemistry in focal adhesions.

In addition to this structural role, integrins regulate numerous cell functions through an outside-in signaling pathway (Clark and Brugge, 1995). For instance, it has been shown that integrin occupancy modulates free cytoplasmic calcium concentration, intracellular pH, phosphatidyl inositol turnover and protein kinase activation. Such messengers control most fundamental biological processes including gene expression, proliferation, apoptosis and differentiation.

Cell-matrix interactions may be reduced by lowering the clustering of integrins (Pomès and Block, 1992) or by modulating integrin affinities through an inside-out signaling pathway. Such a regulation was initially described for integrins such as  $\alpha_{IIb}\beta_3$  on platelets (Bennett and Vilaire, 1979),  $\beta_2$

(Altieri and Edgington, 1988),  $\beta_1$  (Shimizu et al., 1990) and  $\beta_7$  (Crowe et al., 1994). The modification of integrin affinity is due to a conformational change in its extracellular domain generated by the cytoplasmic domain, giving rise to specific epitopes. This switch can be triggered by an extracellular soluble agonist such as thrombin, ADP or phorbol ester, or by monoclonal antibodies which maintain the integrin in a high affinity state (Keizer et al., 1988; Kovach et al., 1992; Kornecki et al., 1990; Arroyo et al., 1992). Similarly,  $\alpha_v\beta_3$  integrins are submitted to activation-deactivation cycles during neutrophil migration (Hendey et al., 1996) and  $\alpha_5\beta_1$  integrins on keratinocytes or  $\alpha_6\beta_1$  integrins on retinal neurons are inhibited during differentiation steps (Adams and Watt, 1990; Neugebauer and Reichardt, 1991). Indeed, integrin affinity changes are involved in critical functions of the cells such as migration, differentiation and metastasis.

Although some integrin chains can be phosphorylated, evidence for a direct control of integrin affinity by phosphorylation of the receptors is poor (Hillery et al., 1991; Shattil and Brugge, 1991; Hibbs et al., 1991). Moreover, inside-out integrin signaling can be inhibited by a dominant negative effect of autonomously expressed  $\beta_1$  or  $\beta_3$  cytoplasmic domains (Chen et al., 1994; Lukashev et al., 1994), suggesting that some cytosolic regulatory proteins, which have yet to be defined, are titrated by overexpression of the  $\beta$  cytosolic tail and are required to alternate the

conformational state of the integrins. Recently Ginsberg's group implicated CD98, as a result of complementation of this dominant suppression (Fenczik et al., 1997). Other effectors may belong to a family of small polypeptides including ICAP-1, cytohesin-1 and  $\beta_3$ -endonexin, interacting with  $\beta_1$ ,  $\beta_2$  and  $\beta_3$ , respectively (Chang et al., 1997; Kolanus et al., 1996; Kashiwagi et al., 1997).

The molecular pathways leading to inside-out activation of integrins are still poorly understood. However, they encompass classical messengers such as cytoplasmic calcium in association with cellular proteins (Marie et al., 1991; Arroyo et al., 1992; Pomiès et al., 1995; Lawson and Maxfield, 1995). Conversely, ligand binding to the purified receptor is dependent solely on either  $Mg^{2+}$  or  $Mn^{2+}$  (Mould et al., 1996). In agreement with this idea, the hindrance of calcium fluxes inhibits T cell  $\beta_1$  integrins (Hartfield et al., 1993) and prevents neutrophil migration (Marks et al., 1991). The inhibition of neutrophil chemokinesis can be reproduced by using inhibitors of the calcium- and calmodulin-dependent serine/threonine phosphatase calcineurin (Hendey et al., 1992). This inhibition was shown to be due to a defect in the inside-out signaling of  $\alpha_v\beta_3$  (Hendey et al., 1996). Similarly, we showed that this phosphatase is also involved in the regulation of  $\alpha_5\beta_1$  activation in CHO cells (Pomiès et al., 1995). Dephosphorylation of a non-identified cellular component increases the  $\alpha_5\beta_1$  affinity for fibronectin. These data suggest that the activation of a serine/threonine protein kinase may diminish the affinity of  $\alpha_5\beta_1$  for its extracellular ligand. Consistent with this idea, a new serine/threonine kinase p59<sup>ILK</sup> was shown to interact with the  $\beta_1$  integrin chain whose overexpression leads to the inhibition of cell adhesion (Hannigan et al., 1996).

Using the solid phase assay previously developed in our laboratory (Pomiès et al., 1995) we studied the protein kinases involved in the control of  $\alpha_5\beta_1$  in CHO cells. Specific protein kinase inhibitors and substrate analogs enabled us to identify the serine/threonine calmodulin-dependent protein kinase II (CaMKII) as the antagonist kinase involved in calcineurin-dependent  $\alpha_5\beta_1$  regulation in vitro. This result was confirmed directly by adding purified CaMKII to the assay. Cell adhesion, fibronectin binding assay on CHO cells and expression of constitutively active CaMKII in the cells demonstrated that CaMKII activity switches  $\alpha_5\beta_1$  to its low-affinity state in the living cells. Thus, the biochemical mechanism disclosed in vitro also occurred ex vivo.

## MATERIALS AND METHODS

### Cell culture

Chinese Hamster Ovary (CHO) cells stably transfected with a modified ecdysone receptor (Ecr CHO cells) were purchased from InVitrogen. CHO clone 15B (CHO15B) and Ecr CHO cells were grown on plates in Minimum Essential Medium with alpha modification ( $\alpha$ -MEM) without nucleoside and supplemented with 7.5% fetal calf serum (v:v) at 37°C, in a humidified 5% CO<sub>2</sub>/95% air atmosphere. In the case of the transfected Ecr CHO cells, Zeocin was introduced into the culture medium according to the manufacturer's instructions. Cells were harvested in phosphate-buffered saline (PBS) supplemented with 1 mM EDTA and 0.05% (w:v) trypsin.

### Transfection of CaMKII in CHO cells

The cDNA encoding a constitutively active calcium/calmodulin

protein kinase II (CaMKII T286D) was a generous gift from Dr H. Schulman (Stanford University School of Medicine). The cDNA was excised from Sr alpha vector as an *EcoRI* fragment and inserted into pIND ecdysone-inducible vector (InVitrogen). Stable Ecr CHO transfectants were obtained by electroporation of  $0.4 \times 10^7$  cells in 400  $\mu$ l of PBS at 340 V with 10  $\mu$ g of pIND-CaMKII T286D vector or with 10  $\mu$ g of pIND vector, and were subsequently cultured in  $\alpha$ -MEM medium supplemented with 7.5% (v:v) fetal calf serum, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin. After 48 hours, G418 was added to the culture medium. The expression of CaMKII T286D was induced by addition of 2-5  $\mu$ M of muristerone A, an ecdysone analog (InVitrogen) for 20 hours at 37°C. As a control, an equal amount of 100% ethanol was added to non-induced cells. Subsequently, the cells were harvested. Among the G418-resistant cells, those capable of expressing CaMKII T286D were selected after an induction step by immunoblot detection using both G301, a polyclonal antibody directed against the calcium/calmodulin-dependent protein kinase II (a generous gift from Dr A. J. Czernik) and the Cb- $\alpha_2$  anti-CaMKII polyclonal antibodies (Gibco BRL).

### Cell lysate

CHO15B cells were harvested from 100 mm tissue culture dishes with trypsin/EDTA and washed once with  $\alpha$ -MEM supplemented with 7.5% (v:v) fetal calf serum. The cells were washed twice with cold PBS and lysed for 1 hour at 4°C with different lysis buffers. Lysis buffers included PBS with 1% detergent (octaethylene monododecyl ether, C<sub>12</sub>E<sub>8</sub>), 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM PMSF, 10  $\mu$ g/ml leupeptin and 10  $\mu$ M pepstatin A. In some experiments NaF, a PP2B inhibitor, and protein kinase inhibitors were added either directly into the lysis buffer, or after cell lysate clarification. Cell lysates were clarified by centrifugation at 15,000 rpm for 15 minutes at 4°C. The protein concentration of the different cell lysates was determined by the micro BCA protein assay (Pierce) according to the manufacturer's instructions.

### Solid phase assay

The binding of  $\alpha_5\beta_1$  integrins onto fibronectin was estimated using a solid phase assay as previously described (Pomiès et al., 1995). A 96-well microtiter plate (Maxisorp Immuno Plate, Nunc) was coated overnight at room temperature with 200  $\mu$ l of fibronectin at 25  $\mu$ g/ml. Fibronectin was purified from bovine plasma as described by Engvall and Ruoslahti (1977). The wells were washed twice with PBS and blocked for 1 hour at room temperature with 300  $\mu$ l of PBS containing 1% (w:v) BSA. Various dilutions of cell lysates (in a final volume of 200  $\mu$ l) were incubated in fibronectin-coated wells for 60 minutes at 37°C under gentle agitation. Unbound proteins were removed by four washes with a washing buffer made of PBS supplemented with 0.1% Tween 20 and 1% (w:v) BSA. The  $\alpha_5\beta_1$  integrins were detected by using a rabbit polyclonal antiserum directed against the human fibronectin receptor ( $\alpha_5\beta_1$ ) from Gibco BRL. A volume of 100  $\mu$ l of this rabbit anti- $\alpha_5\beta_1$  antiserum (dilution of 1/400) was incubated into the wells for 1 hour at 37°C under agitation. After four washes with the washing buffer, 100  $\mu$ l of anti-rabbit IgG-biotin conjugate F(ab')<sub>2</sub> fragment (Boehringer, Mannheim) at a dilution of 1/2,000 was added for 30 minutes at 37°C under agitation. After four washes with the washing buffer, 100  $\mu$ l of streptavidin-horse radish peroxidase conjugate (Boehringer, Mannheim) at a dilution of 1/10,000 was added for 30 minutes at 37°C under agitation. After four additional washes with PBS, a peroxidase substrate was added. When the TMB Peroxidase EIA substrate Kit from Bio-Rad Laboratories was used, the enzyme activity was measured at 450 nm with a plate reader (Dynatech MR5000). When the ABTS Peroxidase EIA substrate kit from Zymed laboratories was used, the enzyme activity was measured at 405 nm.

### Immunoprecipitation

CHO15B cells were depleted in cysteine- and methionine-free  $\alpha$ -

MEM containing 7.5% of fetal calf serum for 1 hour. Metabolic labeling of the proteins was performed overnight with Expre35S [S]-Protein Labeling Mix at 100  $\mu\text{Ci/ml}$  (Dupont NEN, France). The cells were washed three times in PBS at 37°C, lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% (w:v) NP40, 0.5% (w:v) DOC, 0.1% (w:v) SDS, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ ) for 1 hour on ice and centrifuged (15,000 rpm for 15 minutes at 4°C). A preclarification step was performed by incubating the lysate with a non-immune rabbit antiserum for 1 hour at 4°C followed by a subsequent addition of protein A-coupled agarose beads. The removal of the beads allows the elimination of non-specifically bound proteins. To the clarified fraction, either the polyclonal antibody Ru-16 directed against calcium/calmodulin-dependent protein kinase II (a generous gift from Dr Czernik) or a non-immune rabbit antiserum were added. After an overnight incubation step at 4°C, protein A-coupled beads precoated with CHO cell lysate were added and the incubation was followed for 1 hour at 4°C. The beads were washed five times in the lysis buffer, and bound proteins were solubilized in Laemmli's sample buffer. Proteins were resolved by SDS-10% polyacrylamide gel electrophoresis. Autoradiography was performed using X-OMAT AR5 films from Kodak.

### Immunoblot

Homogenized brain tissue from adult rat was solubilized into Laemmli's sample buffer under reducing conditions, total proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose, and the membrane was blocked for 60 minutes at room temperature in TBS (10 mM Tris HCl, pH 8, 150 mM NaCl) containing 5% non-fat milk powder. The CaMKII was detected with the specific polyclonal antibody G301 or Cb- $\alpha_2$  (Gibco, BRL) using the ECL chemiluminescent detection kit (Amersham International), according to the manufacturer's instructions.

### Cell spreading assays

CHO15B cells were harvested from 100 mm culture dishes with Trypsin/EDTA in PBS, then washed three times with  $\alpha$ -MEM containing 7.5% (v:v) fetal calf serum depleted in fibronectin. The cells were incubated for 30 minutes at 37°C with or without inhibitors. Then, either GRGDS peptide (0.5 mg/ml), GRGES peptide (0.5 mg/ml) or the anti- $\alpha_5$  antibody PB1 (4  $\mu\text{g/ml}$ ) were added and the incubation was continued for 15 minutes at 37°C. After incubation, the cells were allowed to spread on fibronectin for 4 hours at 37°C in 35 mm bacteriological dishes coated with fibronectin (the coating was performed by incubating the dishes with a fibronectin solution at a concentration of 1  $\mu\text{g/ml}$  for 2 hours at 37°C and post-coated with 1% (w:v) BSA for 30 minutes at 37°C). The cells were observed at a magnification of  $\times 320$  using an inverted microscope (Zeiss Axiovert 135) equipped with an X32 Ph1, NA 0.60 Achrostat objective lens. The results were quantified by analyzing four different dish areas and about 600 cells were scored as round or attached and spread.

### FACS analysis

CHO15B cells were grown in Petri dishes to 70% confluency in the presence or not of Muristerone A (5  $\mu\text{M}$ ) for 20 hours at 37°C. The cells were harvested and pelleted at room temperature. After two washes with PBS, the cells were fixed in PBS supplemented with 3% paraformaldehyde for 10 minutes at 37°C. The fixed cells were washed twice in PBS, then incubated for 30 minutes at 4°C, either with the specific anti- $\alpha_5$  monoclonal antibody PB1 (1  $\mu\text{g/ml}$ ), or with a control IgG at the same concentration in PBS supplemented with 5% BSA. After two washes in PBS, cells were incubated for 30 minutes at 4°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse F(ab')<sub>2</sub> in PBS and 5% BSA. After two more washes in PBS, 10,000 cells were analyzed on a FACScan (Becton Dickinson) equipped with an Argon laser ( $\lambda=488$  nm).

### Fibronectin binding assays

CHO15B cells were preincubated with or without 10  $\mu\text{M}$  KN-62 for 30 minutes at 37°C. The cells were harvested, washed twice with PBS, and resuspended at a concentration of  $6.4 \times 10^6$  cells/ml in the binding buffer consisting of PBS supplemented with 5% (w:v) BSA, 2 mM  $\text{MgCl}_2$  and 0.2 mM  $\text{CaCl}_2$ , with or without 10  $\mu\text{M}$  KN-62. Samples of  $0.75 \times 10^6$  cells were incubated with increasing amounts of FITC-conjugated fibronectin (molar ratio of 3). In a parallel experiment, 5 mM EDTA and EGTA were added, and the remaining bound fibronectin was taken as the amount of non-specific binding. The incubation of the cells with the labeled substrate was carried out at 28°C for 30 minutes under constant mixing by rotating the tubes end over end at 6 rpm. At the end of the incubation time, samples of  $0.5 \times 10^6$  cells were withdrawn and layered onto a 1 ml sucrose cushion composed of PBS, 15% (w:v) sucrose, 1% (w:v) BSA and 1 mM  $\text{MgCl}_2$ , and centrifuged at 13,000 rpm for 5 minutes. The bottom of the tubes were cut off and the cell pellets solubilized in 200  $\mu\text{l}$  RIPA buffer. After a clarification step by final centrifugation at 13,000 rpm for 5 minutes, the fluorescence in the supernatants was measured at 514 nm in a JY3D spectrofluorometer equipped with a 100  $\mu\text{l}$  quartz cuvette. Quantification of the amount of fibronectin was standardized using an FITC-fibronectin solution of known concentrations in RIPA.

### Reagents

ATP was from Sigma, calmodulin and Autocamtide-2 were from BioMol, KN-62 from Seikagaku, purified Rat CaMKII from Calbiochem and detergents from Fluka.

## RESULTS

### Biological assay of a kinase involved in the control of the interaction between $\alpha_5\beta_1$ and fibronectin in vitro

Using CHO cell lysates, previous studies indicated that the  $\alpha_5\beta_1$ -fibronectin interaction could be followed in vitro in a specific solid phase assay. In this cytosolic environment, the inhibition of the serine/threonine phosphatase calcineurin by sodium fluoride or by an anti-calcineurin monoclonal antibody (Table 1) resulted in the switch of the fibronectin receptor from a high- to a low-affinity conformational state (Pomiès et al., 1995). The most straightforward interpretation was that during cell lysis an activation of specific kinases occurred, initiating some phosphorylation cascade of the inside-out signaling pathway. However, these phosphorylations were specifically counteracted by calcium- and calmodulin-activated calcineurin. Therefore, the inhibition of this phosphatase would result in an increase in the phosphorylation levels,

**Table 1. The binding of  $\alpha_5\beta_1$  in vitro depends on the activity of calcineurin**

Addition to cell lysate	$\alpha_5\beta_1$ binding in vitro (%)
None	100 $\pm$ 5
NaF (70 mM)	32 $\pm$ 8
NaCl (70 mM)	93 $\pm$ 5
Anti-calcineurin (B subunit; 25 $\mu\text{g}/300$ $\mu\text{g}$ protein)	69 $\pm$ 8
Non-specific IgG (25 $\mu\text{g}/300$ $\mu\text{g}$ protein)	95 $\pm$ 2

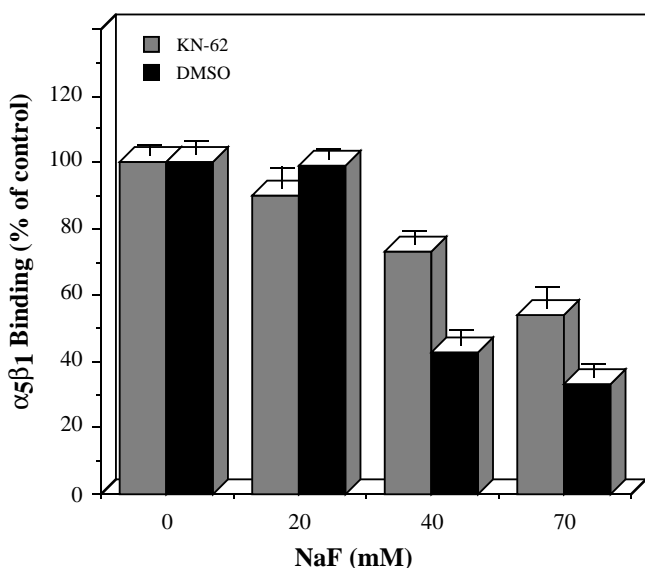
To cell lysates in C<sub>12</sub>E<sub>8</sub> detergents were added 70 mM of either NaF or NaCl or, alternatively, 25  $\mu\text{g}$  of an anti-calcineurin monoclonal antibody (UBI) or control IgG/300  $\mu\text{g}$  of cellular protein. The integrin binding was estimated as described in the experimental section. Each point represents triplicate values.

thereby triggering the conformational switch of  $\alpha_5\beta_1$  to a low-affinity state. If this hypothesis were correct, the binding assay of the fibronectin receptor should be insensitive to the inhibition of calcineurin by sodium fluoride in the absence of any kinase activity.

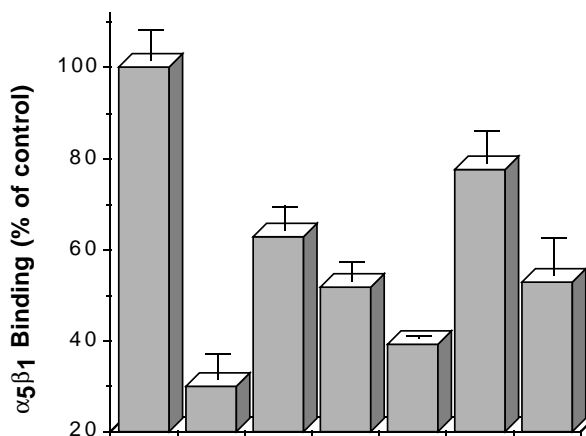
### CaMKII counteracts the calcineurin-induced switch of $\alpha_5\beta_1$ from high- to low-affinity conformational states

Cell lysates were prepared in the nonionic detergent  $C_{12}E_8$ , either in the presence or in the absence of KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine), a specific inhibitor of calcium/calmodulin-dependent kinase II (CaMKII) (Tokumitsu et al., 1990). When KN-62 was added at a concentration of 10  $\mu$ M, sodium fluoride inhibition of  $\alpha_5\beta_1$  binding to fibronectin was reduced (Fig. 1).

It has been established that KN-62 inhibits CaMKII by interacting with the calmodulin-binding site of the enzyme. However, it does not block the calmodulin-independent activity of previously autophosphorylated (activated) CaMKII (Tokumitsu et al., 1990). Therefore, some autophosphorylated kinase may have been present in the cell prior to lysis and may thus have been responsible for the partial release of NaF inhibition in our *in vitro*  $\alpha_5\beta_1$  binding assay. In order to improve the efficiency of KN-62, we preincubated the cell lysates in  $C_{12}E_8$  at 37°C for 30 minutes to reduce the amount of autophosphorylated CaMKII. This preincubation decreased sodium fluoride inhibition of  $\alpha_5\beta_1$  binding to fibronectin by 54% (Fig. 2). This suggested that kinases were rapidly inactivated at 37°C, either due to heat denaturation of the



**Fig. 1.** Effect of KN-62 on  $\alpha_5\beta_1$  binding onto fibronectin *in vitro*. CHO15B cells lysed in  $C_{12}E_8$  were incubated with 10  $\mu$ M of KN-62 (grey columns) or with shuttle (black columns) in the presence of increasing concentrations of NaF. An equal amount of cell lysate (400  $\mu$ g) was incubated in microtiter wells coated with 25  $\mu$ g/ml of fibronectin. Bound fibronectin receptors were detected by the solid phase assay as described in Materials and methods. The control (100%) is defined as the amount of fibronectin receptors bound and detected in the wells in the absence of NaF and kinase inhibitor. Each point represents triplicate values.



Preincubation	-	-	+	+	+	+	-
NaF	-	+	+	+	+	+	+
ATP	-	-	-	+	+	+	-
Calmodulin	-	-	-	-	+	+	-
KN-62	-	-	-	-	-	+	+

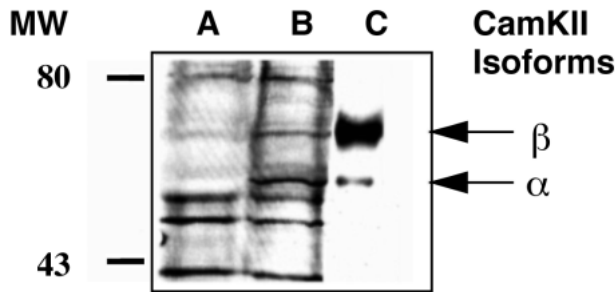
**Fig. 2.** A calmodulin-dependent protein kinase is involved in the regulation of  $\alpha_5\beta_1$  binding *in vitro*. A CHO15B cell lysate was incubated with or without KN-62 (10  $\mu$ M) for 30 minutes at 37°C. Then NaF (70 mM), calmodulin (0.1  $\mu$ M) and ATP (1 mM) were added to the lysate as indicated, and the incubation was continued for 10 minutes. Subsequently, equal amounts of treated cell lysates (400  $\mu$ g) were incubated in microtiter wells coated with 25  $\mu$ g/ml of fibronectin. Bound fibronectin receptors were detected by the solid phase assay as described in Materials and methods. The control (100%) is the binding of the fibronectin receptor in the wells in the solid phase assay without any addition or preincubation. Each point represent triplicate values.

proteins, or because some cellular components such as ATP or other cofactors were used up. Indeed, the subsequent addition of ATP increased the sensitivity of the assay to NaF. The effect of ATP was potentialized by calmodulin (Fig. 2). Sodium fluoride inhibition observed under these conditions (in the presence of ATP and calmodulin after an incubation step) was similar to the inhibition observed when NaF was added during the cell lysis. Moreover, about 70% protection was achieved by 10  $\mu$ M KN-62, whereas this protection was only 45% under standard conditions in which autophosphorylation of CaMKII was not minimized. These results indicated that the decrease in  $\alpha_5\beta_1$  binding upon calcineurin inhibition was augmented by ATP (that favors phosphorylations) and by calmodulin (that favors calcium/calmodulin-dependent kinases). Finally, dephosphorylation in the cell lysate triggered by a preincubation at 37°C for 30 minutes increased the capability of KN-62 to stabilize the fibronectin receptor in its high-affinity state.

To obtain direct evidence that CaMKII belonged to this regulatory pathway, we adopted three different strategies: (1) detection of CaMKII in the cell lysates, (2) analysis of the effect of the addition of purified CaMKII in the assay and (3) analysis of the phenotypes of cells expressing constitutively active CaMKII.

The presence of CaMKII in metabolically labeled CHO15B cell lysates was visualized by immunoprecipitation of the





**Fig. 3.** Detection of CaMKII isoforms in CHO cell lysates. CHO cells were metabolically labeled as described in Materials and methods and lysed in RIPA buffer. After a preclarification step of the lysate by an incubation with a non-immune rabbit antiserum followed by the addition of protein A-coupled agarose beads and centrifugation, the immunoprecipitation was performed overnight at 4°C either with non-immune serum or with the RU-16 polyclonal antibody directed against CaMKII  $\alpha$  and  $\beta$  isoforms. The immune complexes were bound to protein A-coupled sepharose beads for 1 hour at 4°C. After four washes, bound proteins were solubilized in Laemmli's buffer and analyzed by 10% acrylamide-SDS gel electrophoresis. Lanes A and B are the autoradiographs of the immunoprecipitated proteins with non-immune and immune serum, respectively. In the same electrophoreses, proteins from a Rat brain extract were resolved and transferred onto nitrocellulose. Lane C: CaMKII isoforms were detected by western blotting using the anti-CaMKII polyclonal antibody and the ECL chemoluminescence kit. The positions of molecular mass markers (kDa) are indicated (MW).

enzyme with a polyclonal antibody raised against CaMKII. Both  $\alpha$  or possibly  $\delta$  (which comigrates with  $\alpha$ ) and  $\beta$  isoforms of the enzyme were detected (Fig. 3B), although the total amount of the enzyme was low, compared to rat brain extracts. A competition experiment was performed between a putative CaMKII target involved in  $\alpha_5\beta_1$  inside-out signaling and Autocamtide-2, a highly selective peptide substrate of CaMKII. This peptide is derived from CaMKII autophosphorylation site RQETVD and its  $K_m$  is 2  $\mu$ M (Hanson et al., 1989). An excess of 20  $\mu$ M of Autocamtide-2 fully protected the binding assay from inhibition by NaF, indicating that under these conditions calcineurin was no longer required to maintain the integrin in its high-affinity state (Table 2). Another peptide (290-309) containing the CaMKII  $\alpha$  subunit negative regulatory sequence, encompassing the calmodulin binding site together with the autophosphorylation site (Thr-286) (Waxam et al., 1993), was added to the cell lysates. This peptide was shown to block the active site of the enzyme with an  $IC_{50}$  of 80 nM. Again, a large excess of the peptide allowed the integrin binding to immobilized fibronectin in vitro in the presence of NaF, which is in good agreement with KN-62 effects (not shown). Conversely, neither the PKC pseudosubstrate [19-36] (House and Kemp, 1987) nor the negative control molecule KN-04 prevented NaF inhibition of integrin binding (Table 2). Finally, the addition of purified CaMKII to the cell lysate inhibited  $\alpha_5\beta_1$  binding in vitro almost as efficiently as sodium fluoride (Table 2).

### KN-62 controls the $\alpha_5\beta_1$ -dependent cell adhesion and spreading on fibronectin

CHO cell adhesion on low concentrations of fibronectin was monitored in the presence or absence of KN-62. When a low

**Table 2.** CaMKII activity specifically controls  $\alpha_5\beta_1$  binding on fibronectin in vitro

Addition to cell lysate	$\alpha_5\beta_1$ binding in vitro (%)
None	100 $\pm$ 5
Calmodulin (0.15 $\mu$ g/ml) + ATP (0.5 mM)	100 $\pm$ 5
NaF (70 mM)	24 $\pm$ 8
NaF (70 mM) + KN-62 (10 $\mu$ M)	46 $\pm$ 7
NaF (70 mM) + KN-04 (10 $\mu$ M)	20 $\pm$ 5
NaF (70 mM) + Autocamtide-2 (20 $\mu$ M)	90 $\pm$ 16
NaF (70 mM) + PKC peptide inhibitor [19-36] (1.5 $\mu$ M)	25 $\pm$ 2
Purified CaMKII (0.5 $\mu$ g/ml) + Calmodulin (0.15 $\mu$ g/ml) + ATP (0.5 mM)	34 $\pm$ 9

Cell lysates were either preincubated with inhibitors of the KN family, or with calmodulin and ATP with or without purified CaMKII (Calbiochem) for 30 minutes at 37°C prior incubation in fibronectin-coated plastic wells. This preincubation step was omitted when the pseudosubstrates were used. Each point represents triplicate values.

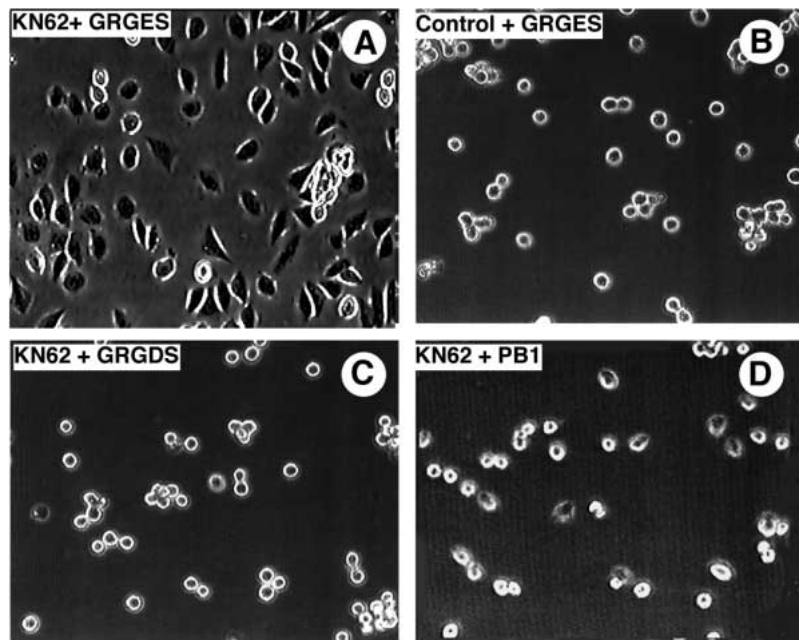
concentration of fibronectin (1  $\mu$ g/ml) was used for coating in cell adhesion assays, a control experiment showed little cell adhesion and spreading, whereas KN-62 treatment resulted in a dramatic increase in cell adhesion and spreading (Fig. 4E). Adhesion and spreading were mediated by the integrin  $\alpha_5\beta_1$ , since they could not be inhibited by the negative control peptide (Fig. 4A), but were fully abolished by adding either the GRGDS peptide (Fig. 4C) or the blocking monoclonal antibody PB1 raised against the  $\alpha_5$  subunit (Fig. 4D). It is noteworthy that the addition of the negative control molecule KN-04 had no effect on cell spreading (not shown).

### CaMKII controls the affinity state of the integrin $\alpha_5\beta_1$

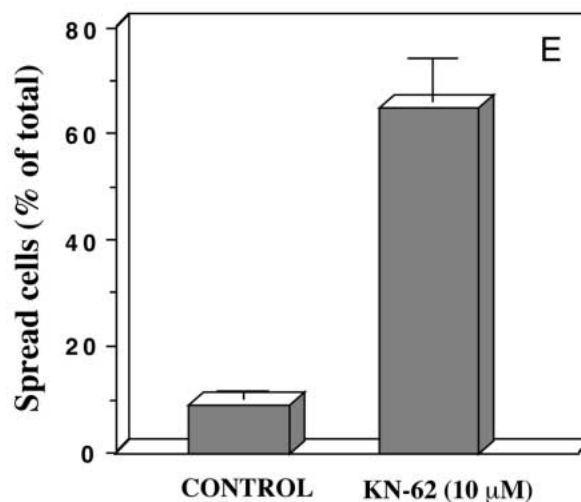
Since integrin clustering can affect receptor avidity, it was important to determine whether CaMKII controlled the integrin affinity or avidity in the living cells. Control CHO15B- or KN-62-treated cells were supplemented with increasing concentrations of FITC-conjugated fibronectin. The specific binding of fibronectin to the integrin was performed as described in Materials and methods and the data were analyzed by the Scatchard method. A typical experiment is shown in Fig. 5. A total number of binding sites ranging from 1.8 to 2.1 $\times$ 10<sup>6</sup> per cell for untreated or KN-62-treated cells and a  $K_d$  value of 0.3  $\mu$ M for control CHO cells were obtained. These values were identical to those previously obtained using <sup>125</sup>I-labeled fibronectin (Akiyama and Yamada, 1995; Marie et al., 1991). Blocking CaMKII activity with KN-62 decreased the  $K_d$  value to 0.05  $\mu$ M, indicating that the kinase readily controlled the affinity of the integrin fibronectin receptor.

### Inducible expression of constitutively active CaMKII results in a defect in cell adhesion

In order to test directly the role of CaMKII on  $\alpha_5\beta_1$ -dependent cell adhesion on fibronectin, we used an ecdysone-inducible mammalian expression system (InVitrogen) to express constitutively active (T286D) CaMKII (Hanson et al., 1989). As shown in Fig. 6A, without induction by the ecdysone analog Muristerone A, the background of CaMKII expression was low and the cells formed a nice spread on plastic dishes coated with fibronectin at a concentration of 2  $\mu$ g/ml. Muristerone A induction resulted in a large increase in CaMKII expression (Fig. 6A) without significant modification of  $\alpha_5\beta_1$  cell surface



**Fig. 4.** CaMKII inhibitors control  $\alpha_5\beta_1$ -dependent cell adhesion on fibronectin. CHO cells were harvested by trypsin/EDTA treatment as described in Materials and methods, then resuspended in  $\alpha$ -MEM containing 7.5% fetal calf serum depleted in fibronectin and incubated in the presence of 10  $\mu$ M KN-62 or shuttle (DMSO) for 30 minutes at 37°C. Samples were withdrawn and further incubated in suspension for 15 minutes at 37°C with 0.5 mg/ml of the GRGDS or GRGES peptides, respectively, or alternatively with 4  $\mu$ g/ml of the monoclonal antibody PB1 directed against the hamster  $\alpha_5$  integrin subunit. Subsequently, the cells were allowed to spread on fibronectin-coated Petri dishes for 4 hours at 37°C. The bacterial plastic dishes were coated for 2 hours with a solution of 1  $\mu$ g/ml of fibronectin solution at 37°C. The phase contrast pictures were obtained at a magnification of  $\times 320$  using an Axiovert 135 Zeiss microscope equipped with a Ph1 achrostat X32, NA 0.60 objective lens. (A) Incubation of the cells with KN-62 and GRGES peptide. (B) Incubation of the cells with shuttle (DMSO) and GRGES peptide. (C) Incubation of the cells with KN-62 and GRGDS peptide. (D) Incubation of the cells with KN-62 and 4  $\mu$ M PB1 antibody. (E) Quantification of control and KN-62-treated cells spreading. Quantification was performed as described in Materials and methods.



expression (Fig. 6B). However, CaMKII expression resulted in a dramatic rounding up of the cells on fibronectin (Fig. 6E). Thus, as expected, high intracellular CaMKII activity and KN-62 had opposite effects. It is noteworthy that, after induction, more than 90% of the cells were still viable.

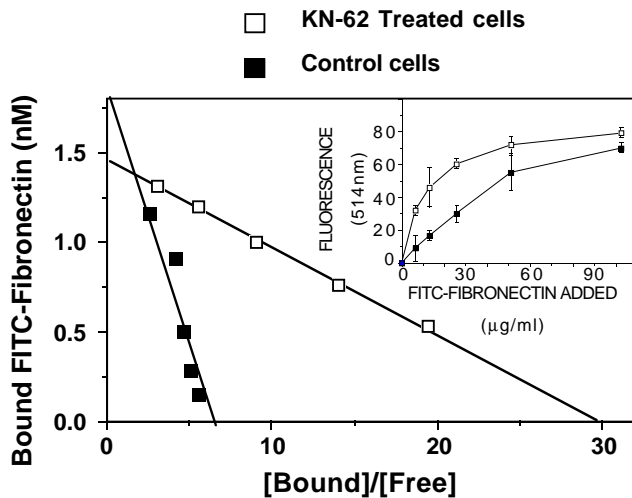
## DISCUSSION

We previously developed an *in vitro* assay to study the interaction of fibronectin with  $\alpha_5\beta_1$  in a cytosolic environment (Pomiès et al., 1995). This allowed us to establish that inside-out  $\alpha_5\beta_1$  signaling was controlled by the calcium/calmodulin serine/threonine-dependent phosphatase calcineurin, which is in good agreement with other data indicating that integrin-dependent neutrophil migration is also controlled by this enzyme (Lawson and Maxfield, 1995; Hendey et al., 1996). Calcineurin seems to maintain low phosphorylation levels associated with the high-affinity state of the  $\alpha_5\beta_1$  integrin.

Therefore, inhibition of the antagonist kinase activity should maintain the integrin in a high-affinity state even under experimental conditions in which calcineurin is inhibited (for instance when sodium fluoride is added). This was the rationale of a biological assay designed to identify the kinase activities involved in the control of  $\alpha_5\beta_1$  affinity.

The specific inhibitor of calcium calmodulin kinase II, KN-62, partially prevented the NaF-induced switch of  $\alpha_5\beta_1$  from a high- to a low-affinity state *in vitro*. Since some protein kinases of the C type (PKCs) were localized in focal adhesions (Jacken et al., 1989), these enzymes might be involved in the modulation of  $\alpha_5\beta_1$  affinity. However addition of calphostin C, a PKC inhibitor with narrow specificity, had no effect (data not shown). Similarly the addition of the PKC inhibitor [19-36] (House and Kemp, 1987) had no significant effect in our binding assay (Table 2), thereby ruling out this hypothesis.

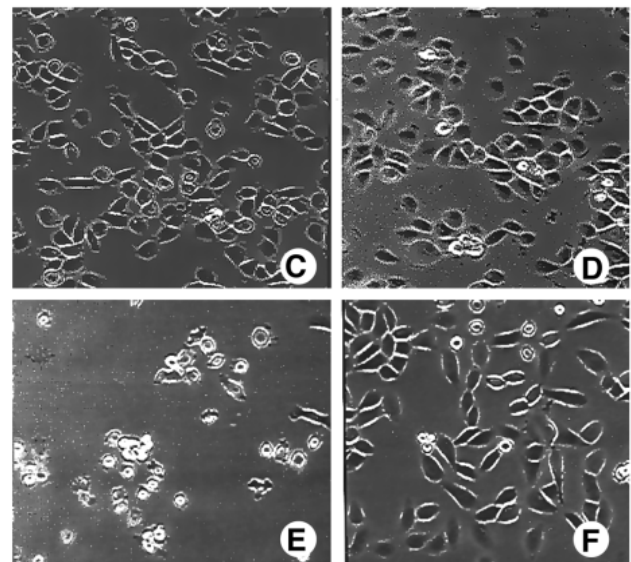
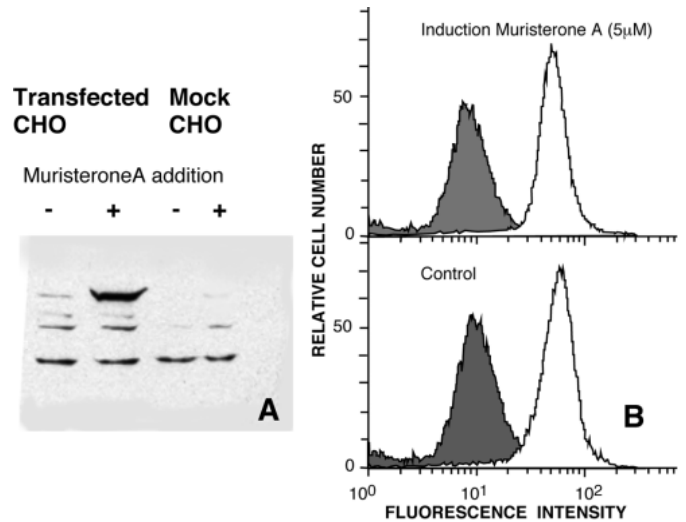
To date, KN-62 has been reported to specifically inhibit calcium/calmodulin protein kinase isoforms (Hidaka and Yokokura, 1996). Since KN-62 could be replaced by the



**Fig. 5.** Specific binding of FITC-conjugated fibronectin on KN-62-treated and control CHO15B cells. CHO15B cells were incubated with increasing amounts of FITC-conjugated fibronectin in the presence or the absence of a mix of 5 mM EGTA and 5 mM EDTA for 30 minutes at 28°C. Binding results (insert) were analyzed by the Scatchard method. The extrapolated intercepts with the y axis represent the concentration of the binding sites in a suspension of  $5 \times 10^6$  cells/ml, and the slopes of the straight lines gave the  $K_d$  values.

pseudosubstrates Autocamtide-2 and peptide 290-309, our present results indicated that CaMKII was involved in this regulatory pathway. The inhibitor KN-62 was shown to block competitively the calmodulin activation step of the kinase, but not the calmodulin-independent activity of the autophosphorylated kinase (Tokumitsu et al., 1990; Cruzalegui et al., 1992). Thus, a partial protection of KN-62 was expected under experimental conditions that allowed partial autophosphorylation of the enzyme. This result was observed. Moreover, preincubation of the lysate at 37°C rendered the binding assay partially insensitive to calcineurin inhibitors, suggesting that kinases were inactivated during the preincubation step, either due to thermal denaturation or to the lack of ATP and calmodulin. Consistent with this hypothesis, additions of ATP and calmodulin synergistically restored the NaF sensitivity of our assay, suggesting that a calmodulin kinase activity was definitely required to switch the integrin to its low-affinity state (Fig. 2). Under these conditions, which are likely to reduce the levels of autophosphorylated CaMKII, KN-62 allowed more than 70% of  $\alpha_5\beta_1$  to stay in a high-affinity state even in the presence of 70 mM of NaF (Fig. 2).

Sodium fluoride, which is routinely used to block calcineurin activity, was reported to inhibit CaMKII *in vitro* (Schworer et al., 1985). Since the addition of this salt in the lysates obviously favored phosphorylations related to the integrin transition towards the low-affinity state, one could suspect that KN-62 either blocked a calcium/calmodulin-dependent kinase distinct from CaMKII, or that the inhibition of CaMKII by NaF was slow as compared to calcineurin. The use of Autocamtide-2, a selective substrate of CaMKII derived from the CaMKII autophosphorylation site (Hanson et al., 1989), fully protected integrin binding to immobilized fibronectin from NaF inhibition (Table 2). Similarly, a peptide containing the CaMKII  $\alpha$  subunit



**Fig. 6.** Phenotype of CHO cells expressing CaMKII (T286D). CHO cells were stably transfected as described in Materials and methods. The phenotype of non-cloned positive cells was analyzed by western blotting of CaMKII, FACS analysis of  $\alpha_5\beta_1$  cell surface expression and cell adhesion assay on fibronectin. (A) Western blot of 50  $\mu$ g of cell lysate proteins from mock transfected cells and CaMKII transfected cells. CaMKII was detected by the G301 antibody using ECL chemiluminescent substrate. Muristerone induction was performed for 20 hours at 37°C at a concentration of 4  $\mu$ M. (B) FACS analysis of CaMKII transfected cells using control mouse IgG (shaded curve) or the anti- $\alpha_5$  monoclonal antibody PB1 (open curve) as described in Materials and methods. Induction was performed for 20 hours at 37°C with 5  $\mu$ M Muristerone A from a stock solution in ethanol. In a parallel experiment (lower panel), ethanol alone was added instead. (C and D) Adhesion of mock transfected CHO cells for 3 hours at 37°C on bacterial plastic dishes coated with 2  $\mu$ g/ml of fibronectin with or without induction with 4  $\mu$ M of Muristerone A, respectively. (E and F) Adhesion of CaMKII T286D-transfected CHO cells for 3 hours at 37°C on bacterial plastic dishes coated with 2  $\mu$ g/ml of fibronectin with or without induction with 4  $\mu$ M of Muristerone A, respectively.

negative regulatory sequence (including the calmodulin binding site and the autophosphorylation site) (Waxam et al., 1993) gave analogous results. Furthermore, preincubation of the cells with

sodium fluoride before cell lysis resulted in cell lysates in which  $\alpha_5\beta_1$  was maximally bound to fibronectin in our solid phase assay even when calcineurin was blocked (not shown). This latter experiment suggested that a long exposure to NaF also resulted in the inhibition of the kinase antagonist to calcineurin. Finally, adding directly purified CaMKII to the lysate reduced  $\alpha_5\beta_1$  binding to 34% of the control (Table 2). Although CaMKII is known to be strongly expressed in the brain, small amounts of the  $\alpha$  or possibly  $\delta$  and  $\beta$  isoforms of the enzyme could be detected in the CHO extracts after immunoprecipitation (Fig. 3).

Since the identification of CaMKII was achieved in an *in vitro* system, it may have no biological significance in living cells. We explored the possible involvement of CaMKII activity in the regulation of the adhesive properties of living CHO cells. Under experimental conditions in which CHO cells attached and spread in an exclusively  $\alpha_5\beta_1$  manner, the inhibition of CaMKII by KN-62 dramatically increased cell adhesion (Fig. 4). The increase in cell adhesion at low fibronectin concentrations induced by KN-62 corresponded to a sixfold increase in the integrin affinity for its substrate, with no significant modification of the number of binding sites on the cells (Fig. 5). Conversely, the expression of constitutively active CaMKII in CHO cells dramatically impaired cell adhesion on fibronectin without modifying integrin surface expression (Fig. 6).

It was previously reported that W7, a potent calmodulin antagonist, blocked the adhesion of HT1080 and RuGli cell lines on a variety of matrix proteins, whereas staurosporine had the opposite effect (Gimond and Aumailley, 1992). On the other hand, KN-62 was also shown to be a potent inhibitor of platelet aggregation (Shiraga et al., 1996), vascular smooth cell migration (Pauly et al., 1995). Altogether, our data strongly support the view that calcium/calmodulin-dependent kinase II controls the  $\alpha_5\beta_1$  inside-out signaling pathway.

It is noteworthy that the same intracellular effectors, calcium and calmodulin, trigger the activating and inactivating pathways of  $\alpha_5\beta_1$ . Thus, the affinity state of the integrin should be the result of the balance between CaMKII and calcineurin activities. Upon the intracellular decrease in calcium corresponding to the resting state of the cell, the residual autophosphorylated CaMKII should favor the low-affinity state of  $\alpha_5\beta_1$ . *Ex vivo* experiments were consistent with this view. Indeed, at the coating concentration of 1  $\mu\text{g/ml}$  of fibronectin, CHO cells did not spread onto the substratum. The previous interpretation of this result was that fibronectin was not concentrated enough to sustain cell spreading. Since KN-62 allowed the spreading of the cells at this fibronectin concentration, one may have to reconsider this explanation. A more realistic interpretation of the data is that  $\alpha_5\beta_1$  was in a low-affinity state on CHO cells in suspension. This was also suggested by previous data (Puzon-McLaughlin et al., 1996). Similarly, Scatchard analysis indicated that CHO cells in suspension had a low-affinity for fibronectin. The switch of the integrins to their high-affinity state was triggered by the inhibition of CaMKII or by a high concentration of insoluble fibronectin. In this model, the default conformational state of  $\alpha_5\beta_1$  should be the low-affinity state, similar to what has been observed for the platelet integrin,  $\alpha_{IIb}\beta_3$ .

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