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Modulation of α5β1 and αVβ3 Integrins on the Cell Surface During Mitosis

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Abstract One of the hallmarks of cells undergoing mitotic division is their rounded morphology and reduced adhesion to the substratum. We have studied and compared the attachment of interphase and mitotic cells to substrata coated with fibronectin and vitronectin. We have found that adhesion of mitotic cells, as compared to interphase cells, is significantly reduced to fibronectin, but is higher to vitronectin. These results correlate well with the expression of α5β1 and αVβ3 integrins, the respective receptors for fibronectin and vitronectin, on the cell surface. Mitotic cells show higher levels of αVβ3 and very low levels of α5β1 proteins on the cell surface as compared to interphase cells. This difference in the levels of these integrins also reflects in the total amounts of fibronectin and vitronectin present on the cell surface of these cells. We have further shown, by flow cytometry, that binding of vitronectin, or the synthetic peptide -GRGDSP-, causes an increase in the intracellular levels of Ca^{2+} in mitotic cells, but no change is seen in the interphase cells. Binding of fibronectin to either of these cells fails to elicit any response. One interesting feature of our results is that the levels of total, i.e., cytoplasmic plus membrane bound, α5β1 and αVβ3 integrins of mitotic and interphase cells remain the same, thus implying an alteration in the distribution of integrin chains between the plasma membrane and the cytoplasm during the conversion of interphase cells into the mitotic phase.

Key words: mitosis, cell adhesion, integrins, extracellular matrix

The mitotic stage in cell cycle is associated with dramatic changes in several properties of the cell, one of the most obvious of which is in its adhesive potential to extracellular matrix (ECM) proteins and cell shape [Vaheri et al., 1977; Pomies and Block, 1992]. Cultured fibroblasts undergoing mitosis are characterized by a distinct rounded morphology and a significantly reduced attachment to the substratum. It is for this reason that mitotic cells can be isolated by the tapping of the culture flask, because interphase cells remain well spread and attached to the substratum. In vitro cell attachment and spreading is mainly mediated by two ECM proteins, fibronectin (FN) and vitronectin (VN), which form a coat on the surface of the culture flask. These proteins are secreted by cells and are also present in the serum. Fibroblasts interact with the ECM proteins via specific heterodimeric molecules called integrins [Hynes, 1992; Giancotti and Mainiero, 1994]. FN binds to cells primarily via the α5β1 integrin and VN primarily via the αVβ3 and αVβ5 integrins. These interactions of the attachment proteins with their respective integrins are mediated by a tripeptide sequence, -RGD-, in the cell binding domain of the ligands [Hemler, 1990]. Therefore, many of the effects of the molecule can be mimicked by -RGD-bearing oligopeptides [Pytela et al., 1987].

Recent reports suggest that some ECM proteins are capable of transmitting signals into cells through integrins [Giancotti and Mainiero, 1994; Juliano and Haskill, 1993; Schwartz, 1992] which result in a number of intracellular biochemical changes. These include tyrosine phosphorylation and the activation of the focal adhesion kinase (FAK) [Schaller et al., 1992; Burridge et al., 1992; Guan et al., 1991; Kornberg et al., 1991], increase in intracellular pH and phospholipid turnover [Schwartz et al., 1991, 1992; Ingber et al., 1990], and calcium flux [Schwartz et al., 1993, 1994]. These signals have been implicated in various functions like the organization of the focal adhesion plaque, gene expression, and cell migration. The hallmark signal of the attachment of fibroblasts to FN is the activation of FAK and the rise in intracellular pH [Burridge et al., 1992; Kornberg et al., 1991;

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Schwartz et al., 1989]. The significance of these changes during mitosis is not yet clear. The alteration in the binding properties of cells during mitosis could be associated with an alteration in the integrin-mediated signaling.

In the present study, therefore, we have investigated the interaction of two adhesive proteins, FN and VN, with mitotic and interphase cells. We examined the attachment of these cells to FN- and VN-coated substrata, and the generation of intracellular signals after the binding. Although adhesion of mitotic cells to FN has been reported earlier [Vaheri et al., 1977; Pomies and Block, 1992], its role in signal transduction in such cells has not been studied. The interaction of VN with these cells has not been looked at earlier.

MATERIALS AND METHODS

Cell Lines

All the experiments were done with the rat fibroblast cell line F111, which was maintained in DMEM containing 10% FCS and antibiotics (penicillin, kanamycin, and streptomycin) in 5% CO_2 and 100% humidified air, and subcultured after every 3–4 days before the cells became confluent. All experiments were done within 10 passages after revival of the cells from liquid nitrogen storage.

Antibodies

Anti-α5 integrin polyclonal antibodies against a 23 amino acid synthetic peptide from C-terminal of rat integrin α5 and anti-β1 integrin polyclonal antibody raised against a 39 amino acid synthetic peptide from C-terminus of chicken integrin β1 were kindly provided by Prof. R.O. Hynes (Massachusetts Institute of Technology). A polyclonal antibody raised against the human placental α5β1 (binds to extracellular domain), anti-rat FN polyclonal antibody, anti-αvβ3 polyclonal antibody, and anti-VN polyclonal antibody were purchased from Telios Pharmaceuticals Inc., USA and GIBCO BRL, USA. FITC-conjugated anti-FN antibody was obtained from Serotec, England. FITC-conjugated anti-rabbit antibody was obtained from Dakopatts, Denmark and Amersham International, England.

FN, VN, and FN Fragments

FN was purchased from Serva Fine Chemicals, Germany. The 120 kDa cell binding domain (CBD) and synthetic peptides -GRGDSP- and -GRGESP- were obtained from Telios Pharmaceuticals Inc.

Collection of Mitotic and Interphase Cells

Mitotic cells were prepared by a variation of the method described by Zieve et al. [1980]. After removal of the free-floating cells by gentle shaking, subconfluent cultures were grown for 8 hr in nocodazole (1 μg/ml). Then the medium was gently aspirated and fresh serum-free medium (with 1 μg/ml nocodazole) was added. The mitotic cells were dislodged by tapping the sides of the culture flask. The medium containing mitotic cells was collected and kept on ice. The remaining attached cells in the flask were harvested using 5 mM EDTA. These interphase cells were washed several times with serum-free medium before use. The purity of mitotic and interphase cell population was determined by staining the cells with acridine orange (25 μg/ml) and observing under a fluorescence microscope.

Cell Attachment Assay

Cell attachment assay was carried out as described by Cunningham and Frederikson [1982]. FN, VN, or CBD fragments of FN were coated on the surface of 96-well culture plates by incubation of different concentrations of these proteins in the wells at 4°C. Uncoated sites in every well were blocked by incubating with 1 mg/ml BSA (Sigma, USA) in PBS for 1 hr at room temperature. Interphase and mitotic cells were prepared as described earlier and suspended in serum-free DMEM at a concentration of 1×10^6 cells/ml. The viability of the cells at this time was >90%, as checked by Trypan blue exclusion. One hundred microliters of cell suspension was added to the coated wells, in triplicate, for each concentration of the ligand. The plate was incubated at 37°C for 1 hr in a CO_2 incubator. Later, the unattached cells were removed by gently flicking the plate; the wells were rinsed with PBS once and the number of attached cells was estimated by the MTT assay.

Colorimetric Estimation of Cell Number–MTT Assay

This was done as described by Mossman et al. [1983]. The percentage of adherent cells was calculated by determining the enzyme activity of 1×10^5 cells (the number of cells that were

originally plated in each well) and comparing that with the residual cells adherent in each well. The mean number of cells adherent to each concentration of the ligand was calculated from the triplicate values obtained for that concentration. The data were plotted as the percent maximum attachment possible at each concentration of the ligand.

Preparation of Cell Lysates

Cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, aprotinin 2 μg/ml, leupeptin 2 μg/ml, PMSF 1 mM) for immunoprecipitation experiments. The lysate was transferred to 1.5 ml microfuge tubes and spun at 10,000 *g* at 4°C for 10 min. The supernatant was collected and incubated with Protein A-Sepharose (Pharmacia, Sweden) suspension in a rotator at 4°C for 30 min. The sample was briefly centrifuged at 1,000 *g*, and the supernatant was collected, protein content was estimated, and lysates stored at −70°C.

Estimation of Proteins

The protein was estimated by Peterson's modification of the Lowry method [1977].

Cell Surface Iodination

Cell surface proteins of interphase and mitotic cells were labeled with ^{125}I according to the lactoperoxidase–glucose oxidase method described by Hynes [1973]. Interphase cells were labeled on the surface of the culture vessel while mitotic cells were labeled in suspension. Both the labeled cells were lysed in RIPA buffer as described above and processed for immunoprecipitation.

Immunoprecipitation

Lysates from labeled or unlabeled cells were normalized on the basis of the amount of TCA-precipitable counts or amount of protein in each assay. Equated amounts of proteins from different cell types were incubated with anti-FN, anti-VN, anti-α5, anti-β1, or anti-α5β1 or anti-αVβ3 antibodies for 1 hr, and the immunoprecipitated proteins were collected by incubation with Protein A-Sepharose beads. Labeled proteins were analyzed by electrophoresis and autoradiography, and unlabeled proteins by Western blotting and chemiluminiscent detection (Amersham, UK).

Electrophoresis

SDS–polyacrylamide gel electrophoresis was carried out by the method described by Laemmli [1970], using a discontinuous buffer system. In order to distinguish the α and β chains of the integrin molecules, nonreducing gels were run (in the absence of β-mercaptoethanol) in most cases, except where mentioned otherwise.

Western Blotting

This was done for the detection of the immunoprecipitated proteins from the unlabeled cell lysates. The proteins were transferred to nitrocellulose paper according to Towbin et al. [1979]. The transferred proteins were detected using the chemiluminiscence detection kits following the manufacturer's instructions (Amersham plc, UK).

Cell ELISA

Interphase and mitotic cells were prepared as described, and approximately 1×10^5 cells of each type in plain DMEM were plated in each well of a 96-well plate (six replicates were used for each cell type). The supernatant medium was removed. The cells were then incubated with FN-, VN-, FNR-, or VNR-specific antibodies (each at a 1:100 dilution in PBS) for 30 min at room temperature. The wells were washed and incubated with HRP-conjugated second antibody at room temperature for 30 min. The wells were washed with PBS, and 100 μl of the substrate (25 μl of 0.1 M citrate phosphate buffer, pH 5.0, 10 μg OPD, 6 μl hydrogen peroxide) was added to each. After color developed, 50 μl of 4 N H_2SO_4 was added to stop the reaction. Aliquots of 50 μl from each well were transferred to another ELISA plate and the absorbence was recorded at 490 nm.

Fluorescence Microscopy

Immunofluorescence. Cells were grown overnight on uncoated glass coverslips in serum-containing medium at 37°C. The coverslips were then rinsed with PBS and fixed with formaldehyde (3.7% formaldehyde in PBS) for 20 min at room temperature. Subsequently, the cells were incubated with primary antibody at a dilution of 1:100 in PBS. The cells were rinsed in PBS and incubated with FITC-conjugated anti-rabbit second antibody (dilution 1:100) for 1 hr at 4°C. The cells were then washed in PBS, mounted in Slow Fade mounting medium (Molecular Probes Inc., USA), and observed under a fluorescence

microscope (DMRB, Lieca, Germany) fitted with epifluorescence optics.

Confocal microscopy. Changes in the intracellular calcium after FN and VN treatment were also studied microscopically. Cells were grown on coverslips and labeled with Fluo-3 without detaching them from the coverslips. After washing with DMEM the cells were mounted in DMEM containing 5 μg/ml of FN or VN. The cells were analyzed using an MRC1000 Confocal Laser Scanning Microscope with a 63.3× fluorescence objective lens (numerical aperture 1.44), and images were scanned within 90 sec of the exposure of the cells to FN or VN. Photographs of the cells were taken on a video hardcopy printer.

Flow Cytometric Analysis

Integrin expression. Interphase and mitotic cells were collected as described earlier. Approximately 1×10^6 cells, in 100 μl PBS, were incubated with primary antibodies to α5β1, αVβ3, FN, or VN at a dilution of 1:100, for 1 hr at 4°C. The cells were washed three times with 200 μl of PBS and further incubated with FITC-conjugated anti-rabbit IgG (dilution 1:50) for 1 hr at 4°C. The cells were again washed three times with PBS to remove traces of the unbound antibody and measured in FACStar Plus flow cytometer using the Lysys II software.

Intracellular calcium. Interphase and mitotic cells were prepared as described. The cells were charged with Fluo-3 (Molecular Probes) as per the manufacturer's instructions using Pluronic F-127 as the loading detergent. Typically, interphase and mitotic cells were treated with 2 μg/ml Pluronic at 37°C for 15 min, then Fluo-3 at a final concentration of 5 μg/ml was added to the cells and the incubation was continued for 30 more min at room temperature. The cells were then washed twice with DMEM and processed further for staining. After loading with Fluo-3 the cells were stained with Hoechst 33342 (2 μg/ml) for 1 hr at room temperature, to stain the DNA of the cells. The cells were washed once again with PBS containing DNA dye and analyzed in a FACStar Plus cytometer equipped with a dual laser system (Becton Dickinson). Hoechst 33342 was excited by a UV laser (350–366 nm) and the Fluo-3 with a 488 nm laser. The following filters were used to detect the specific fluorescence from the cells: a 400LP and a 424DF44 in front of the Hoechst 33342 photomultiplier tube (PMT), and a 525 ± 15 nm filter in front of the Fluo-3 PMT. About 1×10^6 were taken in 0.5 ml DMEM and 5 μg/ml (final concentration) of FN, VN, or -GRDGS- was added to the cells; FACS analysis of the cells was initiated immediately and fluorescence from cells (for both DNA and calcium) was recorded continuously for 5 min. The data were analyzed using the Lysys II software (Becton Dickinson).

RESULTS

Attachment of Interphase and Mitotic Fibroblasts to FN and VN

Cell attachment assays of interphase and mitotic cells were done as described. Interphase cells showed about fourfold higher attachment to FN than mitotic cells (Fig. 1A). A similar difference was observed in the attachment of cells to the cell binding fragment of FN (Fig. 1B), also showing that most of the attachment of the rat fibroblasts to FN is through the -RGD-containing region of FN. The attachment of cells to VN showed a different trend and is shown in Figure 1C. The overall attachment of cells to VN was much lower, as compared to FN, as no more than 20% of cells attached to VN-coated substrata; however, at the lower concentration of VN, mitotic cells showed an almost 3–4-fold higher attachment than interphase cells. These attachment assays clearly showed that mitotic cells can interact with VN more efficiently, whereas interphase cells have a significantly higher affinity for FN than mitotic cells.

Expression of α5β1 and αVβ3 Integrins on Cell Surface

This was studied by immunoprecipitation of ^{125}I-labeled mitotic and interphase cells, as described in Materials and Methods. The cell lysates were immunoprecipitated with α5, β1, and αVβ3 antibodies, electrophoresed under nonreducing (or reducing, if required) conditions, and autoradiographed. The results are shown in Figure 2. The immunoprecipitates with anti-α5 and anti-β1 antibodies are shown in Figure 2A. With both the antibodies we see a much higher amount of α5 and β1 proteins on the cell surface of interphase cells, while the mitotic cells show barely detectable quantities. Immunoprecipitation with anti-αVβ3 antibody, in contrast, shows a higher expression of these integrin chains in mitotic cells and a lower though detectable

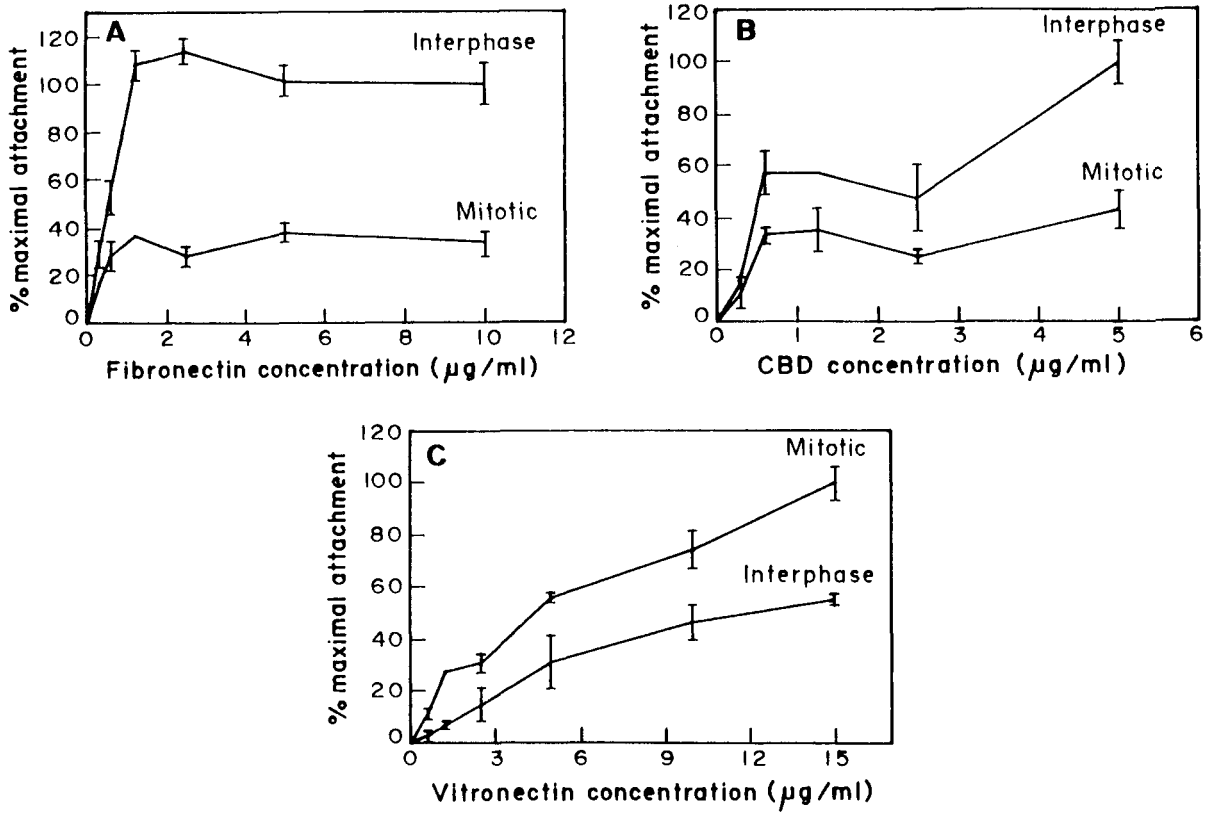

Fig. 1. Interphase cells and nocodazole-arrested mitotic cells were plated on multiwell plates coated with increasing concentrations of FN (A), cell binding domain of FN (B), or VN (C). Cells were allowed to attach for 2 hr and the number of cells attached in each well was calculated by MTT assay. The data have been plotted as percent maximum attached cells at each concentration.

amount on interphase cells (Fig. 2B). These results on the levels of α5β1 and αVβ3 integrins were confirmed by cell ELISA (Table I), immunofluorescence (not shown), and flow cytometric analysis of the cell surface–associated αVβ3 and α5β1 integrins (Fig. 3A,B, respectively).

Total Cellular Expression of α5β1 and αVβ3

In order to check whether the differences in the expression of α5β1 and αVβ3 integrins on the cell surface match with their total cellular levels, we quantitated these integrins by Western blotting of the immunoprecipitates of the total cell lysates done with anti-α5β1 and αVβ3 antibodies. The results of these experiments are shown in Figure 4A and B, respectively. As can be clearly seen, the total amount for both the integrins was equal, thus implying that alteration in the integrin amounts on cell surface is due to changes in the distribution of these molecules between the cytoplasm and the cell surface during mitosis.

Levels of FN and VN on Interphase and Mitotic Cell Surface

The level of VN and FN on mitotic and interphase cell surface was studied by flow cytometric analysis after staining both types of cells in suspension (Fig. 3C,D). Our results clearly showed that VN levels on mitotic cells were higher than on interphase cells, but FN levels were higher on interphase cells than in mitotic cells.

We confirmed these results on attached cells by immunofluorescence microscopy and immunoprecipitation of cell surface–labeled FN and VN on interphase and mitotic cells. Interphase cells showed a distinct fibrillar pattern of fluorescence for FN, but stain faintly and diffusely for VN; mitotic cells exhibit bright fluorescence for VN but are poorly stained with anti-FN antibodies (data not shown). Immunoprecipitation of the surface-labeled cells also showed the same quantitative pattern of distribution on mitotic and interphase cells (not shown).

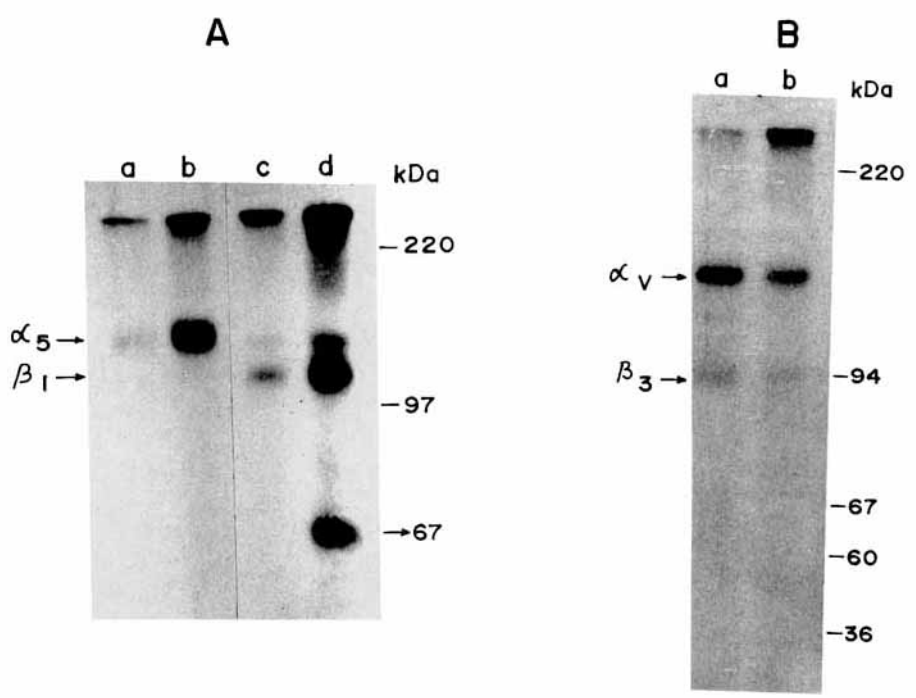

Fig. 2. ^{125}I surface-labeled interphase and mitotic cells were lysed in RIPA buffer and the extract immunoprecipitated with α5 and β1 (**A**) and αVβ3 (**B**) antibodies. Immunoprecipitates were electrophoresed under nonreducing conditions, dried, and autoradiographed. In A, *lanes a* and *b* show the α5 immunoprecipitation, and *c* and *d* show β1 immunoprecipitation. Lanes a and c in A represent mitotic cells, and b and d show interphase cells. In B, *lane a* is mitotic cells and *b* represents interphase cells.

TABLE I. Interphase and Mitotic Cells Incubated With α5β1 and αVβ3 Antibodies for 2 Hr*

	αVβ3 integrin	α5β1 integrin
Interphase cells	0.601	0.861
Mitotic cells	1.521	0.432

*The bound antibodies were estimated by peroxidase-conjugated anti-IgG incubation and OPD assay. Optical density for equal number of cells is shown for different ligands.

Effect of FN and VN Binding on Mitotic and Interphase Cells

As shown earlier, there is a distinct change in the expression of integrins on the cell surface during mitosis, which is reflected in their attachment to FN and VN. The downregulation of the α5β1 level was expected as these cells adhere poorly to FN. However, the marginal increase in the αVβ3 levels was intriguing. The αVβ3 integrin has been reported to cause calcium fluxes after ligand binding, and there are other reports of changes in intracellular calcium during G_2/M transition. We therefore studied the role of αVβ3 on mitotic cells in promoting intracellular calcium fluxes after binding to VN by FACS analysis and confocal microscopy.

For FACS analysis, interphase and mitotic cells were labeled in suspension with HO-33342 and the Ca^{2+}-sensitive dye Fluo-3 as described earlier. No significant differences in the dye uptake were noticed as measured by fluorimetry of the labeled cells. The changes in the levels of intracellular calcium were monitored continuously for 5 min in the flow cytometer with or without incubation of labeled cells with VN, FN, or -GRGDSP-, as described in Materials and Methods. Mitotic cells showed a clear increase in calcium levels after about 60 sec of adding VN or RGDS, and the increased levels did not come down up to 5 min after adding the ligands, the recording of which time is shown in Figure 5B (b and c, respectively). Addition of up to 25 μg/ml of FN failed to show any response up to 5 min (Fig. 5B, d). Interphase cells failed to show any increase after the addition of any of the ligands (Fig. 5A, b,c,d). Mitotic cells also showed higher basal levels of intracellular cytosolic calcium (compare a of Fig. 5A and B). The percentage of cells showing change in calcium level is shown in Table II.

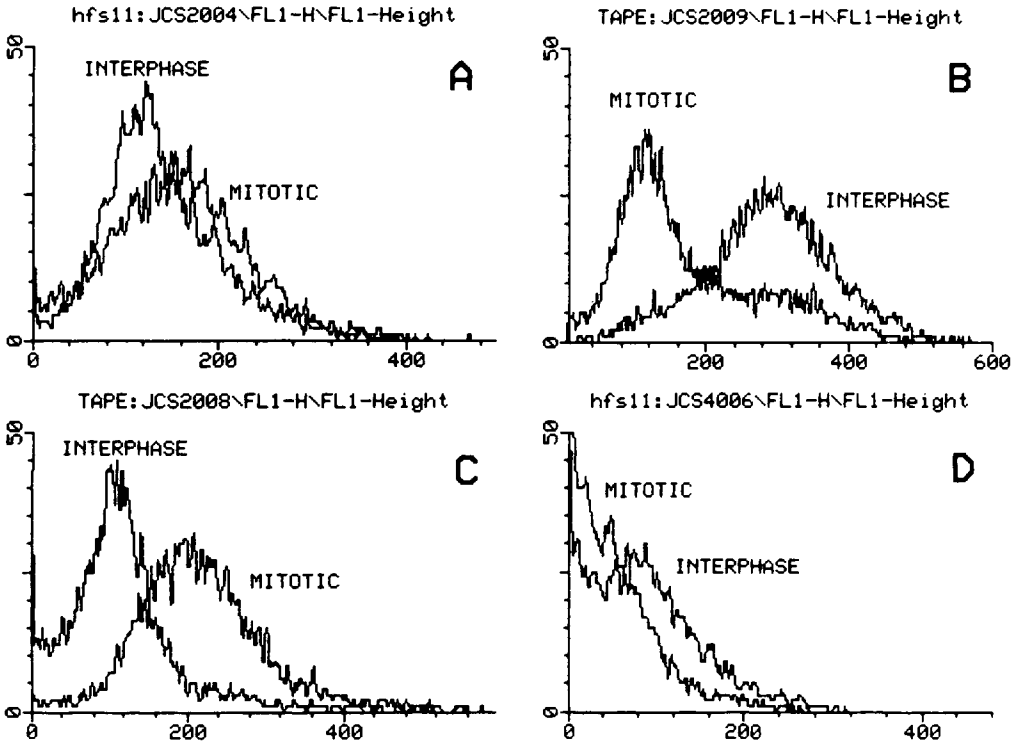

Fig. 3. Flow cytometric analysis of the cell surface levels of FN, VN, and α5β1 and αVβ3 integrins in isolated mitotic and interphase cells. Cells were stained with specific primary antibodies followed by FITC-conjugated anti-rabbit antibodies, and fluorescence was measured by FACS. **A:** αVβ3. **B:** α5β1. **C:** VN. **D:** FN.

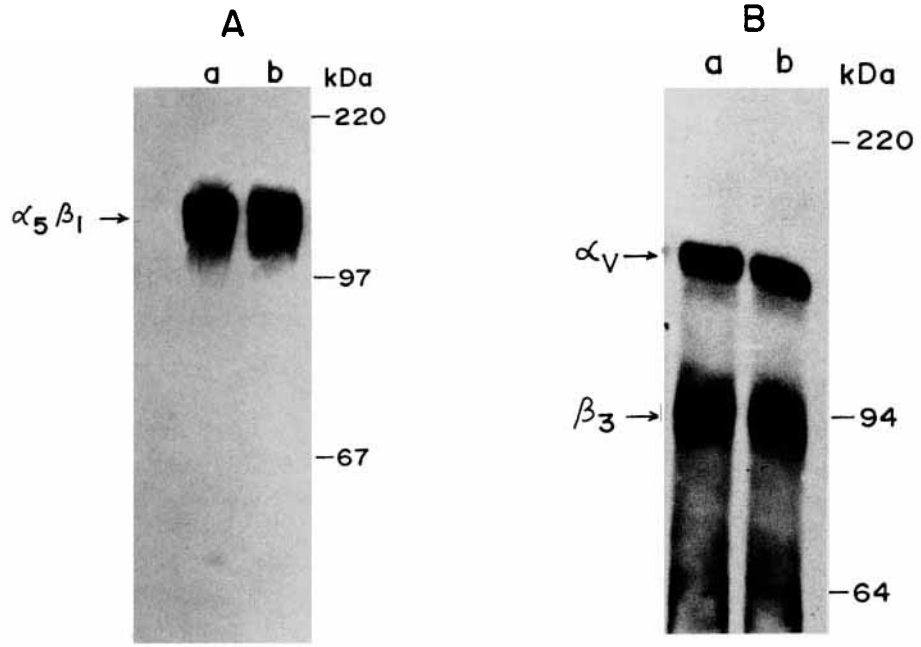

Fig. 4. RIPA lysates of mitotic and interphase cells were immunoprecipitated with (**A**) α5β1 and (**B**) αVβ3 antibodies, electrophoresed under reducing conditions, and then Western blotted with the same antibody as described in Materials and Methods. In both A and B, *lane a* represents interphase cells and *lane b* mitotic cells.

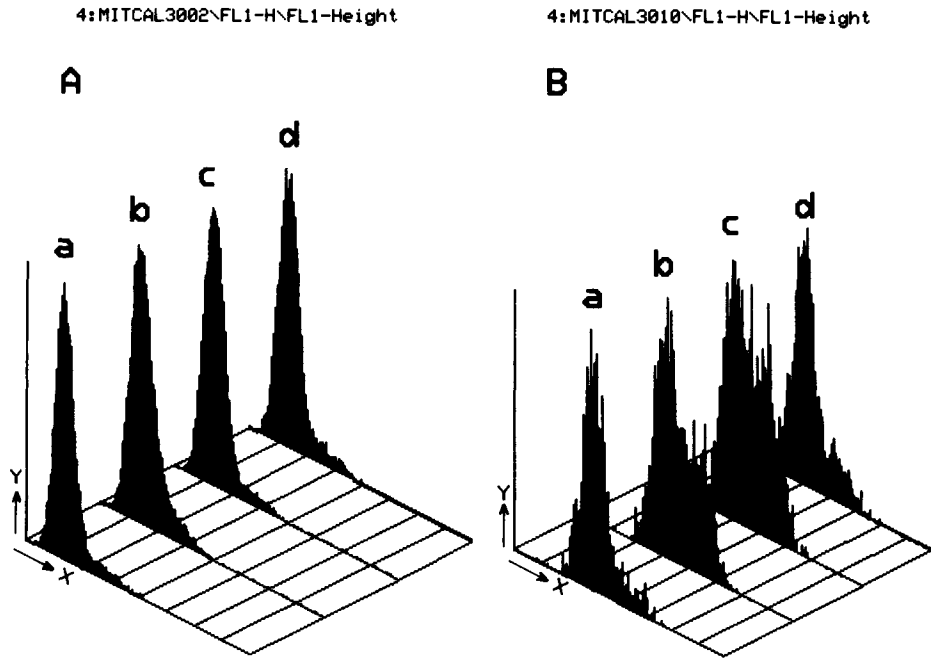

Fig. 5. FACS analysis of interphase (A) and mitotic cells was done after labeling them with calcium-sensitive dye, Fluo-3, as described. The calcium-specific fluorescence is shown on the X axis, and the Y axis represents the number of cells in each recording. In both A and B, *a* shows the basal level of fluorescence, and *b–d* show fluorescence of cells up to 5 min after the addition of VN, -GRGDSP-, and FN, respectively.

TABLE II. Interphase and Mitotic Cells Analyzed by Flow Cytometry for Their Ability to Mobilize Calcium in the Intracellular Pool as Described*

Cell treatment	% positive cells	
	Interphase cells	Mitotic cells
Control (untreated)	12	20
Vitronectin (10 μg/ml)	18	46
Fibronectin (25 μg/ml)	12	24
RGDS (10 μg/ml)	11	45

*The percent positive cells indicates the number of cells which exhibit higher than baseline fluorescence after the addition of ligands, as shown in Figure 5.

We also confirmed these results by staining mitotic and interphase cells with Fluo-3 and Hoechest 33342 simultaneously followed by FACS analysis. We found that mitotic cells (with 4 N DNA) showed a clear increase in calcium after VN treatment, but interphase cells did not show any significant changes in the level of calcium after VN treatment (data not shown).

In order to rule out possible artifacts that may have arisen in cells that were treated in suspension, we also studied the changes in intracellular calcium levels in cells that were attached to their substrata. Cells growing on coverslips were labeled with Fluo-3 and their calcium levels were monitored under a confocal microscope as described. The results from cells treated with VN and FN are shown in Figure 6A and B, respectively. After VN treatment the increase in calcium levels of mitotic cells is very clear, whereas in the FN treatment the mitotic cell does not show any higher increase than the other interphase cells.

DISCUSSION

Integrins have been reported to play a significant role in several functions, such as cell attachment, spreading, and migration on specific substrata of the ECM; control of cell differentiation and proliferation; tumor growth and metastasis; wound healing; cell–cell interactions; and others [Hynes, 1992]. Many of these functions are regulated by intracellular signals, generated as a result of the interaction between specific ECM proteins and their specific integrin receptors. Signaling from different types of integrins has been shown to cause different biochemical changes in the cytoplasm, e.g., the interaction of FN with the α5β1 receptor leads to the elevation of the intracellular pH and phosphorylation of FAK [Burridge et al., 1992; Lipfert et al., 1992; Guan et al., 1991; Schaller et al., 1992], whereas

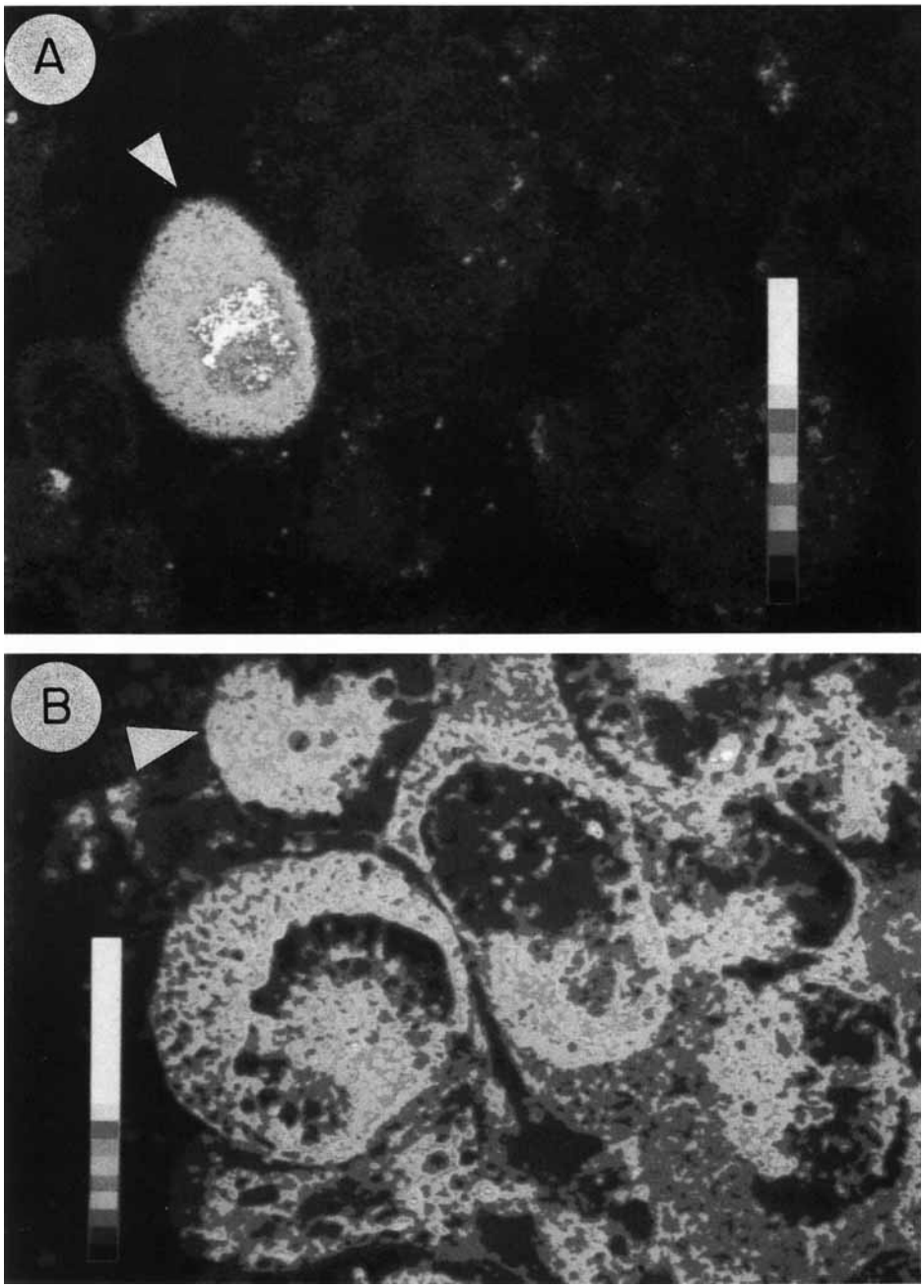

Fig. 6. Pseudocolor imaging done on a confocal image of fibroblasts stained with Fluo-3 and exposed to VN (**A**) or FN (**B**) for 90 sec. Arrowheads show the cells in mitotic phase. The pseudocolor bars are shown; red indicates the highest levels of calcium while dark blue indicates the lowest.

the interaction of VN with the αvβ3 causes a Ca^{2+} flux in the cells and the activation of protein kinase C (PKC) [Davies et al., 1993], IL-3, etc. [Bianchine, 1992].

Both types of interactions, viz., α5β1 to FN and αVβ3 to VN, also support the adhesion and spreading of different cell types [Chen et al., 1985; Singer et al., 1988; Felding-Habermann and Cheresh, 1993; Underwood and Bennet, 1989]. These observations indicate that the biological consequences of the ECM–integrin interactions are dependent on the cell type as well as their stage of differentiation.

A comparitive assessment of ECM–integrin interactions on the same cell type would provide better insights in the overall response of cells to

different ECM proteins. Mitotic cells offer a good model system to compare these functions of integrins because these cells show reduced adhesion to specific substrata and also are very responsive to extracellular signals [Dunphy, 1994]. It was in this context that we studied the adhesive potential of interphase and mitotic cells to FN and VN through the α5β1 and αVβ3 integrins, respectively, and compared the potential of these interactions to modulate the levels of intracellular Ca^{2+}.

The fourfold reduction in the adhesion potential of mitotic cells to FN, and to the CBD of FN, in comparison to interphase cells (Fig. 1) reflects a loss of α5β1 activity in the mitotic cell surface. This loss was probably due to the significant reduction in the level of α5β1 integrin from the surface of the mitotic cells in comparison to interphase cells as shown by our results on the cell surface levels of α5β1 using several protocols: immunoprecipitation of surface-iodinated cell lysate (Fig. 2), flow cytometric analysis (Fig. 3), and cell ELISA using anti-α5β1 antibodies (Table I) of mitotic and interphase cells. We also noticed the associated reduction in the FN protein on the surface of these cells as shown by cell ELISA (not shown) and FACS analysis (Fig. 3D). A similar loss of cell surface FN and the α5β1 integrin has been reported in the transformed cells as well [Plantefaber and Hynes, 1989; Chen et al., 1984; Hynes, 1981]. It has been shown that after oncogenic transformation, the changed morphology of cells is associated with loss of cell surface FN and α5β1 integrin, and it is restored by the addition of exogenous FN [Hynes and Wike, 1975; Yamada et al., 1976; Ali et al., 1977]. The significance of the rounding up, and the associated loss of FN from the surface of mitotic cells, in comparison to the well spread phenotype and normal levels of cell surface FN during interphase, could also be assessed in the same light. An interesting observation about the reduced levels of α5β1 on the cell surface was that the total cellular level (i.e., surface and cytoplasmic) of the protein remained the same, as evidenced by Western blotting of the immunoprecipitate of total cell lysates with the α5β1 antibody (Fig. 4).

Vitronectin is the other major matrix protein present in serum and substrata of culture vessels [Preissner, 1991]. VN promotes the adhesion of many cell types, and the major receptors for VN on the cell surface are αVβ3, αIIbβ3, and αVβ1 [Felding-Habermann and Cheresh, 1993]. In our attachment assays, it was found that mitotic cells adhered to VN at least twice as much more than interphase cells, especially at the lower concentrations (Fig. 1), although the adhesion to VN was much less than that to FN. Immunoprecipitation of the surface-iodinated mitotic and interphase cells and flow cytometric analysis with anti-αVβ3 antibodies revealed that the αVβ3 integrin level is higher on mitotic cell surface than on interphase cell surface, although the difference was not as dramatic as for the α5β1 integrin; this was especially true for the amounts of the αV chain. Western blotting of the total cell lysates showed that the total amounts of αV and β3 chains remain unchanged in both mitotic and interphase cells (Fig. 4). The higher levels of the αVβ3 integrin on *mitotic* cells is supported by higher levels of vitronectin present on these cells, as shown by cell ELISA and FACS analysis (Fig. 3).

While using different protocols for the measurement of integrin or FN/VN levels on the cells, both while attached to their substrata as well as in suspension, the levels of α5β1 integrin and FN on interphase cells and αVβ3 integrin and VN on mitotic cells were higher. However, in quantitative terms, different protocols showed variation in the contents. Thus it is difficult to precisely quantitate the differences in the levels of α5β1 and αVβ3 integrins and their corresponding ligands, but their qualitative increase and/or decrease in the respective cell type is clear.

From our results it appears that the loss of the α5β1 integrin from the surface of the mitotic cells is primarily responsible for their reduced adhesion of the cells to their substrata. The significance of elevation of the αVβ3 integrin was not clear. Based upon reports available earlier that interaction of VN with its receptors causes an increase in the intracellular Ca^{2+} in a number of cell types, we studied the response of mitotic and interphase cells to VN binding by a two-color FACS analysis. As can be seen in Figure 5, cells arrested in mitosis showed an increase in the intracellular Ca^{2+} after binding to VN and -RGD- peptides, but FN failed to cause any change; interphase cells, on the other hand, did not respond to any of the ligands. Schwartz [1993, 1994] has reported that calcium fluxes could occur in cells via both αVβ3 and αVβ1 integrins. In the rat fibroblasts, however, the total quantity of the β1 chains on the mitotic cell surface is minimal (Fig. 2, lane c), therefore the role of αVβ1 in the increase of intracellular

calcium can be ruled out. The low response of interphase cells could be due to the lower levels of the αV chains or also because of a functional modification. It has been reported earlier that the level of intracellular Ca^{2+} increases when the cells transit from G2 to mitosis [Means, 1994]. Increased Ca^{2+} levels have been shown to activate the cdc2 kinase, which is vital for driving the cells through mitosis, involving a calmodulin-dependent kinase called CAM-II kinase [Lu and Means, 1993]. The role of cell–VN interaction in the regulation of intracellular Ca^{2+} therefore could be very important in the progress of cells from G2 to mitosis.

One of the more interesting observations of our work is that although we observed marked differences in the cell surface level of α5β1 and αVβ3 integrins on mitotic and interphase cells, the total cellular amounts remained the same. Such a situation can arise out of two possibilities: (1) differences in the post-translational modification of the two integrins, α5β1 and αVβ3, can bring about differences in partitioning between the plasma membrane and the cytoplasm; (2) the level of α5β1 integrin can be lowered by its endocytosis during the G2/M transition and, subsequently in the postmitotic phase, restored by exocytosis. Such endocytosis of the α5β1 integrin has been reported under specific conditions [Bretscher, 1989; Altankov and Grinnel, 1995]. Keeping in line with the first possibility, Pomies and Block [1992] have reported that there are no differences in the cell surface levels of the α5β1 integrin in mitotic and interphase cells, but marked changes are seen in the adhesion of mitotic cells to FN. Similar findings have been noticed by Hynes (personal communication). Loss of affinity of the α5β1 integrin has been reported by the phosphorylation of the cytoplasmic ends of these chains [Aneskievich et al., 1991]. In the case of the rat fibroblasts undergoing mitosis, such post-translational modification of the integrins could make them incompetent for binding FN.

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