Journal of Cellular Biochemistry 77:517–528 (2000)

ARTICLES

Modulation of α5β1 Integrin Functions by the Phospholipid and Cholesterol Contents of Cell Membranes

P. Gopalakrishna, S.K. Chaubey, P.S. Manogaran, and Gopal Pande*

Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

Abstract Several modifications of the α5β1 integrin, which alter its intracellular and extracellular interaction with fibronectin and other proteins, have been reported. However, the significance of the lateral mobility of integrin molecules in the plasma membrane, as a regulator of their distribution and function, is poorly understood. We examined this problem by increasing the cholesterol content of plasma membranes, and consequently modifying the fluidity of membrane phospholipids, in rat fibroblasts. Under these conditions, the clustering of α5β1 integrin molecules in focal adhesions, their adhesion to the cell-binding domain of fibronectin, and their association with the cytoskeletal protein talin were significantly enhanced as compared to control cells. However, the activation of MAP-kinase pathways by the association of fibronectin with α5β1 integrin, and its association with integrin-linked kinase (ilk), were suppressed. The treated cells also showed distinct changes in shape, and their actin stress fiber network was more dense and thick as compared to control cells. The changes in fluidity of phospholipids occurred differentially and fluidity of phosphatidyl-ethanolamine increased, while that of phosphatidyl-choline was reduced. Our results suggest that proteins in focal adhesions could be partitioned in specific lipid domains, which regulate specific aspects of α5β1 integrin functions. J. Cell. Biochem. 77:517–528, 2000.

Key words: cell adhesion; cell signaling; cytoskeleton; membrane fluidity

The regulation of integrin functions is possible in various ways: phosphorylation of tyrosine and serine residues in the cytoplasmic tails of β and α subunits and associated molecules, glycosylation of the extracellular amino acids, and association of integrin subunits with divalent cations and with other cytoplasmic or membrane proteins have all been documented as important modifiers of integrin function [Giancotti and Ruoslahti, 1999; Hughes and Pfaff, 1998]. Different types of integrins exhibit different modifications and, therefore, regulate their properties in a cell-type specific manner [Aplin et al., 1998]. One of the properties of α5β1 integrin molecules, particularly well-exhibited in tissue culture cells, is to aggregate at specific positions in the plasma membrane and support cell-anchorage by interaction with extracellular matrix (ECM) proteins outside the cells, and with cytoskeletal proteins inside the cells. These positions are called "focal adhesion complexes" or "focal contacts," and the interaction of integrins with other proteins at these points plays an important role in the regulation of cell architecture and other structural features of the cell [Burridge and Chrzanowska-Wodnicka, 1996; Bershadsky et al., 1996; Jockusch and Rudiger, 1996; Aplin et al., 1998; Ingber, 1997]. The importance of integrin-lipid crosstalk in the formation of focal contacts is at present poorly understood.

The lateral movement of integrins in the plasma membrane and their interaction with other proteins during the process of cell attachment and spreading are necessary for the formation and stability of focal contacts [Plopper and Ingber, 1993; Burridge and Charzanowska Wodnicka, 1996]. It has also been shown that movement and clustering of other membrane proteins, such as N-cadherin, Fc receptor, and MHC antigens, occur when cells make contact with components of the extracellular matrix or the membrane of another cell [Katz et al., 1998;

*Correspondence to: Gopal Pande, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India. E-mail: gpande@ccmb.ap.nic.in

Received 6 September 1999; Accepted 5 November 1999

Kunz et al., 1996; Weir and Edidin, 1986; McCloskey and Poo, 1986]. The redistribution of integrin molecules in the plasma membrane has also been reported: for example, the α5β1 integrin was shown to be more laterally mobile in cells that locomote as compared to cells that are stationary [Duband et al., 1988]. Similarly, the lateral mobility of LFA-3 molecules (in artificial lipid bilayers coated on glass surfaces) was shown to be important for the binding of these molecules to cells that were expressing the CD2 molecule, the natural ligand of LFA-3 [Chan et al., 1991]. These studies demonstrated the importance of the lateral mobility of integrin molecules for their adhesive function; however, they did not provide insights into the intracellular phenomena that this property of integrins affected. Miyamoto et al. [1995, 1996] showed that clustering of integrins by antibodies, or the ligand, was capable of inducing intracellular signaling pathways, but the role of the fluidity of the membrane lipids in the process was not discussed.

The lipid bilayer organization and function in membranes are dependent on the interplay between the constituent proteins and phospholipids [Schroeder et al., 1991]. It was predicted and subsequently demonstrated that plasma membranes are composed of patchwork structures, referred to as membrane domains, whose boundary and internal constitution are defined by, and decisive for, the nature of lipids and proteins therein [Edidin, 1997]. The incorporation of cholesterol in animal cell membranes adds further to the complexity of the organization of these domains, as it can either increase or decrease the lateral mobility of the phospholipids in the membrane, depending upon the phospholipid microenvironment [Yeagle, 1985; Schroeder et al., 1991; Liscum and Underwood, 1995; Gimpl et al., 1997]. It has also been shown that the lateral mobility of different phospholipids in the plasma membrane is affected differently by cholesterol [Julien et al., 1993]. Recently it was shown that the membrane caveolae-associated protein, caveolin, plays a role in integrin-associated adhesion and signaling [Wei et al., 1999]. Since caveolin's distribution in the membrane is strongly regulated by the membrane lipids, it is possible that membrane lipids regulate integrin function also.

In this report, we exploited the effects of plasma membrane cholesterol levels on the fluidity of phospholipids in the membrane, and studied the properties of α5β1 integrin under conditions of altered phospholipid fluidity. Elevation of membrane cholesterol levels was achieved by incorporation of exogenous cholesterol in the plasma membranes of rat fibroblasts; these cells were subjected to biochemical, microscopic, and immunochemical investigations. We found a significant increase in the adhesion of treated cells to fibronectin matrices, although there was no difference in the quantity, or phosphorylation, of α5β1 integrin molecules on the cell surface. After spreading on the substratum, the treated cells looked much more stretched and flat in appearance as compared to the control cells, and the localization of talin and α5β1 integrin in the focal contacts was significantly increased. These cells also showed a much denser network of actin stress fibers than the control cells.

Significantly, activation of MAP-kinase after attachment to fibronectin, and the association of the integrin with integrin-linked kinase (ilk), were reduced. Measurement of the lateral mobility of fluorescently tagged phosphatidylethanolamine (PE) and phosphatidyl-choline (PC) molecules in the plasma membrane, by fluorescence recovery after photobleaching (FRAP), showed that PE fluidity was about fivefold higher, but PC fluidity was about twofold lower, in cholesterol-treated cells as compared to untreated cells. In summary, we have shown that increased membrane cholesterol levels result in the increased interaction of α5β1 integrin with fibronectin and cytoskeletal proteins but reduced potential for cell signaling, and we propose that these changes in integrin behavior are associated with the domain-specific changes in phospholipid fluidity caused by cholesterol.

MATERIALS AND METHODS

Cell Lines and Chemicals

All experiments were done with a normal rat fibroblast cell line, F111. Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin, and streptomycin (GIBCO Life Technologies, Gaithersburg, MD). For the purpose of microscopy, cells were grown on glass coverslips under the same conditions. All experiments were done within the first five passages after revival of cells from

cryopreservation. Anti-α5 integrin polyclonal antibody directed against the cytoplasmic domain of α5 chain was a gift from Prof. Richard Hynes; anti-ilk monoclonal antibody was provided by Dr. S. Dedhar; the MAP-kinase assay kit was from New England Biolabs (Beverly, MA); rhodamine-labeled phalloidin, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycerophosphoethanolamine (NBD-PE), and di-phenyl-hexatriene (DPH) were from Molecular Probes (Eugene, OR); tissue culture grade cholesterol, cholesterol oxidase, and all other chemicals for estimation of cholesterol, and anti-talin antibodies were from Sigma (St. Louis, MO); the Western blot chemiluminescence detection kit was from Boehringer (Mannheim, Germany). All other buffers were prepared from analytical grade reagents available from standard companies.

Cholesterol Incorporation and Estimation in Plasma Membranes

Cholesterol was incorporated in the cells using the protocol of Arbogast et al. [1976]. Briefly, exponentially grown cells were washed with plain DMEM and further incubated with plain DMEM for 12 h in the same conditions as described above. After 12 h, in one set of cells the DMEM was replaced with DMEM containing 1.25 μg/ml, 2.5 μg/ml, 5.0 μg/ml, or 10.0 μg/ml of tissue culture grade cholesterol. All cells were further incubated for 12 h, after which they were processed for various studies. Thus, all our studies were done with three types of cells: control untreated cells (cells incubated in DMEM + 10% FCS throughout the experiment), starved cells (cells incubated in plain DMEM throughout the experiment), and treated cells (cells grown for 12 h in plain DMEM followed by DMEM containing different quantities of cholesterol).

Estimation of membrane cholesterol was done according to cholesterol oxidase method of Hartel et al. [1998]. In this method, sonicated cell preparations are incubated with exogenous cholesterol oxidase. The method is based upon the principle that >95% of cytoplasmic cholesterol is in the form of cholesterol ester, and therefore cholesterol oxidase does not react with it; thus, all activity of cholesterol oxidase is on the cholesterol associated with plasma membrane. The action of cholesterol oxidase converts all the membrane cholesterol to cholest-4-ene-3-one, which is estimated by the measurement of fluorescence emission in a spectrofluorimeter with λ_{ex} =325 nm and λ_{em} = 415 nm. The quantity of cholest-4-ene-3-one produced in the reaction reflects the quantity of cholesterol present in the plasma membrane.

Cell Adhesion Assays

These were performed as previously described [Anilkumar et al., 1996]. Briefly, cells were detached form their substrata in low concentrations of trypsin in the presence of trypsin inhibitor and washed thrice with cold PBS, and 1×10^5 cells were plated in serum-free DMEM. Adherent cells were estimated after 1 h by the MTT assay.

Immunoprecipitation and Western Blotting

Cell lysates for immunoprecipitation were prepared in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, aprotinin 2 μg/ml, leupeptin 2 μg/ml, and 1 mM PMSF). For Western blotting, lysates were prepared in NP40 buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1.0% NP40, aprotinin 2 μg/ml, leupeptin 2 μg/ml, 1 mM PMSF, and 0.02% sodium azide). The reactions were done as follows: a) integrin immunoprecipitation; equated cell lysates in RIPA buffer were treated with the anti-α5 polyclonal antibody and the immunoprecipitates were analyzed as described earlier [Anilkumar et al., 1996]; b) association of talin with the α5β1 integrin; immunoprecipitation of the α5β1 integrin was done as above, and the immunoprecipitates were Western-blotted using the anti-talin monoclonal antibody (Sigma); the blotted antibody was detected using the chemiluminescence detection kit; and c) expression and association of ilk with integrins; for the total expression of ilk, cell lysates were prepared in RIPA buffer and immunoprecipitated and blotted with the anti-ilk antibody; for the estimation of ilk associated with integrin, RIPA lysates were immunoprecipitated with anti-α5 polyclonal antibody and blotted with anti-ilk antibody [Hannigan et al., 1996]. The blots were detected using a chemiluminescence detection kit.

MAP-Kinase Assays

One million cells in DMEM were plated in triplicates on surfaces (one well each of a six-well tissue culture plate) coated with 5 μg/ml

fibronectin, and lysates were prepared after 15 min. The activation of MAP-kinase enzyme was detected using the ingredients of the MAP-kinase detection kit as described by the manufacturers (New England Biolabs).

Staining of Cells and Microscopy

The morphology of cells was studied by phase contrast microscopy, using unfixed cells. Cells were photographed with a Nikon inverted microscope at a magnification of 400×, using a phase contrast lens.

For immunostaining with anti-integrin and anti-talin antibodies, cells were grown on glass coverslips and the cells fixed in 3% formaldehyde at room temperature for 30 min. The cells were washed thrice with PBS and treated with chilled acetone (at −70°C) for 3 min. Cells were then washed in PBS and incubated with either anti-α5 polyclonal or anti-talin monoclonal antibody at 4°C for 1 h. The cells were washed in PBS and then incubated with TRITC-labeled anti-rabbit IgG (for the integrin antibody) or FITC-labeled anti-mouse IgG (for the anti-talin antibody) at 4°C for 30 min. For visualization of the actin stress fibers, the acetone-permeabilized cells were incubated with rhodamine-labeled phalloidin for 30 min at room temperature and washed in PBS. All the stained cells were mounted on a microscope slide in 50% glycerol and visualized under a confocal micrscope (Meridian-Ultima), using suitable filters and a 40× Plan-Apochromatic objective lens. Optical sectioning of cells was done as per standard procedure, and sections of 0.5 μm thickness, corresponding to 1.0–1.5 μm from the adhesive substratum of the cells, were analyzed and displayed for integrin and talin localization; for the actin stress fibers, cell sections only from the middle of individual cells were analyzed.

Fluorescence Recovery After Photobeaching (FRAP) Analysis

Cells grown on coverslips (in a subconfluent condition) were stained with 20 μg/ml NBD-PE or 5 μg/ml NBD-PC in plain DMEM by (prepared by diluting the 1 mg/ml stock solutions made in 99% ethanol) at 37°C in 5% CO_2 for 15 min, after which the cells washed in plain DMEM and mounted on a glass slide in plain DMEM. Cells were subjected to FRAP analysis within 15 min after staining was over, and recovery time of NBD fluorescence was determined using the software provided by the manufacturers of the confocal microscope (Meridian). At least 25 recordings for fluorescence recovery were done from a minimum of five cells from each cell sample. Lateral mobility of PE molecules was calculated as average of all the recordings from a particular cell type.

DPH Fluorescence Anisotropy

Measurement of membrane fluidity was done by estimating fluorescence polarization of 1,3 diphenyl, 1,3,5 hexatriene (DPH), using the method of Revathi et al. [1994]. Cells, while they were still attached to their substrata, were incubated at 37°C with 1 μM DPH (final concentration) in DMEM for 20 min. Unincorporated dye was removed by repeated washings with plain DMEM. Labeled cells were suspended at a concentration of 2×10^6/ml in PBS, and fluorescence of the incorporated dye was measured at 360 nm (λ_{ex}) and 430 nm (λ_{em}) at room temperature, with constant stirring of cells, using a Hitachi F-4000 spectrofluorimeter, equipped with polarizer attachment. Fluorescence anisotropy values were calculated using the equation

$$P = (I_{VV} - GI_{VH}/I_{VV} + GI_{VH})$$

where I_{VV} and I_{VH} are fluorescence intensities observed with the emitted light polarizer in vertical or horizontal position, respectively, with respect to the excitation polarizer. G is the grating correction factor and is equal to I_{HV}/I_{HH}.

RESULTS

Changes in Cell Shape

After the increase in cholesterol levels of plasma membranes, F111 cells exhibited some remarkable changes in cell shape. These changes in cell morphology are shown in Figure 1. All cells were photographed at the same magnification; cholesterol-treated cells appear more elongated and stretched in comparison to untreated or serum-starved cells. This change in cell shape was best visualized in cells treated with 5.0 μg/ml cholesterol, where the effect was seen in a majority of cells. At higher concentrations (10 μg/ml), cell toxicity was seen; at lower concentrations (1.25 and 2.5 μg/ml), changes in cell morphology were less pronounced.

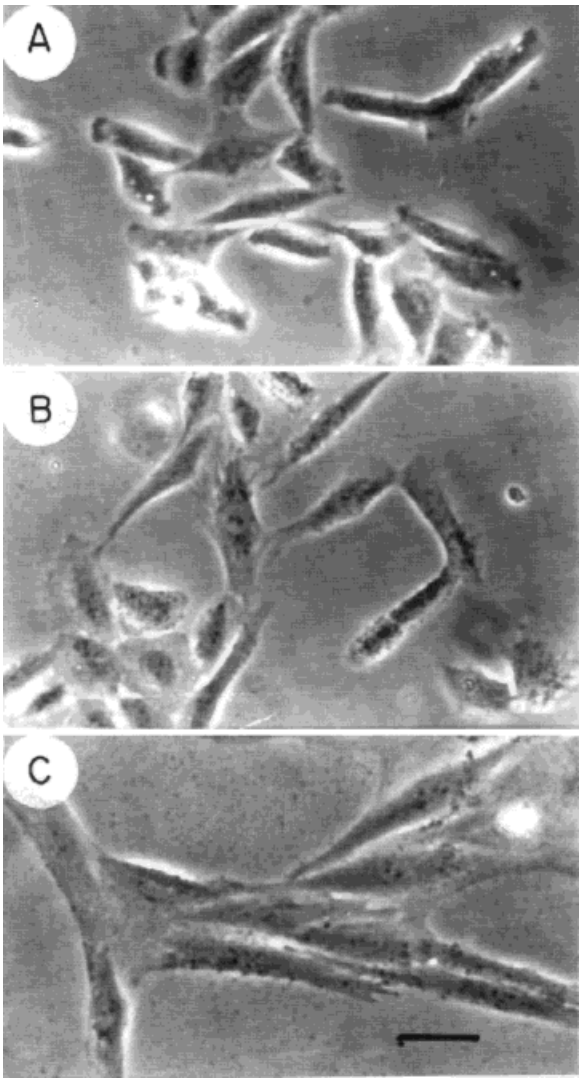

Fig. 1. Phase-contrast pictures of F111 cells, showing morphology of cells before and after treatment with cholesterol. **A:** Cells incubated in DMEM and 10% FCS. **B:** Cells that have been incubated in plain DMEM for 24 h. **C:** Cells incubated first in plain DMEM for 12 h, followed by 12-h incubation in 5 μg/ml cholesterol in plain DMEM. All photographs are at same magnification; bar, 2 μm.

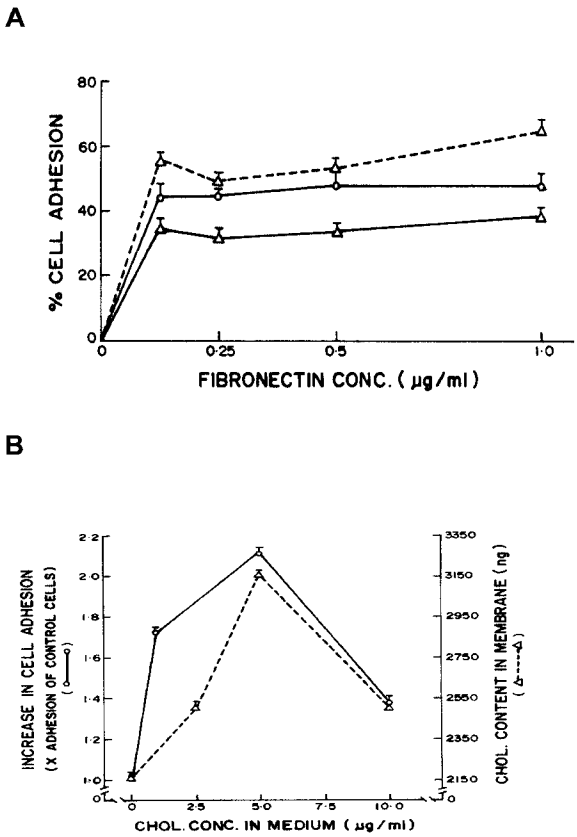

Fig. 2. Effect of membrane cholesterol levels on adhesion of cells to the cell-binding domain of fibronectin. **A:** Comparison of adhesion of untreated, normally grown cells (open triangles with solid line), serum-starved cells (open circles), and cholesterol (2.5 μg/ml)-treated cells (open triangles with dashed line). X-axis shows the concentration of 120-kDa fragment of fibronectin coated on the wells. Y-axis shows percent of total cells plated adhering to the well after 1 h. Each point shows the results from the average of cell adhesion in three wells. **B:** Correlation between levels of membrane cholesterol and cell adhesion of fibronectin. Membrane cholesterol levels were estimated as described after incubation of cells with different concentrations of cholesterol in DMEM, and the effect of treatment of these cells on adhesion to fibronectin was measured as fold increase over the adhesion of untreated cells.

Membrane Cholesterol Concentration and Cell Adhesion to Fibronectin

We correlated effect of increase in cholesterol content of membranes with their potential to adhere to the cell-binding domain of fibronectin. The results are shown in Figure 2. As can be seen in Figure 2A, the adhesion of cholesterol-treated cells is higher than in cells that were grown in complete medium or under serum-deprived conditions. In Figure 2B, the correlation of the actual concentration of membrane cholesterol and cell adhesion is shown. The data are plotted as a function of cholesterol concentration in the medium. As can be seen, both cell adhesion and level of membrane cholesterol rise until the concentration of cholesterol in the medium is under 5 μg/ml. At that concentration, the rise in membrane cholesterol levels is about 1.5-fold, while the rise in cell adhesion is about 2-fold as compared to cells where no cholesterol treatment was given. However, with 10 μg/ml cholesterol, there was a drop in the incorporation of cholesterol in

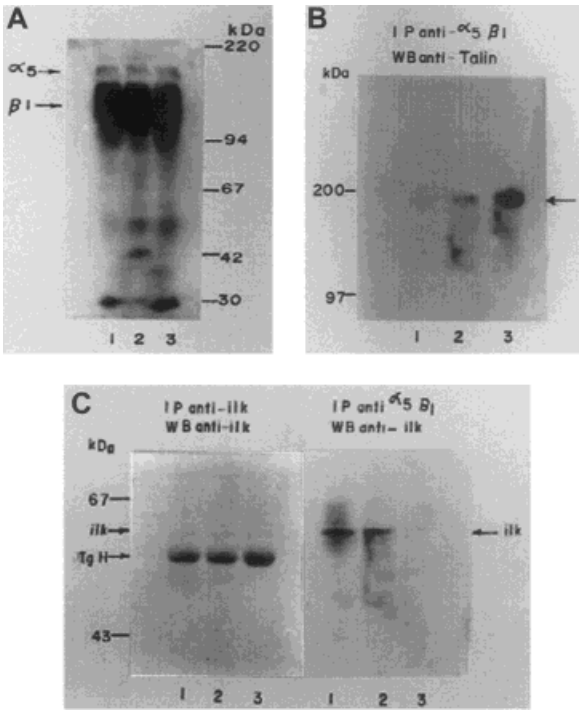

Fig. 3. Expression of α5β1 integrin (**A**) and its association with talin (**B**) and ilk (**C**). **Lanes 1–3** represent untreated, serum-starved, and cholesterol-treated cells, respectively. Cell lysates, containing equated protein contents, were immunoprecipitated with polyclonal anti-α5 antibody, as described, in all except C, left, where anti-ilk antibody was used for immunoprecipitation. In A, the immunoprecipitates were Western-blotted as such with the same anti-α5 antibody that was used for immunoprecipitation. In B and C, the immunoprecipitates were equated for integrin content and blotted with anti-talin monoclonal (B) or anti-ilk monoclonal (C) antibodies. IP, immunoprecipitating antibody; WB, Western blotting antibody.

membranes and also in the adhesion of cells to fibronectin. Based on these results, we decided to do all experiments with 5 μg/ml cholesterol in the medium for all other experiments.

Expression of α5β1 Integrins in Cells

Considering the differences in adhesion to fibronectin between cell types, we compared the levels of integrin expression in cells. The results are shown in Figure 3A. No differences could be seen in the integrin content of these cells. It was noteworthy that proteins from all the cell types were solublized using RIPA buffer while the cells were attached to the substratum, so that any artifactual difference in integrin levels due to the insolubility of integrins, especially from the focal contact regions, was avoided. We also checked for the tyrosine and serine phosphorylation of these integrins, and no detectable phosphorylation of either residue could be seen in any of the cells (data not shown).

Association of Talin and Ilk With α5β1 Integrin Molecules

We examined the effect of cholesterol treatment of cells on the association of talin (a cytoskeletal protein) and the cytoplasmic enzyme known to associate avidly with the cytoplasmic domain of α5β1 integrin molecule [Hanningan et al., 1996]. Cell lysates prepared in RIPA buffer were immunoprecipitated with the anti-α5 polyclonal-antibody, and the immunoprecipitates were analyzed by Western blotting with either anti-talin (Fig. 3B) or the anti-ilk monoclonal antibody (Fig. 3C, right). It can be seen that the quantity of talin associated with the α5 immunoprecipitate from cholesterol-treated cells is significantly higher than that seen in the control or starved cells (Fig. 3B). On the other hand, although the expression of ilk in all three cell types is equal (Fig. 3C, left), its association with α5β1 integrin is significantly less in cholesterol-treated cells as compared to the control and serum-starved cells (Fig. 3C, right). These results put together suggest that an increase in membrane cholesterol level facilitates the partitioning of integrin molecules in the membrane that allows the association of only talin with the integrin molecules and excludes ilk. The correlation of loss of ilk binding to the α5β1 integrin molecules in cholesterol-treated cells, and their higher adhesion potential to fibronectin, was consistent with the observations made earlier about the role of ilk in the regulation of cell adhesion [Hanningan et al., 1996].

Activation of MAP Kinase

Activation of MAP-kinase after adhesion of cells to fibronectin was essentially done according to the procedure described by Chen et al. [1994]. All the reagents for the assay were used from the MAP-kinase assay kit of New England Biolabs. The results of the assay are shown in Figure 4. The activation of the enzyme can be clearly seen in control and starved cells, although it is not very robust (compare with the control lane). However, no activity was seen in the cells treated with cholesterol (Fig. 4, lane 3). This clearly shows that the

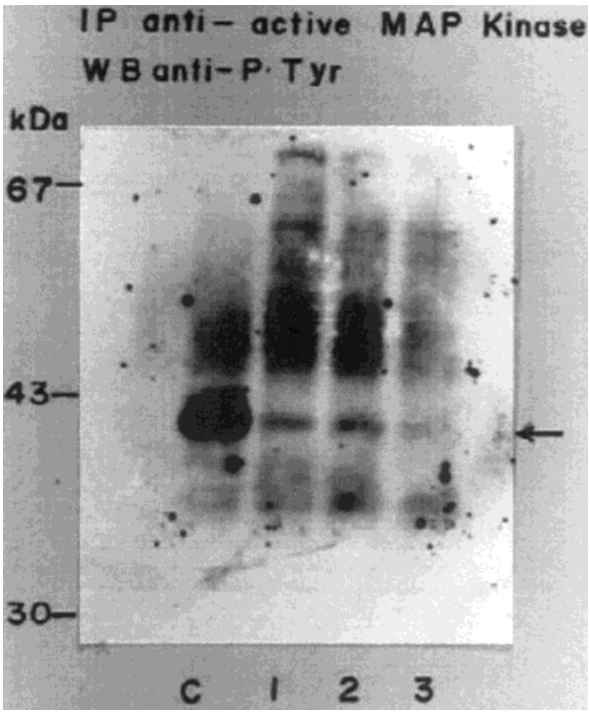

Fig. 4. Activation of MAP-kinase after adhesion of cells to FN. Cholesterol-treated/untreated cells were plated on FN-coated surfaces for 10 min, and MAP-kinase activity (arrow) was estimated as described in Materials and Methods. The control activity of the enzyme is shown in **lane C**; untreated, serum-starved, and cholesterol-treated cells are shown in **lanes 1–3**, respectively. IP, immunoprecipitating antibody; WB, Western blotting antibody.

transduction of signals for MAP-kinase activation through fibronectin-integrin interaction is impaired in cells with higher membrane cholesterol.

Organization of Focal Adhesions and Actin Cytoskeleton

We studied the morphological effects of cholesterol treatment and starvation of cells on the organization of focal adhesion complexes and actin cytoskeleton. For this purpose we stained the cells with anti-α5 and anti-talin antibody, and for the actin cytoskeleton organization with rhodamine-phalloidin, as described in Materials and Methods. The cells were examined by confocal microscopy. FITC and TRITC fluorescence was visualized using appropriate filters. The results are shown in Figure 5. The actin filament organization in the treated cells (Fig. 5C) was more prominent and dense in comparison to the control and starved cells (Fig. 5A,B, respectively); the latter two cell types did not show any major differences between each other. For the sake of clarity and to ensure that uniform intensity of fluorescence was observed, only single optical sections of all cells (each of 0.5 μm thickness) are displayed. The staining intensity of the anti-α5 and anti-talin antibodies in cholesterol-treated cells (Fig. 5F,I, respectively) was also much brighter as compared to the control and starved cells (Fig. 5D,G for control cells and Fig. 5E,H for starved cells). In all the cells only the most proximal section to the substratum has been shown, to highlight the fluorescence of the integrin and associated talin molecules. These results should be viewed in conjunction with those shown in Figure 3A,B, where it can be seen that levels of α5β1 integrin in all cells are the same; however, the associated talin is significantly higher in the cholesterol-treated cells. We analyzed the localization of these proteins by two-color immunofluorescence: the brighter staining for integrin and talin in cholesterol treated cells, in Figure 5, indicates that higher quantities of both these proteins are localized in the focal adhesion complexes of these cells as compared to the control or starved cells. It was of interest to us as to what facilitates the more efficient localization of integrin and talin molecules in the focal adhesions in cholesterol-treated cells as compared to untreated cells, and we therefore measured the lateral mobility of molecules in the plane of the membrane.

Lateral Mobility of Membrane Phospholipids

One of the most abundant molecules in the plasma membrane are the membrane phospholipids, and they exhibit both lateral and rotational movement in the lateral plane of the membrane. The lateral movement of the phospholipids governs the overall fluidity of the membrane bilayers and can be measured by using a fluorescently tagged phospholipid assay. We used the method of fluorescence recovery after photobleaching (FRAP) for measuring the mobility of NBD-PE and NBD-PC molecules, as described in Materials and Methods. Another criterion of movement of molecules in the membrane is the overall fluidity of membrane: the fluidity of membranes was measured by the fluorescence anisotropy of DPH, as described. The results of PE/PC lateral mobility, and membrane fluidity, are shown in Table I. As can be seen, the mobility of

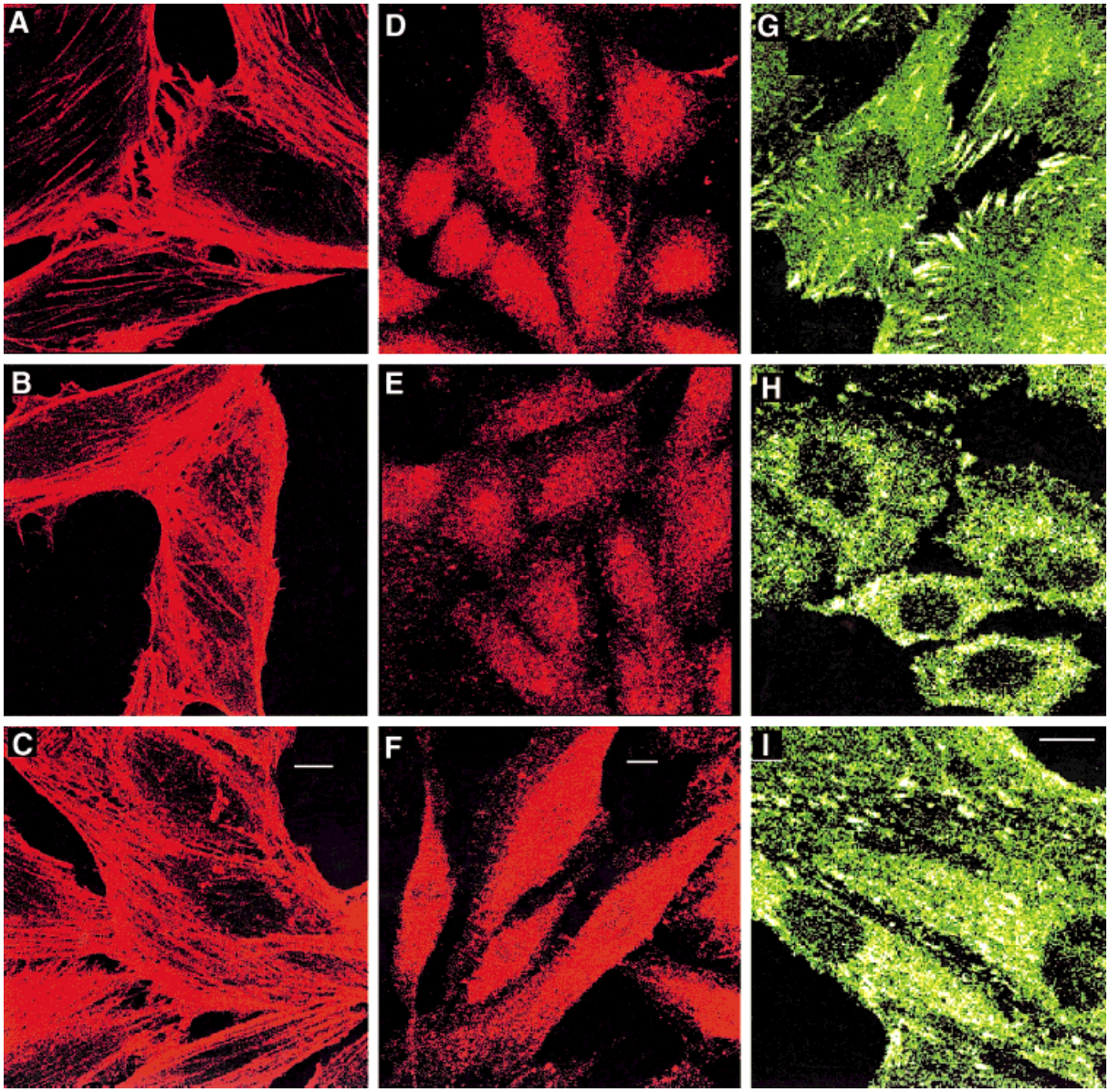

Fig. 5. Photomicrographs of cells stained with rhodamine-labelled phalloidin (**A–C**), anti-α5β1 antibody (**D–F**), and anti-talin antibody (**G–I**). Cells were plated on glass coverslips and allowed to adhere and spread. After 24 h they were further incubated with or without cholesterol-containing medium, as described. Cells were fixed in formaldehyde, followed by permeabilization with cold acetone (only for phallodin and talin staining), and then stained with the respective reagents. Normal untreated cells are shown in A, D, and G; serum-starved cells are shown in B, E, and H; and cholesterol-treated cells are shown in C, F, and I.

NBD-PE molecules increased while that of NBD-PC molecules decreased in cells with increased cholesterol. The figures given in Table I are averages from repeated measurements from one experiment; a similar difference between the mobility of the probes was seen in three independent experiments. The data for membrane fluidity have been averaged from five independent experiments. As can be seen, no significant effect of cholesterol was seen on the overall fluidity of membranes, either in serum-starved or high-cholesterol conditions.

DISCUSSION

The multifarious functions of integrins in cells are regulated by their numerous modifications by enzymes and their associations with other membrane and cytosolic proteins [Aplin

TABLE I. Lateral Mobility of NBD-PE, NBD-PC, and Fluorescence Anisotrophy of DPH in Normal and Cholesterol-Treated F111 Cells[a]

Cell type	NBD-PE mobility ($cm^2\ sec^{-1}$)	NBD-PC mobility ($cm^2\ sec^{-1}$)	Anisotrophy (P)[b]
Untreated	$4.266 \pm 0.68 \times 10^{-10}$ (14)	$6.60 \pm 4.60 \times 10^{-9}$ (22)	0.30 ± 0.041
Starved	$8.63 \pm 3.41 \times 10^{-10}$ (9)	$5.14 \pm 3.00 \times 10^{-9}$ (18)	0.32 ± 0.047
Treated (5.0 μg)	$21.18 \pm 5.62 \times 10^{-10}$ (13)	$1.99 \pm 0.86 \times 10^{-9}$ (6)	0.30 ± 0.038

[a]In parentheses, number of recordings done.
[b]Values are means of five observations.

et al., 1998]. In general, integrins can initiate a variety of signal transduction events, which include activation of serine/threonine kinases, alteration in cellular phospholipid and calcium levels, an increase in the tyrosine phosphorylation in a subset of proteins, etc. These events are associated with formation of large assemblies of molecules in the cell membranes, which can be best visualized by fluorescence microscopy. Focal contacts in the cell membrane contain a variety of structural proteins (such as talin, vinculin, and α-actinin), signaling molecules (such as ilk and focal adhesion kinase (FAK), and adapter molecules (e.g., paxillin, tensin, and P130cas). These proteins facilitate the linkage of integrin cytoplasmic tails to the actin cytoskeleton and the formation of focal contacts. This process can also be regulated by the actin cytoskeleton-organizing proteins, i.e., the Rho-family GTPases such as Cdc-42 and Rac1 [Aplin and Juliano, 1999; Barry et al., 1997].

In spite of our understanding of the importance of integrin-associated proteins in the formation and function of focal contacts, very little is known about the role of the lipid components of the plasma membrane in the process. A few reports have addressed this question and it has been shown, using liposomes, that the fluidity of membrane lipids affects the LFA-3-mediated binding of CD2 molecules on the cell surface [Chan et al., 1991] and the functions of α5β1 integrins in epithelial cells [Duband et al., 1988]. In the present paper, we studied the effect of an increase in membrane cholesterol content of α5β1 integrin distribution and function in rat fibroblasts. Our results show some remarkable changes in the behavior of integrin molecules under conditions of increased membrane cholesterol. The most obvious change is in the morphology of the cells, which appear elongated and stretched, and therefore flatter, and more adherent to FN matrices when membrane cholesterol is increased. These cells also show larger-sized focal adhesion points, which is possibly due to increased localization of integrin and talin molecules in these points as compared to untreated cells. We also saw a higher association of the integrin-linked kinase (ilk) with, and lowered activation of, MAP-kinase (Erk-1/Erk2) upon attachment to FN in cholesterol-treated cells. Since there is no change observed in the quantitative levels of these proteins, these changes could be facilitated by the indirect consequence of the altered lateral mobility of integrins and their associated molecules in the membrane, which could occur as a direct consequence of alteration in membrane cholesterol levels. The following aspects of alterations in integrin behavior, under increased membrane cholesterol conditions, need to be considered specifically.

Integrin Clustering and Binding to FN

The role of clustering of integrin molecules and its various effects on integrin mediated functions have been described earlier [Miyamoto et al., 1995, 1996]. Based on these reports, it is possible to delineate functions that are ligand-occupancy-mediated, vs. those that are clustering-induced. It is proposed that ligand occupancy, by itself, facilitates receptor targeting to focal contacts, and receptor clustering alone is capable of accumulating tensin and FAK and tyrosine phosphorylation of the integrin molecules. When both phenomena occur together, large immobile accumulations of integrins and cytoskeletal molecules are seen at focal contacts. Miyamoto et al. [1995, 1996] also suggested that due to the differential regulation of integrin function by these mechanisms, a single integrin molecule could function selectively in translocation, signaling, or

multiple cytoskeletal protein binding. In our study, we found a similar selective effect on integrin function subsequent to changes in membrane cholesterol levels. We have shown that higher membrane cholesterol levels lead to a stronger binding of α5β1 integrin to FN and also increased association of the cytoskeletal proteins talin and actin; however, the MAP-kinase signaling pathway (at erk1/erk2 level) is inhibited. In these conditions, integrin clustering is increased. These results suggest that the clustering/ligand binding capacity of integrin molecules can be influenced by the composition and organisation of membrane lipids and thus, besides the protein-protein interactions, the lipid molecules in the membrane also play a role in the clustering and FN binding by α5β1 integrins.

Another interesting aspect of our results is the decreased association of the integrin-linked-kinase-ilk with the α5β1 integrin in the cholesterol-treated cells. Since these cells show reduced binding to FN, our results corroborate the findings by Hannigan et al. [1996] that increased association of ilk with α5β1 integrins decreases their binding potential to FN.

MAP-Kinase Activation by Integrins

One of the most prominent aspects of integrin signaling is the activation of the MAP-kinase cascade [Aplin et al., 1998; Lin et al., 1997; Renshaw et al., 1997]. The involvement of the erk-1/erk-2 kinases in this process was documented early on [Morino et al., 1995]. More recently, it was shown that the activation of these kinases can be achieved through ras-dependent and ras-independent pathways and also through the rho family of proteins [Clark and Hynes, 1996; Barry et al., 1997; Clark et al., 1998; Aplin and Juliano, 1999]. In conditions of high membrane cholesterol, we find that activation of both erk-1 and erk-2 is suppressed. It is not possible to assign a specific role that cholesterol may play in the activation process; however, a recent report [Green et al., 1999] showed that cholesterol levels in membrane affect the assembly of signaling complexes, particularly for the αv integrins. Hence the deficiency in activation of erk1/erk2 in high-cholesterol cells could be due to a blockage in the assembly of the components of ras-dependent MAP-kinase activation.

Organization of the Actin Cytoskeleton

One of the obvious differences between cells with higher membrane cholesterol and those grown in normal or serum-free medium is that cells with high membrane cholesterol appear flatter and more stretched than their normal or serum-starved counterparts (Fig. 1). This observation correlated well with quality of actin cytoskeleton in these cells, i.e., they showed a much thicker and more prominent actin cytoskeleton in comparison to the normal and serum-starved cells (Fig. 5). Since it has been proposed that integrin clustering alone can influence the cytoskeletal organization of cells [Miyamoto et al., 1995; La Flamme et al., 1992], it is likely that the formation of integrin-cytoskeletal complexes is more stimulated in cells with higher cholesterol. The better association of talin with α5β1 integrin, and a more robust actin cytoskeleton in these cells, provide the evidence for this contention (Figs. 3B, 5). MAP-kinase activation, however, is dependent on both clustering, and efficient ligand binding does not show a similar increase.

Lateral Mobility of Molecules in the Membrane

According to the fluid-mosaic-model of membrane structure, one of the critical factors that govern the lateral motion of protein molecules in the plasma membrane is the "fluidity" of the lipid components [reviewed in Yeagle, 1985; Edidin, 1997]. The capacity of membrane lipids, especially cholesterol, to form microdomains or raft like structures in the membrane could play an important role in the regulation of integrin functions [Green et al., 1999; Edidin 1997; Varma and Mayor, 1998]. It has been shown that levels of membrane cholesterol can significantly affect membrane architecture and fluidity (lateral mobility) of phospholipids [Liscum and Underwood, 1995; Julien et al., 1993]. In our experiments, we found that increase in membrane cholesterol leads to a differential effect on the lateral mobility of NBD-tagged PE and PC molecules, as checked by FRAP experiments. Whereas the mobility of NBD-PE molecules increased, that of NBD-PC molecules decreased; interestingly, there was no significant difference in the overall fluidity of the plasma membranes, as shown by the fluorescence anisotropy of DPH (Table I). This indicates that the effect of increased cholesterol in the membrane is rather subtle on the mobility

of phospholipids. The influence of this change on mobility of phospholipids and on integrin clustering could either be direct, i.e., the integrin molecules occupy a more rigid or a more fluid region of the membrane, or it could mediated through the association of integrins with other proteins which are so regulated. The reported association of caveolin with integrins [Wei et al., 1999] suggests that the second possibility is more likely.

In summary, we have shown that increase in membrane cholesterol levels affects α5β1 integrin functions in many ways, and brings into focus the importance of membrane lipids in regulating selective aspects of integrin-mediated cell adhesion and signaling steps. Our results also show that it is possible to distinguish between signals induced by integrin clustering and those induced by ligand binding.

REFERENCES

Anilkumar N, Bhattacharya AK, Manogaran PS, Pande G. 1996. Modulation of α5β1 and αvβ3 integrins on the cell surface during mitosis. J Cell Biochem 61:338–149.

Aplin AA, Juliano RL. 1999. Integrin and cytoskeletal regulation of growth factor signalling to the MAP kinase pathway. J Cell Sci 112:695–706.

Aplin AE, Howe A, Alahari SK, Juliano RL. 1998. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules and selectins. Pharmacol Rev 50:197–263.

Arbogast LY, Rothblatt GH, Leslie MH, Cooper RA. 1976. Cellular cholesterol ester accumulation induced by free cholesterol rich lipid dispersions. Proc Natl Acad Sci USA 73:3680–3684.

Barry ST, Flinn HM, Humpheries MJ, Critchley DR, Ridley AJ. 1997. Requirement of Rho in integrin signaling. Cell Adhes Commun 4:14989–14994.

Bershadsky A, Becker E, Lyubimova A, Geiger B. 1996. Involvement of microtubules in the control of adhesion dependent signal transduction. Curr Biol 6:1279–1289.

Burridge K, Charzanowska-Wodinicka M. 1996. Focal adhesions contractility and signaling. Annu Rev Cell Dev Biol 12:463–518.

Chan PY, Lawrence MB, Dustin ML, Fergeuson LM, Golan DE, Springer T. 1991. Influence of receptor lateral mobility on adhesion strengthening between membranes containing LFA-3 and CD-2. J Cell Biol 115:245–255.

Chen Q, Kinch MS, Lin TH, Burridge K, Juliano RL. 1994. Integrin-mediated cell adhesion activates mitogen-activated protein kinases. J Biol Chem 269:31229–31233.

Clark EA, Hynes RO. 1996. Ras activation is necessary for the integrin-mediated activation of extracellular-signal regulated kinase-2 and cytosolic phospholipase A2 but not cytoskeletal organization. J Biol Chem 271:14814–14818.

Clark EA, King WG, Brugge JS, Symons M, Hynes RO. 1998. Integrin-mediated signals regulated by the members of the Rho family of GTPases. J Cell Biol 142:573–576.

Duband JL, Nuckolls GH, Ishihara A, Hasegawa T, Yamada KM, Theiry JP, Jacobson K. 1988. Fibronectin receptor exhibits high lateral mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar streaks in immobile cells. J Cell Biol 107:1385–1396.

Edidin M. 1997. Lipid microdomains in cell surface membranes. Curr Opin Struct Biol 7:528–532.

Giancotti F, Ruoslahti E. 1999. Integrin signaling. Science 85:1028–1032.

Gimpl G, Burger K, Fharenholz F. 1997. Cholesterol is modulator of receptor function. Biochemistry 36:10959–10974.

Green JM, Zhelesnyak A, Chung J, Lindberg FG, Sarafati M, Frazier WA, Brown EJ. 1999. Role of cholesterol in formation and function of a signaling complex involving alpha V beta 3, integrin-associated protein (CD47) and heterodimeric G proteins. J Cell Biol 146:673–682.

Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, Bell JC, Dedhar S. 1996. Regulation of cell adhesion and anchorage-dependent growth by a new ββ1 integrin-linked protein kinase. Nature 379:91–96.

Hartel S, Diehl HA, Flavio-Ojeda S. 1998. Methyl β cyclodextrins and liposomes as water soluble carriers for cholesterol incorporation into the membranes and its evaluation by a microenzymatic fluorescence assay and membrane fluidity sensitive dyes. Anal Biochem 258: 277–284.

Hughes PE, Pfaff M. 1998. Integrin affinity modulation. Trends Cell Biol 8:359–364.

Ingber DE. 1997. Tensegrity: the architectural basis of cellular mechano-transduction. Annu Rev Physiol 59: 575–599.

Jockusch BM, Rudiger M. 1996. Crosstalk between cell adhesion molecules: vinculin as a paradigm for regulation by conformation. Trends Cell Biol 6:311–315.

Julien M, Tournier JF, Tocanne JF. 1993. Differences in the transbilayer and lateral motion of fluorescent analogs of phosphatidyl choline and phosphatidyl ethanolamine in the apical plasma membrane of bovine endothelial cells. Exp Cell Res 208:387–397.

Katz BZ, Levenberg S, Yamada KM Geiger B. 1998. Modulation of cell-cell adherans junctions by the clustering of N-cadherin cytoplasmic tail. Exp Cell Res 243:415–424.

Kunz S, Ziegler V, Kunz B Sonderegger P. 1996. Intracellular signaling is changed after clustering of neural cell adhesion molecules axonin-1 and Ng-CAM during neuritic fasciculation. J Cell Biol 135:253–267.

La Flamme SE, Akiyama SK, Yamada KM. 1992. Regulation of fibronectin receptor distribution. J Cell Biol 117: 437–447.

Lin TH, Aplin AE, Shen Y, Schaller M, Romer L, Aukhil I, Juliano RL. 1997. Integrin-mediated activation of MAP-kinase is independent of FAK: evidence for dual integrin signaling pathways in fibroblasts. J Cell Biol 136:1385–1395.

Liscum L, Underwood KW. 1995. Intracellular cholesterol transport and compartmentation. J Biol Chem 270: 15443–15446.

McCloskey MA, Poo MM. 1986. Contact induced redistribution of membrane components: local accumulation and development of adhesion. J Cell Biol 102: 2185–2196.

Miyamoto S, Akiyama SK, Yamada KM. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science 267:883–885.

Miyamoto S, Teramoto H, Gutkind JS, Yamada KM. 1996. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP-kinase activation: roles of integrin aggregation and occupancy of receptors. J Cell Biol 135:1633–1642.

Morino N, Mimura T, Hamasaki K, Tobe K, Ueki K, Kikuchi K, Takehara K, Kadowaki T, Yazaki Y, Nozima Y. 1995. Matrix/integrin interaction activates the mitogen-activated protein kinase, $p44^{erk-1}$ and $p42^{erk-2}$ J Biol Chem 270:269–273.

Plopper G, Ingber DE. 1993. Rapid induction and isolation of focal adhesion complexes. Biochem Biophys Res Commun 193:571–578.

Renshaw MW, Ren XD , Schwartz MA. 1997. Growth factor activity of MAP-kinase requires cell adhesion. EMBO J 16:5592–5599.

Revathi CJ, Chattopadhyay A, Srinivas U. 1994. Change in membrane organization induced by heat shock. Biochem Mol Biol Int 32:941–950.

Schroeder F, Jefferson JR, Kier AB, Knittel J, Scallen TJ, Wood WG, Hapala I. 1991. Membrane cholesterol dynamics: cholesterol domains and kinetic pools. Proc Soc Exp Biol Med 196:235–252.

Varma R, Mayor S. 1998. GPI-anchored proteins are organised in sub-micron domains at the cell surface. Nature 394:798–801.

Wei Y, Xiuwei Y, Liu Q, Wilkins JA, Chapman HA. 1999. A role for caveolin and the urokinase receptor in integrin-mediated adhesion and signaling. J Cell Biol 144:1285–1294.

Weir ML, Edidin M. 1986. Effects of cell density and extracellular matrix on the lateral diffusion of major histocompatibility antigens in cultured fibroblasts. J Cell Biol 103:215–222.

Yeagle PL. 1985. Cholesterol and cell membrane. Biochim Biophys Acta 822:267–287.