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A Cell-free System to Study Regulation of Focal Adhesions and of the Connected Actin Cytoskeleton

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Assembly and modulation of focal adhesions during dynamic adhesive processes are poorly understood. We describe here the use of ventral plasma membranes from adherent fibroblasts to explore mechanisms regulating integrin distribution and function in a system that preserves the integration of these receptors into the plasma membrane. We find that partial disruption of the cellular organization responsible for the maintenance of organized adhesive sites allows modulation of integrin distribution by divalent cations. High Ca^{2+} concentrations induce quasi-reversible diffusion of β1 integrins out of focal adhesions, whereas low Ca^{2+} concentrations induce irreversible recruitment of β1 receptors along extracellular matrix fibrils, as shown by immunofluorescence and electron microscopy. Both effects are independent from the presence of actin stress fibers in this system. Experiments with cells expressing truncated β1 receptors show that the cytoplasmic portion of β1 is required for low Ca^{2+}-induced recruitment of the receptors to matrix fibrils. Analysis with function-modulating antibodies indicates that divalent cation-mediated receptor distribution within the membrane correlates with changes in the functional state of the receptors. Moreover, reconstitution experiments show that purified α-actinin colocalizes and redistributes with β1 receptors on ventral plasma membranes depleted of actin, implicating binding of α-actinin to the receptors. Finally, we found that recruitment of exogenous actin is specifically restricted to focal adhesions under conditions in which new actin polymerization is inhibited. Our data show that the described system can be exploited to investigate the mechanisms of integrin function in an experimental setup that permits receptor redistribution. The possibility to uncouple, under cell-free conditions, events involved in focal adhesion and actin cytoskeleton assembly should facilitate the comprehension of the underlying molecular mechanisms.

INTRODUCTION

Focal adhesions are regions of the ventral portion of the plasma membrane of adherent cultured cells, which are in tight contact with the underlying extracellular matrix (ECM).[1] Adhesion at these sites is mediated by clustered integrin receptors, which anchor bundles of actin microfilaments at their cytoplasmic face. Focal adhesions have provided an ideal experimental model for studying the links between the ECM and the cytoskeleton.

A large number of intracellular proteins colocalize with integrins at these sites and seem to be important both for signaling and cytoskeletal reorganization (Jockusch *et al.*, 1995; Craig and Johnson, 1996). Several observations suggest that tyrosine phosphoryla-

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[1] Abbreviations used: CEF, chicken embryo fibroblast; ECM, extracellular matrix; F-actin, filamentous actin; FCS, fetal calf serum; G-actin, globular (monomeric) actin; HCB, high-calcium buffer; Ig, immunoglobulin; LCB, low-calcium buffer; mAb, monoclonal antibody; Rh-actin, rhodamine–G-actin; VPM, ventral plasma membrane.

tion is involved in integrin-mediated signaling (Schaller and Parsons, 1993), and that integrin clustering is an important event to trigger tyrosine phosphorylation and recruitment of several proteins at the adhesive sites (Miyamoto *et al.*, 1995). A large amount of information has accumulated on the possible role of several focal adhesion components (reviewed in Clark and Brugge, 1995) by expressing wild-type and mutant proteins in cells, as well as studying the molecular interactions between purified proteins in vitro. Yet little is known about the mechanism of assembly and regulation of focal adhesions and the role of the numerous proteins colocalizing with these adhesive structures. The setup of cell-free systems would be of great advantage to the exploration of focal adhesion dynamics and for a better understanding of the relationships between adhesion and actin organization in a living cell. Successful attempts have already been made in this direction. They include studies in which receptor-stimulated actin polymerization has been achieved in permeabilized neutrophils (Redmond *et al.*, 1994) and platelets (Hartwig *et al.*, 1995). Furthermore, permeabilized Swiss 3T3 cells have been used to show the involvement of activated RhoA GTP-binding protein in the stimulation of phosphorylation of $p125^{FAK}$ and paxillin (Seckl *et al.*, 1995). Crowley and Horwitz (1995) have used permeabilized chicken fibroblasts to show an ATP-dependent destabilization of focal adhesions during cell detachment. More recently, McKay *et al.* (1997) have shown that moesin, ezrin, and radixin can reconstitute actin polymerization and focal complex formation in response to activation of Rho and Rac in serum-starved Swiss 3T3 cells permeabilized with digitonin.

In this paper we describe the use of a cell-free system to study the regulation of integrin distribution and function. We have used a modification of the lysis-squirting technique (Nermut *et al.*, 1991; Cattelino *et al.*, 1995) for the preparation of detergent-free ventral plasma membranes (VPMs) obtained from adherent chicken embryo fibroblasts (CEFs). Our recent work has shown that VPMs contain well-structured focal adhesions and stress fibers, as detected by both morphological and biochemical criteria (Cattelino *et al.*, 1995, 1997). Two important advantages of this system are the maintenance of the adhesive receptors within their natural lipidic environment (i.e., the adherent portion of the plasma membrane of cells spread on ECM) and the accessibility to the cytoplasmic side of the adhesive membrane, without need for detergents that may affect the environment of the adhesive receptors. By using this system, we show that changes in calcium concentrations can affect integrin behavior within VPMs. In particular, we observe a correlation between integrin localization and the functional state of the receptors, which can be reversibly modulated either by changes in free calcium ion concentration $[Ca^{2+}]$ or by function-modulating anti-integrin $\beta 1$ monoclonal antibodies (mAbs). Our results also show that $[Ca^{2+}]$-induced integrin redistribution is dependent on the presence of the $\beta 1$ cytoplasmic domain, whereas it is independent from the presence of filamentous actin (F-actin) and focal adhesions in this experimental system, implicating uncoupling of events relevant to focal adhesion assembly under cell-free conditions. Moreover, under conditions in which focal adhesions are preserved but new actin polymerization is inhibited, we observe exogenous actin specifically recruited to focal adhesion sites. These findings, together with the ability to reconstitute α-actinin binding to actin-depleted VPMs, attest the value of the system for further analysis of the molecular mechanisms regulating integrin function and focal adhesions.

MATERIALS AND METHODS

Cell Culture

CEFs were isolated from 10-d-old embryos and cultured in DMEM containing 5% fetal calf serum (FCS), 1% chicken serum, 100 U/ml penicillin and streptomycin, 20 mM glutamine, at 37°C, 5% CO_2. CEFs up to the eighth passage were used for experiments.

Antibodies

The production and characterization of the polyclonal antibody $\beta 1$-cyto raised against a peptide from the cytoplasmic domain of the integrin $\beta 1$ subunit and of the mAb TASC against chick integrin $\beta 1$ subunit have been previously described (Tomaselli *et al.*, 1988; Neugebauer and Reichardt, 1991). The polyclonal antibody AAL20 against actin was a generous gift from Dr. C. Chaponnier (University of Geneva, Geneva, Switzerland). The mAb CSAT against the chick integrin $\beta 1$ subunit (Neff *et al.*, 1982) was a generous gift from Dr. A.F. Horwitz (University of Illinois, Urbana, IL). The mAb TS2/16 against the human integrin $\beta 1$ subunit (Hemler *et al.*, 1984) was a generous gift from Dr. Guido Tarone (University of Torino, Torino, Italy). The mAbs against α-actinin, vinculin, tensin, and talin were purchased from Sigma-Aldrich Italy (Milan, Italy). The mAb against paxillin was from Zymed Laboratories (San Francisco, CA). The mAbs M2D5 (immunoglobulin G [IgG]) and X1E8 (IgM) were obtained by injecting VPM preparations into mice and by subsequent screening of the hybridoma clones by immunofluorescence. M2D5 recognizes both human and chicken fibronectin by immunofluorescence and Western blotting, whereas X1E8 colocalizes with phalloidin along acting stress fibers by immunofluorescence.

Preparation of VPMs

VPMs were prepared from CEFs grown in 100-mm-diameter culture dishes or on glass coverslips using a modification of the lysis squirting technique (Nermut *et al.*, 1991), as described by Cattelino *et al.* (1995). For biochemical analysis, VPMs were prepared from cells cultured in 100-mm dishes, and the jet of buffer (20 mM HEPES-KOH, pH 7, 0.3 mM PMSF) was obtained by forcing it through a pipette by air pressure.

Transfections

The plasmid coding for the $\beta 1$TR construct corresponding to the human integrin $\beta 1$ subunit missing the cytoplasmic domain (Retta *et al.*, 1998) was kindly provided by Dr. G. Tarone. CEFs were plated

on glass coverslips in 15-mm wells and cultured 18 h before transfection. Subconfluent cells were transfected with liposomal transfection reagent Dosper (Boehringer Mannheim, Mannheim, Germany), using 3 μg of plasmid and 6 μl of Dosper/well. The Dosper and the DNA were diluted separately in 20 mM HEPES, 150 mM NaCl (pH 7.4) to a final volume of 25 μl each. The Dosper was added to the DNA dropwise and incubated 15 min at room temperature. The mixture was added to the wells containing cells with 1 ml of fresh medium with 5% FCS. After 6 h, cells were washed and cultured in fresh medium for an additional 14 h with 5% FCS.

Cell-free Assay

VPMs prepared from cells grown on 13-mm-diameter glass coverslips were incubated for the indicated times at 37°C in 25 μl of low-calcium buffer (LCB, containing 125 mM K-acetate, 2.5 mM $MgCl_2$, 12 mM glucose, 25 mM HEPES-KOH, pH 7, 50 nM free Ca^{2+}) or high-calcium buffer (HCB, containing 125 mM K-acetate, 2.5 mM $MgCl_2$, 12 mM glucose, 25 mM HEPES-KOH, pH 7, 1 mM free Ca^{2+}) per coverslip. EGTA-$CaCl_2$ buffers were used to obtain defined [Ca^{2+}], according to published procedures (Bers *et al.*, 1994). Buffering conditions different from those specified here have been described, when used, in RESULTS. For biochemical analysis, 10 ml of the same buffers for each 100-mm dish were used. In some experiments, VPMs were preincubated for 3 min at 0°C in HCB containing 4 μM fusion protein corresponding to the full-length gelsolin (Way *et al.*, 1989), a generous gift from Dr. Michael Way (European Molecular Biology Laboratory, Heidelberg, Germany). After incubation at 37°C, VPMs were immediately processed for immunofluorescence or for biochemical analysis. Untreated VPMs kept at 0°C were used as controls for immunofluorescence and biochemical analysis.

Biochemical Analysis

VPMs were prepared from CEFs cultured overnight in serum-free medium on 100-mm culture dishes. For each experiment, two dishes were solubilized with 0.25 ml of SDS-PAGE loading buffer (Laemmli, 1970), containing 1 mM sodium orthovanadate, 10 mM NaF, and 10 μg/ml antipain, chymostatin, leupeptin, and pepstatin. Equal volumes of lysate were analyzed by SDS-PAGE on 5–13% acrylamide gels. After blotting, filters were incubated overnight at 4°C with primary antibodies at the following dilutions: affinity-purified anti-paxillin, 1 μg/ml; anti-actin/ascites, 1:2000; anti-talin/ascites, 1:100; anti-vinculin/ascites, 1:200; affinity-purified anti-tensin antibody, 1 μg/ml; β1-cyto antibody, 6 μg/ml IgG. Monoclonal and polyclonal antibodies were detected using 0.2 μCi/ml ^{125}I-sheep anti-mouse IgG or ^{125}I-protein A, respectively (Amersham, Arlington Heights, IL). Filters were exposed to Amersham Hyperfilm-MP.

Reconstitution of α-Actinin Binding to VPMs

Purified α-actinin was obtained from smooth muscle (chicken gizzard) as previously described (Feramisco and Burridge, 1980). For reconstitution experiments, VPMs were incubated for 10 min at 37°C in a buffer with low ionic strength (2.5 mM $MgCl_2$, 12 mM glucose, 25 mM HEPES-KOH, 5% bovine serum albumin, pH 7) with either high (1 mM free Ca^{2+}) or low (50 nM free Ca^{2+}) [Ca^{2+}]. This was done to induce integrin redistribution. When indicated, the buffer also contained 4 μM gelsolin to remove endogenous actin. VPMs were then washed once for 2 min at room temperature with 1 ml of the same buffer (without gelsolin) and further incubated for 10 min at 37°C in 20 μl of the same buffer per 13-mm coverslip. When indicated, 100 μg/ml purified α-actinin was present during this incubation. Samples were then washed for 2 min at room temperature in 1 ml of the same buffer without α-actinin and fixed for immunofluorescence.

Reconstitution of Actin Binding and Polymerization on VPMs

Actin purified from rabbit muscle (Pardee and Spudich, 1982) was a kind gift from Dr. Tony Hyman (European Molecular Biology Laboratory), and it was labeled with 5- and 6-carboxytetramethyl-rhodamine succinimidyl ester (Molecular Probes, Junction City, OR) as previously described (Kellogg *et al.*, 1988). Two cycles of assembly-disassembly were performed. The labeled globular actin (G-actin) was stored in aliquots at −80°C in 10 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 1 mM DTT, pH 8, at a concentration of 26 μM. For reconstitution experiments, control or treated VPMs were incubated for 2 min at 37°C in LCB containing 1% BSA and 1 μM rhodamine–G-actin (Rh-actin). When indicated, the buffer also contained 100 nM cytochalasin D and 100 μg/ml DNase I.

Immunofluorescence

After treatment of cells or VPMs under the different experimental conditions, samples were fixed with 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with 0.2% gelatin in PBS. Coverslips were then incubated for 60 min at room temperature with the following primary antibodies: anti-vinculin/ascites, 1:200; β1-cyto, 26 μg/ml IgG; mAb M2D5/hybridoma supernatant, 1:25; anti-α-actinin mAb/ascites, 1:300. In the case of TASC, samples were incubated for 20 min at 0°C with 20 μg/ml purified antibody before fixation. Primary antibodies were detected after incubation for 40 min with FITC- and TRITC-conjugated secondary antibodies, as indicated (Boehringer Mannheim; Chemicon, Temecula, CA; Jackson ImmunoResearch, West Grove, PA). F-actin was revealed by incubation with FITC- or TRITC-conjugated phalloidin (Sigma). For staining with the lipophilic carbocyanine dye $DiIC_{16}$ (Molecular Probes), at the end of the experiment VPMs were incubated at room temperature for 5 min with 4 μg/ml $DiIC_{16}$ and washed twice at 0°C. Cells were observed using a Zeiss (Thornwood, NY) Axiophot microscope.

Immunoelectron Microscopy

After treatment of VPMs for 10 min at 37°C in LCB, they were fixed, permeabilized, and incubated with first antibodies as described in the preceding paragraph. First antibodies were β1-cyto, 10 μg/ml IgG; mAb X1E8/hybridoma supernatant, 1:2; mAb M2D5/hybridoma supernatant, 1:25; anti-actin polyclonal antibody AAL20/IgG fraction, 1:100. For staining with the TASC mAb, intact cells were incubated 20 min at room temperature with 20 μg/ml of purified IgG before preparation of VPMs. The secondary antibodies used were anti-rabbit IgG conjugated to 18-nm colloidal gold particles, anti-mouse IgG conjugated to 6-nm colloidal gold particles, and anti-mouse IgM conjugated to 6-nm colloidal gold particles (Jackson ImmunoResearch). The samples were postfixed in 1% glutaraldehyde, incubated in 1% osmium, and stained with 10% uranile acetate. Samples were then dehydrated and coated with a carbon thread evaporator unit (Balzer Union, Principality of Liechtenstein). They were then floated off the coverslip with 4.8% hydrofluoric acid (Miller *et al.*, 1991), collected on copper grids, and viewed with a Hitachi (Tokyo, Japan) H7000 transmission electron microscope.

RESULTS

[Ca^{2+}] Regulates the Distribution of β1 Integrins by Affecting Receptor Function in a Cell-free System

We have used VPMs prepared from adherent fibroblasts with the aim of setting up an in vitro system to study the regulation of focal adhesions and integrin function under cell-free conditions. For this purpose, we have treated VPMs under different experimental

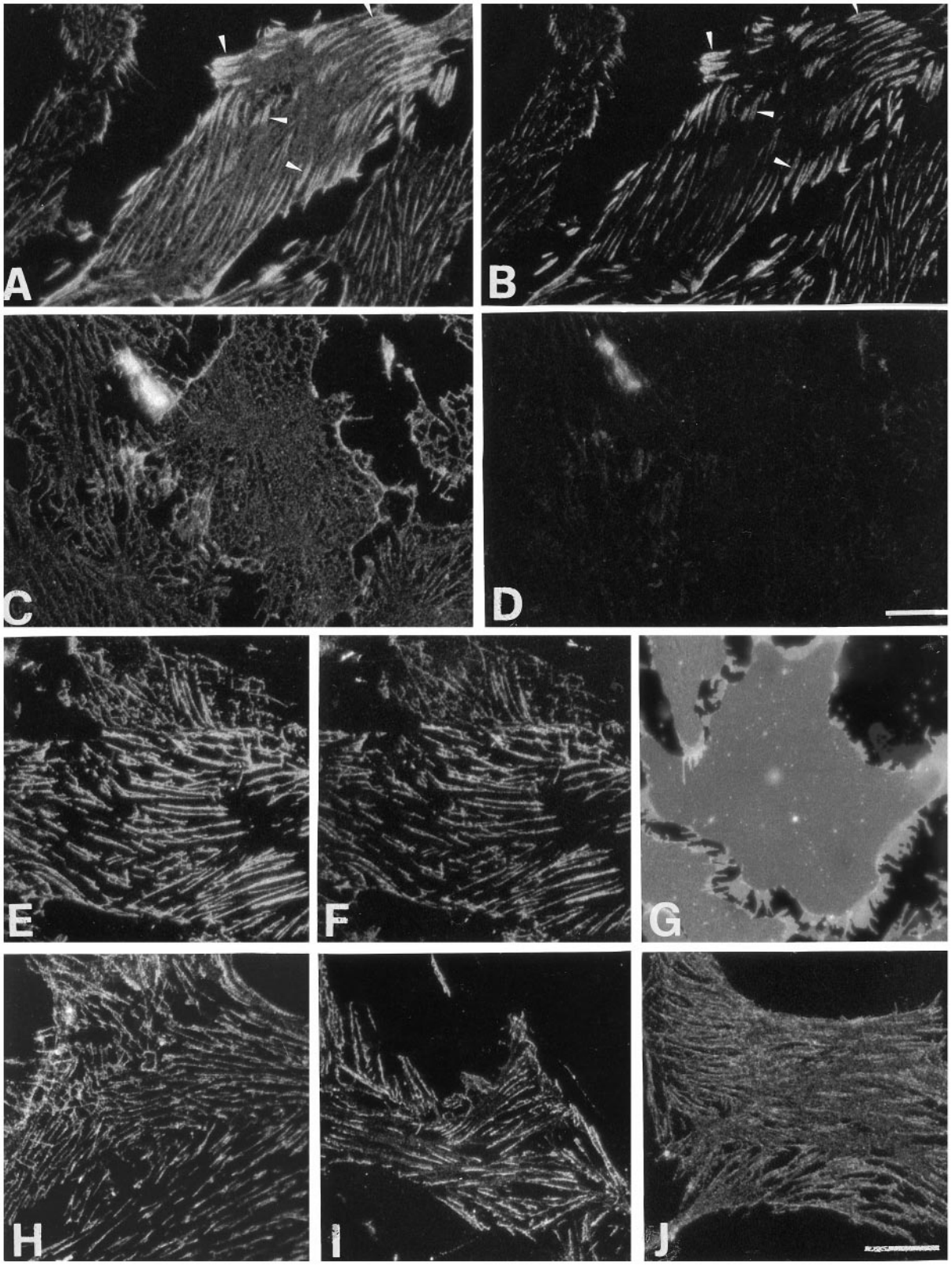

Figure 1.

conditions to identify parameters that would affect integrin distribution and/or function in vitro. At the end of each experiment, cells were fixed for analysis by immunofluorescence, as described in MATERIALS AND METHODS.

Among the experimental parameters examined were ionic composition, ionic strength, divalent cations, pH, and temperature. Surprisingly, preliminary analysis showed that VPMs prepared from CEFs in the presence of divalent cations appeared well preserved also after several hours of incubation at 37°C, as detected by staining with the lipophilic dye $DiIC_{16}$ (our unpublished results). We then used immunofluorescence to look for conditions that would affect integrin distribution upon incubation of VPMs at 37°C. Control, untreated samples were obtained by fixing the VPMs immediately after preparation on ice. Staining of control VPMs with the polyclonal antibody β1-cyto showed that the receptors were both localized into focal adhesions and diffuse throughout the ventral surface of the adherent fibroblasts (Figure 1A). We then tested the effect of the incubation of the VPMs at 37°C for 15 min in different buffers. Among the parameters analyzed, variations in $[Ca^{2+}]$ in the presence of 2.5 mM $MgCl_2$ showed interesting effects on the distribution of the β1 receptors in vitro. When VPMs were incubated for 15 min at 37°C in a buffer containing $[Ca^{2+}] \geq 10$ μM, β1 integrins appeared predominantly diffuse on the membrane, and their distribution became more homogeneous after incubation at 37°C with 1 mM free Ca^{2+} (Figure 1C). The diffusion of the integrin β1 subunits at high $[Ca^{2+}]$ was not caused by disassembly of the α/β heterodimers, because biochemical analysis showed coprecipitation of the α subunits with β1 under these conditions (our unpublished results).

The β1 receptors showed a dramatically different distribution when VPMs were incubated at low $[Ca^{2+}]$. In fact, after incubation at 37°C for 15 min in LCB (containing 50 nM free Ca^{2+}), β1 integrins were found concentrated along fibrillar structures (Figure 1E), which were generally thinner and longer compared with focal adhesions (Figure 1, compare E with A). In this situation, the diffuse integrin staining observed in control VPMs disappeared, suggesting that all β1 integrins had redistributed into the fibrillar structures. To control whether the distribution observed after low $[Ca^{2+}]$ treatment was due to disruption of the membranes, we used the lipophilic fluorescent dye $DiIC_{16}$ to stain VPMs treated at 37°C for 15 min in LCB (Figure 1G). Membrane integrity was not affected by this treatment. These findings strongly indicate that the distinct patterns observed at different $[Ca^{2+}]$s are due to actual changes in receptor distribution, rather than to a modification of the structure of the plasma membrane. Moreover, although 15 min incubation was used as a standard experimental condition, both high and low $[Ca^{2+}]$-induced effects were observed already after 3 min at 37°C, whereas incubation at 0°C under different $[Ca^{2+}]$ values did not affect integrin distribution (our unpublished results).

To investigate the reversibility of the $[Ca^{2+}]$-mediated effects on β1 distribution, VPMs treated for 15 min at 37°C in HCB were incubated for an additional 15 min in LCB. Under these conditions, β1 integrins were found concentrated into fibrillar structures (Figure 1H). In contrast, β1 receptors concentrated into fibrillar structures by 15 min incubation at 37°C in LCB were not able to diffuse when VPMs were incubated for a further 15 min in HCB (Figure 1I). These results show that high $[Ca^{2+}]$-induced β1 integrins diffusion is quasi-reversible, because it can be affected by low $[Ca^{2+}]$; it should be emphasized though that the fibrillar distribution of integrins observed at low $[Ca^{2+}]$ is different from that of typical focal adhesions observed in whole cells or in untreated VPMs (Figure 1, compare E with A). On the other hand, low $[Ca^{2+}]$ treatment somehow irreversibly locks β1 integrins into the fibrillar pattern.

Integrin receptors may be expressed in different activation states on the surface of the cell. We tested the hypothesis that the different $[Ca^{2+}]$ values were affecting β1 integrin distribution by modulating the affinity of the receptors for their ECM ligands. For this purpose we compared the distribution of the total population of β1 receptors, using the antibody β1-cyto, with the distribution obtained by staining with the mAb TASC, which recognizes activated, high-affinity β1 receptors (Neugebauer and Reichardt, 1991). In control VPMs, the β1-cyto antibody recognized the receptors concentrated into focal adhesions, as well as those diffused throughout the membrane (Figure 1A), whereas the mAb TASC only recognized the receptors localized in focal adhesions (Figure 1B). Furthermore,

Figure 1 (facing page). Calcium-dependent redistribution of β1 integrins in VPMs. VPMs from adherent CEFs were fixed after preparation on ice (A and B) or after incubation for 15 min at 37°C in HCB (C and D), in LCB (E–G), in HCB followed by 15 min at 37°C in LCB (H), or in LCB followed by 15 min at 37°C in HCB (I). (J) VPMs were treated as in I, except that 200 μg/ml integrin β1 function-blocking antibody CSAT was present during the last 15 min incubation at 37°C in HCB. For staining with the anti-integrin β1 TASC mAb (B, D, and F), VPMs were incubated with a 20 μg/ml concentration of this antibody for 20 min at 0°C before fixation. VPMs were then fixed and processed for immunofluorescence. (A, C, E, and H–J) staining with the β1-cyto polyclonal antibody. Primary antibodies were revealed by FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG, respectively. (G) VPMs were stained with the lipophilic carbocyanine dye $DiIC_{16}$. The same fields are shown in A and B, in C and D, and in E and F. Arrowheads in A and B point to focal adhesions, where colocalization of β1-cyto and TASC staining is visible. Bars, 10 μm.

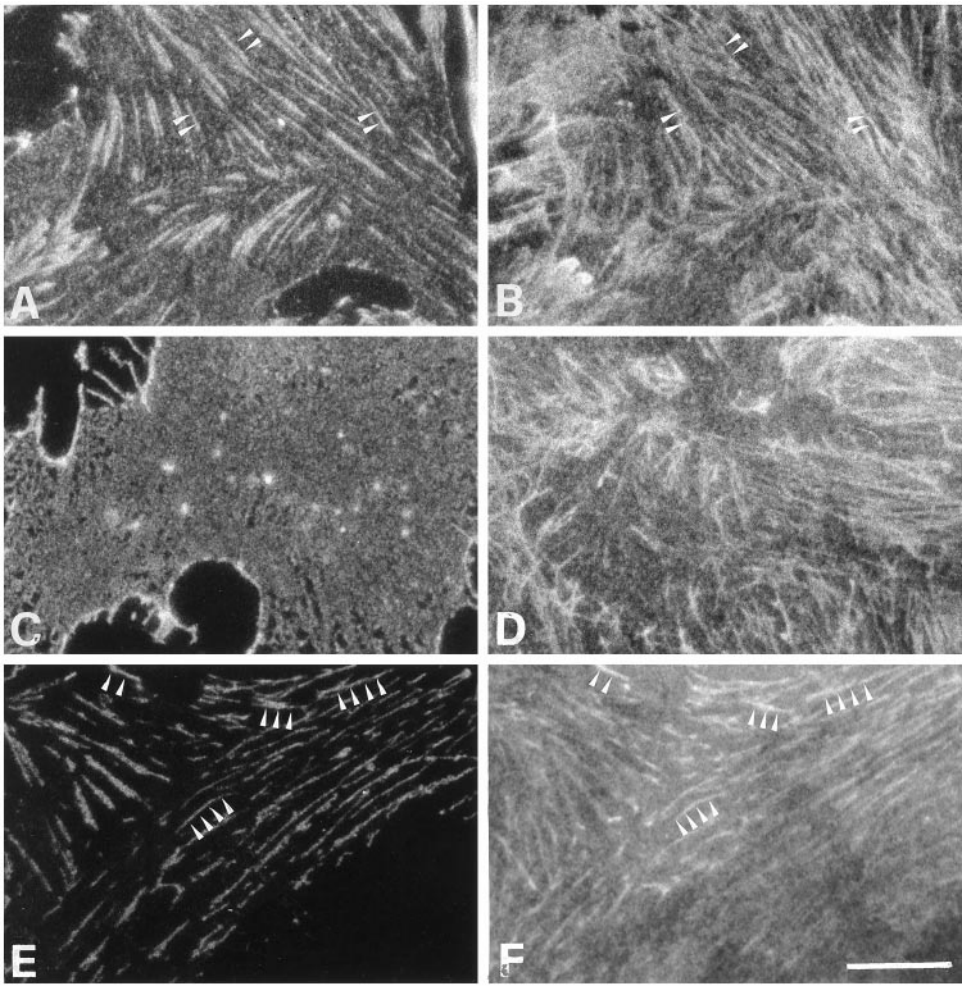

Figure 2. Localization of β1 integrins and ECM fibrils after treatment of VPMs at different calcium concentrations. VPMs from CEFs were fixed immediately after preparation on ice (A and B), after incubation for 15 min at 37°C in HCB (C and D), or after incubation for 15 min at 37°C in LCB (E and F). Fixed VPMs were processed for immunofluorescence using the β1-cyto antibody (A, C, and E) and the M2D5 mAb recognizing fibronectin (B, D, and F). Primary antibodies were revealed by FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG. The same fields are shown in A and B, C and D, and E and F. Arrowheads in A, B, E, and F point to sites where colocalization of integrins with ECM fibrils is visible. Bar, 10 μm.

TASC did not recognize the diffuse β1 receptors in VPMs treated with HCB (Figure 1, compare D with C), whereas it was able to recognize the LCB-induced fibrillar pattern (Figure 1, compare F with E). These results show that under cell-free conditions the $[Ca^{2+}]$ is able to affect β1 integrin distribution, possibly by regulating the ability of the receptors to recognize their extracellular ligands.

When VPMs that had been incubated at low $[Ca^{2+}]$ were further incubated at high $[Ca^{2+}]$ in the presence of the function-blocking mAb CSAT, receptor clustering induced by low $[Ca^{2+}]$ could be partially reversed (Figure 1J), because a clear diffuse staining for integrins could be observed together with the fibrillar pattern. This result indicates that the presence of the function-blocking antibody can disrupt low $[Ca^{2+}]$-mediated integrin distribution, probably by interfering with receptor-ligand binding.

These results indicate that high $[Ca^{2+}]$-induced receptor diffusion within the ventral surface of the cell may be explained by inhibition of integrin function, whereas low $[Ca^{2+}]$-induced receptor concentration along fibrils may be explained by receptor activation.

Low $[Ca^{2+}]$ Induces Relocation of β1 Integrins along ECM Fibrils

CEFs in culture are known to produce their own ECM, which they deposit and organize on the substrate. The low $[Ca^{2+}]$-induced redistribution of β1 integrins into fibrillar structures suggested a possible relocalization of the receptors along ECM fibrils underlying the VPMs. We therefore analyzed the distribution of the receptors and of ECM in control and experimentally treated VPMs. In control VPMs fixed after preparation at 0°C, ECM fibrils, stained by the mAb M2D5 recognizing chicken fibronectin, colocalized only partially with β1-positive focal adhesions (Figure 2, A and B, arrowheads). After incubation for 15 min at 37°C in HCB, β1 integrins were predominantly diffuse on the membrane (Figure 2C), whereas ECM fibrils could still be observed (Figure 2D). Incubation for 15 min at 37°C

in LCB induced a redistribution of β1 integrins to sites that strikingly corresponded to the ECM fibrils (Figure 2, E and F, arrowheads).

We used immunoelectron microscopy to obtain more details on the nature of the fibrillar structures where β1 integrins localize at low [Ca^{2+}]. Figure 3 shows different "en face" views of VPMs treated with LCB. Bundles of F-actin were visible as electron dense structures that were abundantly decorated both by the mAb X1E8 (Figure 3A, small gold particles), and by a polyclonal anti-actin antibody (Figure 3B, large gold particles). Both antibodies stained phalloidin-positive actin stress fibers by immunofluorescence (our unpublished results). β1 integrins were concentrated along fibrils, which sometimes ran parallel to the F-actin bundles (Figure 3A, large gold particles), but most of the times were clearly separated from them (Figure 3B, small gold particles). These structures correspond to ECM fibrils, because they could be stained with the mAb M2D5 recognizing fibronectin (Figure 3C, small gold particles) and were often heavily decorated by anti-integrin β1 antibodies (Figure 3C, large gold particles). As expected from the diffuse ECM staining observed around fibrils by immunofluorescence (see Figure 2, B, D, and F), diffuse ECM staining could be observed also at the ultrastructural level (Figure 3C, small gold particles). Ultrastructural analysis of VPMs incubated in HCB showed a diffuse distribution of the β1 receptors (our unpublished results), confirming the data obtained by immunofluorescence. These results show that the fibrillar structures decorated by integrins upon incubation at low [Ca^{2+}] correspond to sites of fibrillar accumulation of ECM, which are distinct from actin stress fibers.

The Distribution of Integrins Lacking the Cytoplasmic Domain of the β1 Subunit Can Be Affected by an mAb Modulating Integrin Function but Not by Low [Ca^{2+}]

To study whether the cytoplasmic portion of β1 may play a role in receptor redistribution, we transfected CEFs with a human β1TR construct corresponding to the human integrin β1 subunit lacking the entire cytoplasmic domain. The distribution of β1TR on VPMs was analyzed by immunofluorescence using the mAb TS2/16, specific for the human β1 subunit. As expected, we found that in contrast to the endogenous β1 receptor (Figure 4A), β1TR was not clustered in focal adhesions but showed a diffuse staining on VPMs fixed immediately after preparation at 0°C (Figure 4B). After incubation in LCB, β1TR was found predominanlty diffuse (Figure 4D), with no clear colocalization with the endogenous receptor (Figure 4C). As expected, β1TR had a diffuse localization after incubation of VPMs in HCB (our unpublished results). In contrast, β1TR did clearly concentrate along ECM fibrils if VPMs were incubated 15 min at 37°C in the presence of the human-specific stimulatory mAb TS2/16. This effect was observed either at high [Ca^{2+}] (Figure 4F), with the endogenous receptor diffuse on the membrane (Figure 4E), or at low [Ca^{2+}], with the endogenous β1 receptors colocalizing with β1TR along ECM fibrils (our unpublished results). These results show that the mechanism of low [Ca^{2+}]-mediated β1 integrin redistribution along fibrils in vitro requires the cytoplasmic domain of the receptor, whereas receptors lacking the cytoplasmic domain retain the ability to redistribute upon activation by stimulatory antibodies.

β1 Integrin Redistribution on VPMs Does Not Require the Accumulation of Focal Adhesion Components

Integrin receptors colocalized with vinculin into focal adhesions of control, untreated VPMs (Figure 5, A and D, respectively). Interestingly, incubation for 15 min at 37°C either in HCB (Figure 5, B and E) or in LCB (Figure 5, C and F) led to loss of vinculin staining from VPMs (Figure 5, E and F). All the effects observed on integrin and vinculin distribution were negligible when VPMs were incubated at 0°C under the same experimental conditions (our unpublished results).

To confirm this result and to extend the analysis to other focal adhesion components, we performed immunoblot analysis on lysates from control and experimentally treated VPM preparations. Control samples solubilized immediately after preparation at 0°C (Figure 6, lanes 1 and 3), were compared with VPMs incubated 15 min at 37°C in LCB (Figure 6, lane 2), or HCB (Figure 6, lane 4), respectively. The results show that incubation at 37°C with either LCB or HCB did not affect the recovery of the integrin β1 subunit compared with the respective controls, as expected for a transmembrane protein. Actin was also largely retained on VPMs under all conditions analyzed. In contrast, the recovery of several cytosolic components of the focal adhesions, such as tensin, talin, vinculin, and paxillin, was dramatically decreased after incubation at 37°C at either high or low [Ca^{2+}] (Figure 6, compare lanes 2 and 4 with lanes 1 and 3, respectively). These biochemical data were confirmed by immunofluorescence with antibodies specific for the different focal adhesion proteins (our unpublished results).

There is an apparent incongruence between the complete removal of vinculin (Figure 5, E and F) and other focal adhesion proteins from VPMs treated 15 min at 37°C, as revealed by immunofluorescence, and the incomplete removal of the same proteins, as detected by biochemical analysis (Figure 6, lanes 2 and 4). This finding can be explained by considering that on coverslips treated for immunofluorescence, a mi-

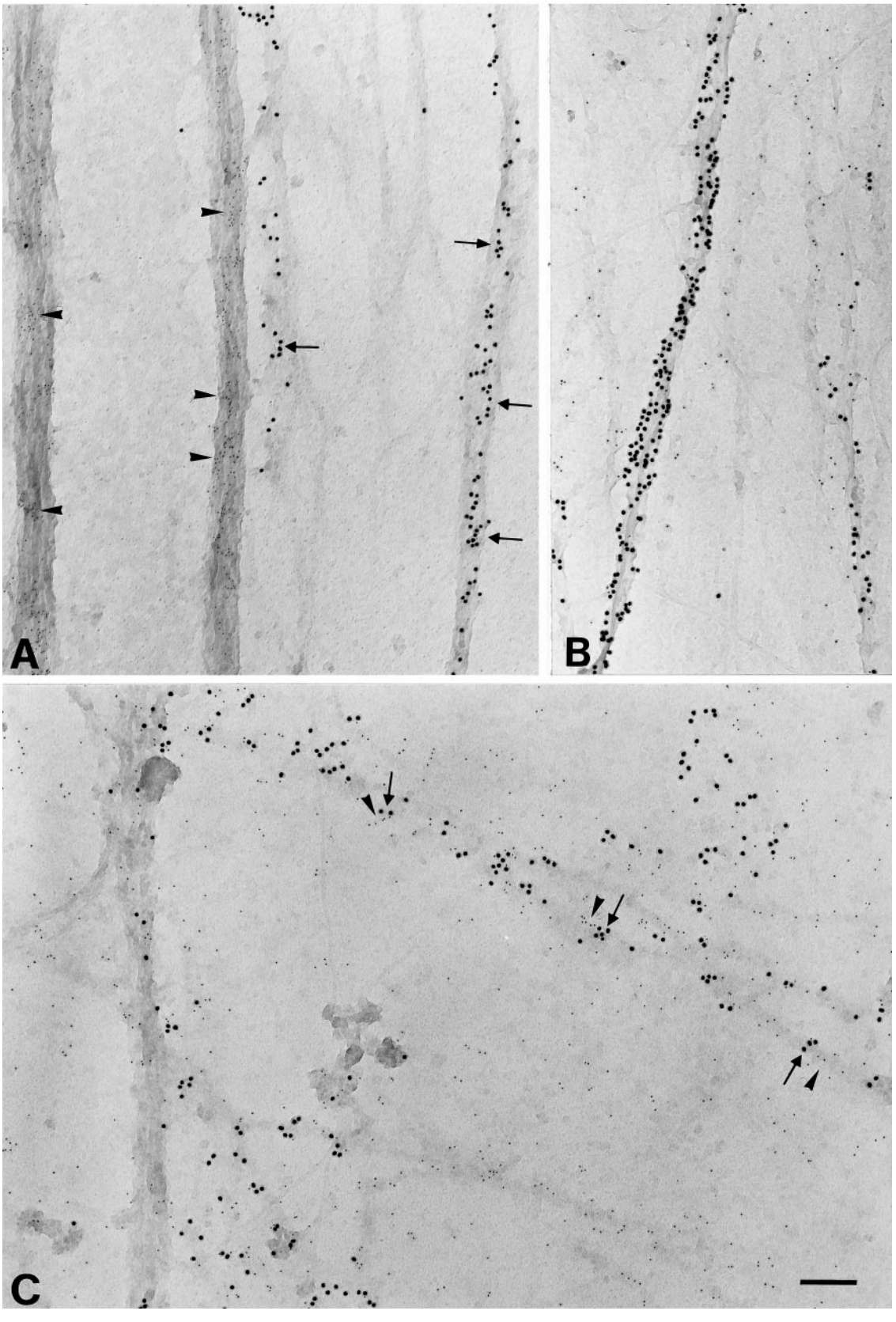

Figure 3.

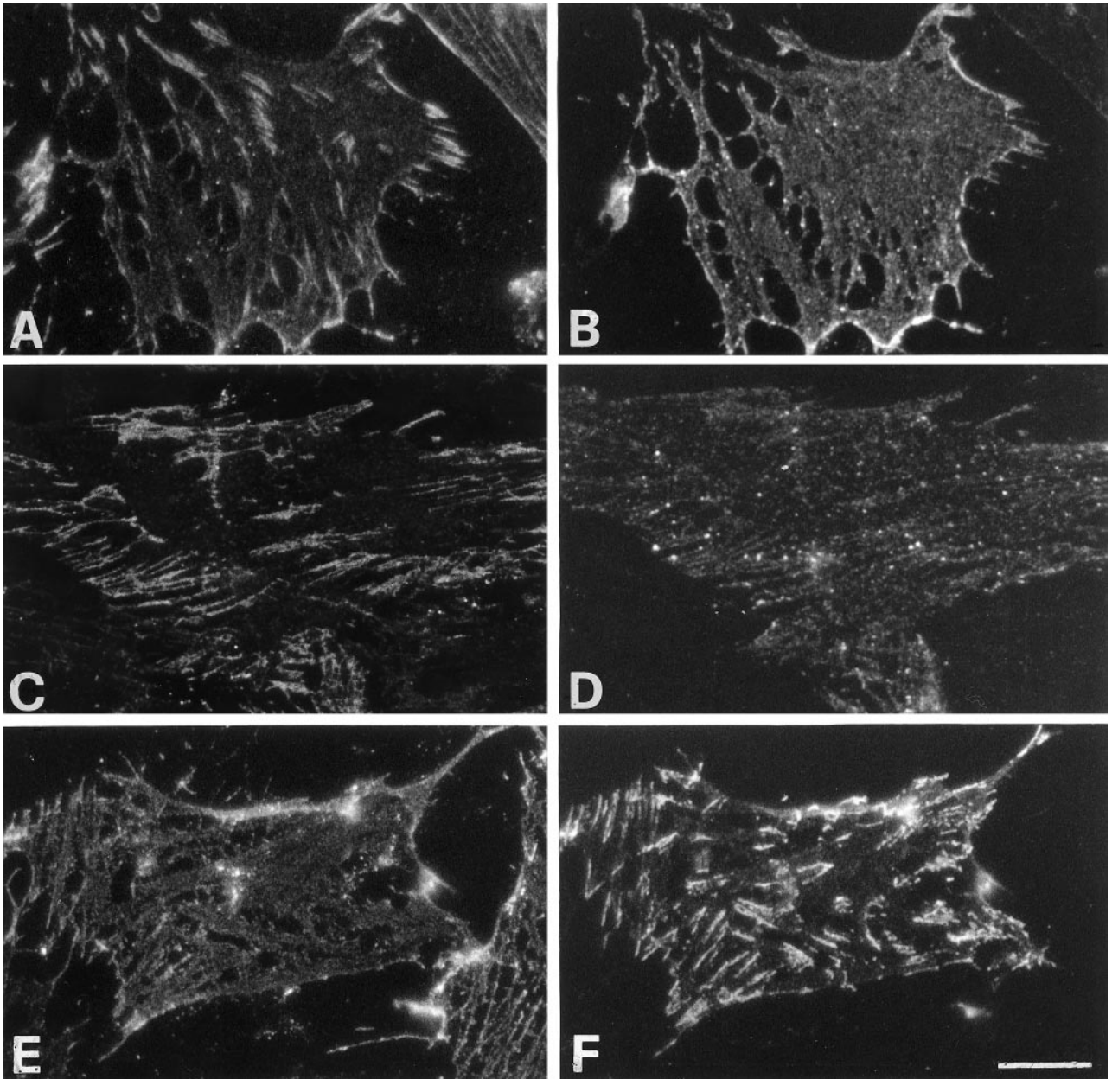

Figure 4. Low [Ca^{2+}] does not affect the localization of integrin β1 receptors lacking the cytoplasmic domain. CEFs were transiently transfected to express the truncated β1TR construct corresponding to the human integrin β1 subunit lacking the cytoplasmic domain. After 20 h culture, VPMs were prepared at 0°C and immediately fixed (A and B) or incubated for 15 min at 37°C in LCB (C and D) or in HCB containing 40 μg/ml activating mAb TS2/16 specific for the human β1 subunit (E and F). After fixation, the samples were incubated with the β1-cyto antibody and FITC-conjugated anti-rabbit IgG to detect the endogenous chicken β1 subunit (A, C, and E), whereas the truncated human β1 subunit was detected with the anti-human β1 mAb TS2/16, revealed by TRITC-conjugated anti-mouse IgG (B, D, and F). The same fields are shown in A and B, C and D, and E and F. Bar, 10 μm.

Figure 3 (facing page). Ultrastructural localization of β1 integrins along ECM fibrils at low [Ca^{2+}]. VPMs were incubated for 15 min at 37°C in LCB and processed for immunoelectron microscopy as described in MATERIALS AND METHODS. (A) Double labeling with the β1-cyto antibody (large, 15-nm gold; arrows) and the X1E8 mAb decorating actin stress fibers (small, 6-nm gold; arrowheads). (B) Double labeling with the anti-β1 TASC mAb (small, 6-nm gold) and the AAL20 polyclonal antibody against actin, decorating actin stress fibers (large, 15-nm gold). (C) Fibrillar structures are labeled both by the β1-cyto antibody (large gold particles) and by the mAb M2D5 recognizing ECM (small gold particles). In C, a few examples are shown of colocalization of β1 integrins (arrows), with ECM (arrowheads) along fibrils. Bar, 200 nm.

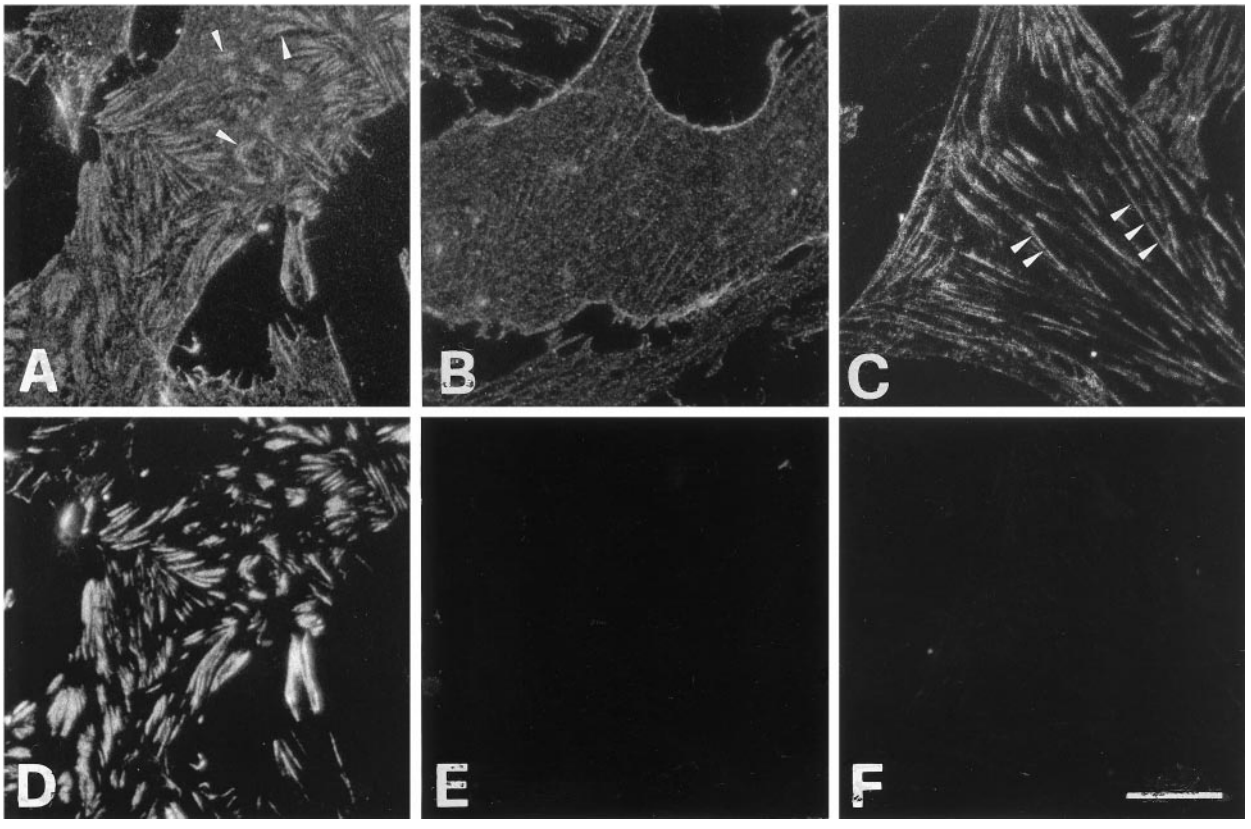

Figure 5. Loss of vinculin from focal adhesions during [Ca^{2+}]-induced integrin redistribution. VPMs from adherent CEFs were fixed after preparation on ice (A and D) or after incubation for 15 min at 37°C in HCB (B and E) or in LCB (C and F). VPMs were then processed for immunofluorescence using the β1-cyto polyclonal antibody (A–C) and the anti-vinculin mAb (D–F). The same fields are shown in A and D, B and E, and C and F. Arrowheads in A and C point to focal adhesions and fibrillary structures, respectively. Bar, 10 μm.

nor fraction of incompletely disrupted cells are found, in which these proteins remain in focal adhesions even after incubation at 37°C. Considering that for biochemical analysis VPMs are prepared from large dishes, it is conceivable to think that a small fraction of cells may escape rupture or complete extraction, thus justifying the amount of focal adhesion components still detectable after treatment at 37°C.

Altogether, these data imply that low [Ca^{2+}]-induced relocation of diffuse receptors along ECM fibrils in VPMs pretreated with high [Ca^{2+}] (see Figure 1H) did not require the accumulation of the analyzed focal adhesion components on the cytoplasmic side of the membrane.

Stress Fibers Are Not Required for Maintenance of β1 Integrins Into Focal Adhesions and for Receptor Redistribution under Cell-free Conditions

To investigate the role of actin on [Ca^{2+}]-mediated β1 integrin redistribution in vitro, we selectively disassembled the stress fibers by using the actin capping and severing protein gelsolin (Figure 7). Incubation of VPMs for 3 min at 0°C in the presence of 4 μM recombinant gelsolin was sufficient to disassemble most, if not all, stress fibers, as revealed by F-actin staining with fluorescent phalloidin (Figure 7D). Some F-actin could still be detected only in areas at the border of VPMs, where residues of the dorsal plasma membrane were probably preventing efficient access of gelsolin (Figure 7, E and F, asterisks). In VPMs maintained at 0°C, β1 integrins could be detected in focal adhesions even in the absence of detectable stress fibers (Figure 7, compare C and D with A and B, respectively). Furthermore, high [Ca^{2+}]-induced diffusion of the receptors (Figure 7E) and subsequent low [Ca^{2+}]-induced concentration along ECM fibrils (Figure 7G) were not affected by lack of stress fibers (Figure 7, F and H, respectively). These results show that stress fibers are not required for the redistribution of β1 integrins under cell-free conditions. Moreover, removal of stress fibers from VPMs does not affect maintenance of β1 integrins into focal adhesions at 0°C.

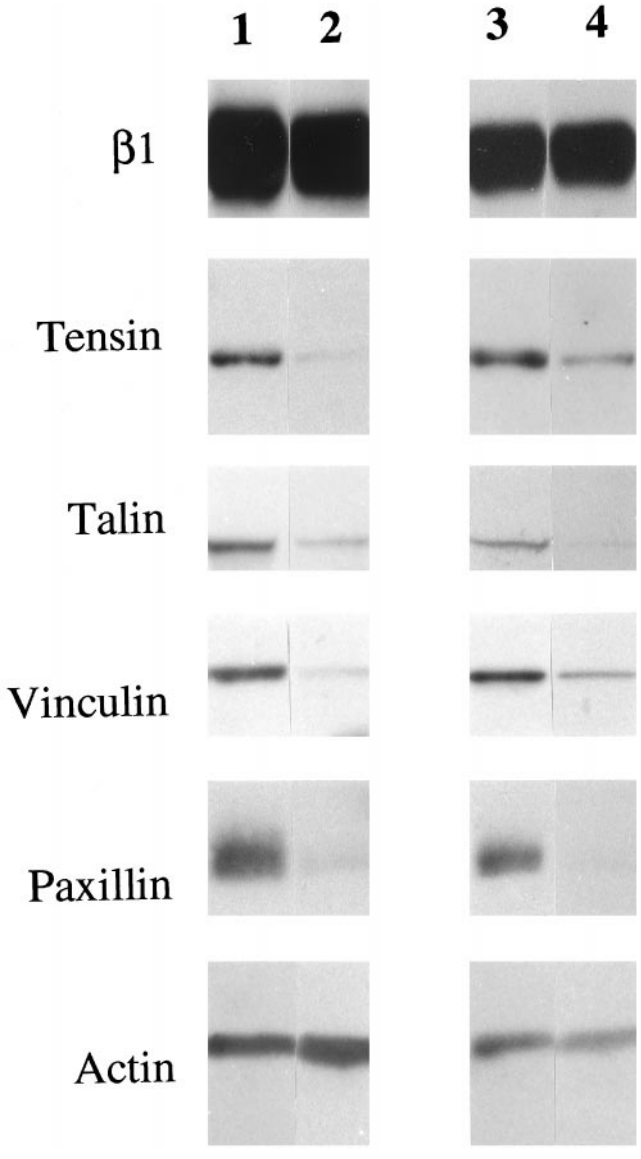

Figure 6. Loss of focal adhesion components from VPMs after incubation at 37°C. VPMs were extracted in SDS-PAGE buffer immediately after preparation on ice (lanes 1 and 3) or after incubation for 15 min at 37°C in LCB (lane 2) or in HCB (lane 4). Equal volumes of the lysates were run on a 5–13% polyacrylamide gradient gel and processed for immunoblotting using the following primary antibodies: β1-cyto (β1), anti-tensin, anti-talin, anti-vinculin, anti-paxillin, and anti-actin. Primary polyclonal and monoclonal antibodies were revealed by ^{125}I-protein A or ^{125}I-sheep anti-mouse IgG, respectively. Loss of focal adhesion components was evident after incubation of VPMs at 37°C in the presence of either low (lane 2) or high (lane 4) [Ca^{2+}]; β1 integrins and actin were not significantly affected by either treatment.

Reconstitution of α-Actinin Binding to F-Actin-depleted VPMs: [Ca^{2+}]-mediated Redistribution under Cell-free Conditions

We have used purified α-actinin with the aim of reconstituting some of the molecular interactions required for the assembly of focal adhesions. This protein can bind to integrin and F-actin. Incubation of VPMs at 37°C in the presence of purified α-actinin in high [Ca^{2+}] buffer led to the accumulation of the exogenous protein along stress fibers (Figure 8, G and H). The signal detected after incubation with exogenous α-actinin was much stronger when compared with the signal given by the endogenous α-actinin left after incubation at 37°C in the absence of the exogenous protein (Figure 8, B and D). In both cases, the pattern of distribution for α-actinin was different from that for integrins (Figure 8, compare B with A and F with E).

To test whether binding of α-actinin to β1 integrins could be reconstituted in the described cell-free system, we repeated the experiments on VPMs depleted of F-actin (Figure 9D). After incubation with gelsolin at 37°C at high [Ca^{2+}], no endogenous α-actinin could be detected on the membranes (Figure 9E). Incubation of these membranes for 10 min at 37°C in the presence of purified α-actinin at high [Ca^{2+}] resulted in a strong, diffuse signal on the membranes (Figure 9F), which was similar to that observed for β1 integrins (Figure 9C).

Treatment of VPMs at 37°C with gelsolin at low [Ca^{2+}] resulted also in complete removal of the endogenous α-actinin (Figure 9J), and further incubation with purified α-actinin at low [Ca^{2+}] resulted in a fibrillar staining (Figure 9K), which overlapped with the staining for the β1 receptors (Figure 9H). Moreover, binding of exogenous α-actinin to gelsolin-treated VPMs at high [Ca^{2+}], followed by incubation at 37°C at low [Ca^{2+}] in the absence of the exogenous protein, resulted in the redistribution of both prebound α-actinin and integrins along ECM fibrils (Figure 9, I and L).

These data provide evidence for the reconstitution of the binding of a focal adhesion component to β1 receptors in VPMs. Moreover, the [Ca^{2+}]-induced modulation of the distribution of partially reconstituted adhesive complexes under cell-free conditions illustrates the possibility of using this system for further reconstitution studies.

Recruitment of Exogenous Actin to Focal Adhesion Sites on VPMs

To start investigating the relationships between focal adhesions and actin assembly in our cell-free system, we performed a number of experiments using Rh-actin. We found that incubation of freshly prepared VPMs with 1 μM Rh-actin for 2 min at 37°C, a condition that preserved the localization of vinculin into focal adhesions (Figure 10A), resulted in the accumulation of the exogenous actin in fibrillar structures on the membranes (Figure 10D). Under these conditions, exogenous actin colocalized with stress fibers (Figure

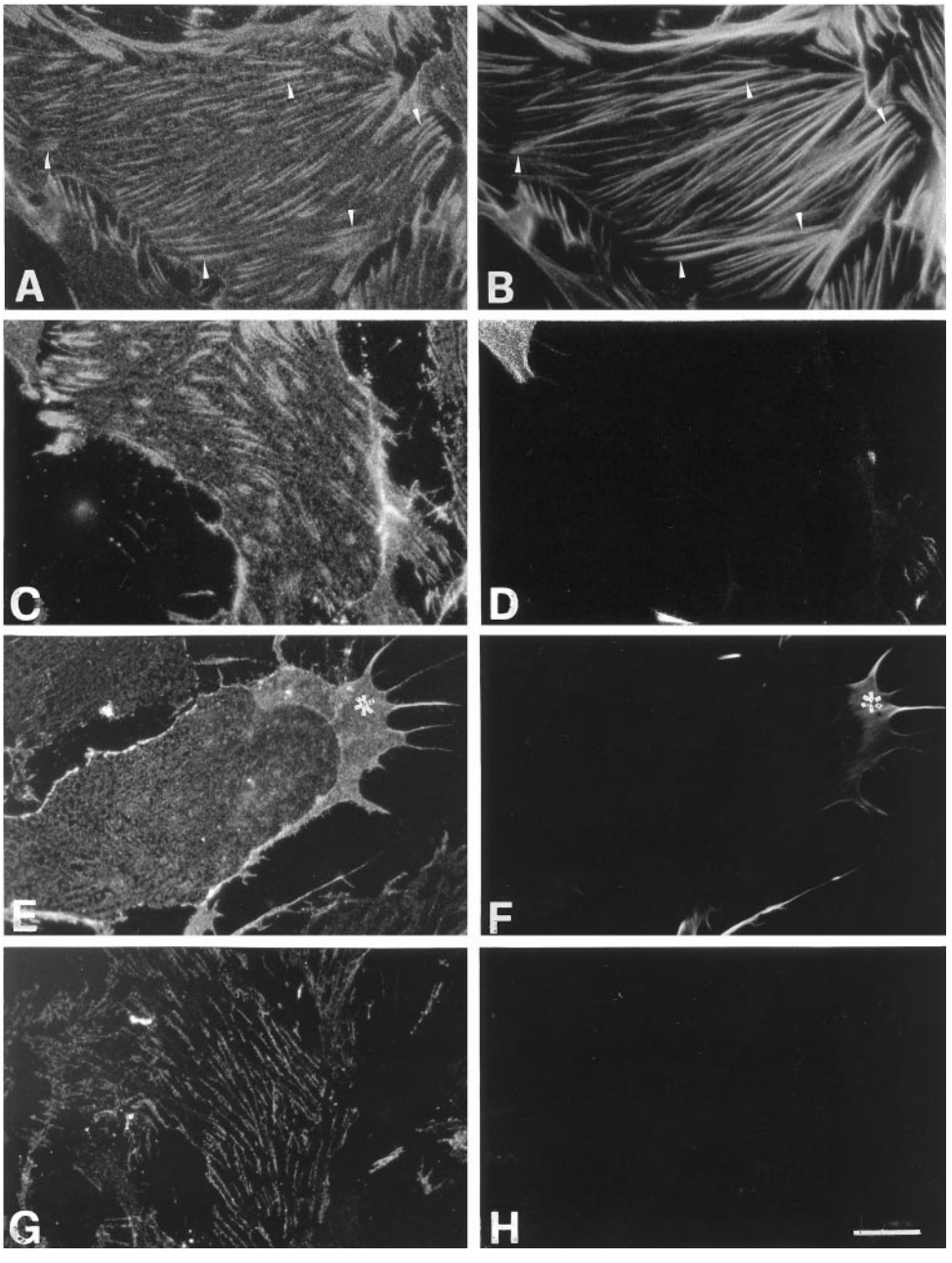

Figure 7. Maintenance of β1 integrins in focal adhesions and their calcium-dependent redistribution are not affected by gelsolin-induced stress fiber disassembly under cell-free conditions. VPMs from adherent fibroblasts were fixed immediately after preparation on ice (A and B) or were incubated 3 min at 0°C with 4 μM gelsolin to disrupt F-actin (C–H). After the treatment with gelsolin, VPMs were either fixed (C and D) or incubated 15 min at 37°C in HCB (E–H). VPMs were then fixed (E and F) or incubated for a further 15 min at 37°C in LCB (G and H). After fixation, all samples were processed for immunofluorescence with the β1-cyto antibody and TRITC-conjugated anti-rabbit IgG (A, C, E, and G). FITC-phalloidin (B, D, F, and H) was used to stain F-actin. The same fields are shown in A and B, C and D, E and F, and G and H. Arrowheads in A and B show colocalization of integrins in focal adhesions (A) with the tip of actin stress fibers (B). Asterisks in E and F show areas at the periphery of the VPM where a portion of the dorsal plasma membrane is present and some F-actin is still visible. Bar, 10 μm.

10, compare B and E). When Rh-actin was added to VMPs in the presence of cytochalasin D and DNase I (which inhibit polymerization from the barbed and pointed end of actin, respectively), exogenous actin accumulated specifically to focal adhesion sites (Figure 10, C and F), whereas binding along stress fibers was not detected. The same result was obtained by incubation with cytochalasin D only (our unpublished results). Because the short incubation with cytochalasin D and DNase I did not evidently affect endogenous stress fibers (our unpublished results), this result indicates that accumulation along stress fibers requires new actin polymerization and is somehow different from binding of exogenous actin to focal contacts.

Exogenous actin accumulated along stress fibers on VPMs preincubated for 15 min at 37°C at low [Ca^{2+}] (Figure 10J) and had a distribution distinguishable from the fibrillar pattern observed for β1 (Figure 10G). Cytochalasin D and DNase I completely prevented actin recruitment on VPMs under these conditions (Figure 10K). Because incubation for 15 min at 37°C in low [Ca^{2+}] resulted in the absence of detectable levels

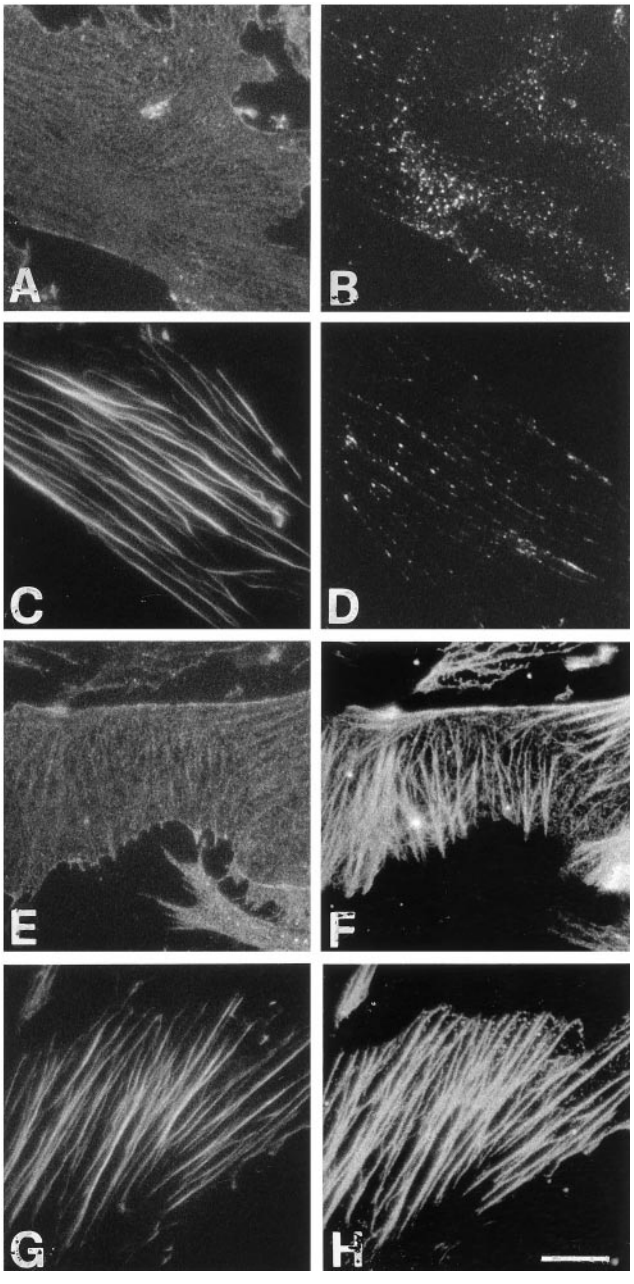

Figure 8. Reconstitution of the binding of α-actinin to VPMs in the presence of actin stress fibers. After a first incubation for 10 min in low-ionic-strength, high-[Ca^{2+}]–containing buffer, VPMs were incubated for a further 10 min in the same buffer in the absence (A–D) or presence (E–H) of purified α-actinin, as described in MATERIALS AND METHODS. After fixation, F-actin was detected with FITC-phalloidin (C and G), β1 integrins were detected with the β1-cyto antibody (A and E), and α-actinin was detected with a specific mAb (B, D, F, and H). Same fields are shown in A and B, C and D, E and F, and G and H. Bar, 10 μm.

of several focal adhesion proteins on VPMS (e.g., vinculin; see Figure 5F), these data indicate that focal adhesion complexes are required to specifically recruit actin under conditions in which new actin polymerization is inhibited.

With the aim of starting to analyze the minimal requirements for the recruitment of actin at adhesive sites, we tried to reconstitute Rh-actin binding to partially reconstituted adhesive complexes in the presence of actin polymerization inhibitors. Incubation of VPMs containing exogenous α-actinin were prepared as described in the previous paragraph (see Figure 9L) and incubated for 2 min at 37°C with 1 μM Rh-actin in the presence of cytochalasin D and DNase I. Under these conditions, detectable levels of Rh-actin could not be observed on the membranes (Figure 10L). This result suggests that complexes between active β1 integrins and α-actinin are not sufficient to recruit exogenous actin to sites of interaction between the cell and the ECM.

DISCUSSION

Four major findings have been described in this study by using VPMs as a cell-free system to study integrin function. First, we found that [Ca^{2+}] affects β1 integrin localization in a cytoskeleton-independent manner, and that a correlation exists between the pattern of distribution of the receptors and their activation state. Second, the relocalization of the receptors in the presence of low [Ca^{2+}] requires the cytoplasmic portion of the integrin β1 subunit. Third, focal adhesions represent a site for specific recruitment of exogenous actin under conditions that prevent actin polymerization along stress fibers. Finally, the use of the cell-free system has allowed the partial reconstitution of a [Ca^{2+}]-modulated receptor complex in F-actin–depleted VPMs.

The analysis of the mechanisms involved in focal adhesion formation and regulation is complicated by the fact that several events may concomitantly occur during assembly and disassembly of a focal adhesion in the living cell. The possibility of uncoupling some of these events would facilitate the analysis of the mechanisms involved. The presence of accessible integrin receptors in an intact lipid bilayer in VPMs makes this an ideal system for the experimental manipulation of integrin function and distribution.

Most studies on the regulation of β1 integrins by divalent cations have involved either solubilized receptors or cell binding assays with intact cells (Humphries, 1996). By using VPMs, we have been able to correlate modifications of the localization of the receptors with changes in their functional properties. Integrin α and β subunits contain binding sites for divalent cations, which can positively (Mg^{2+} or Mn^{2+}) or negatively (Ca^{2+}) affect integrin–ligand affinity (Tuckwell *et al.*, 1992). Modulation of integrin-mediated cell adhesion to ligands by these cations suggests that the ratio between Mg^{2+} and Ca^{2+} is

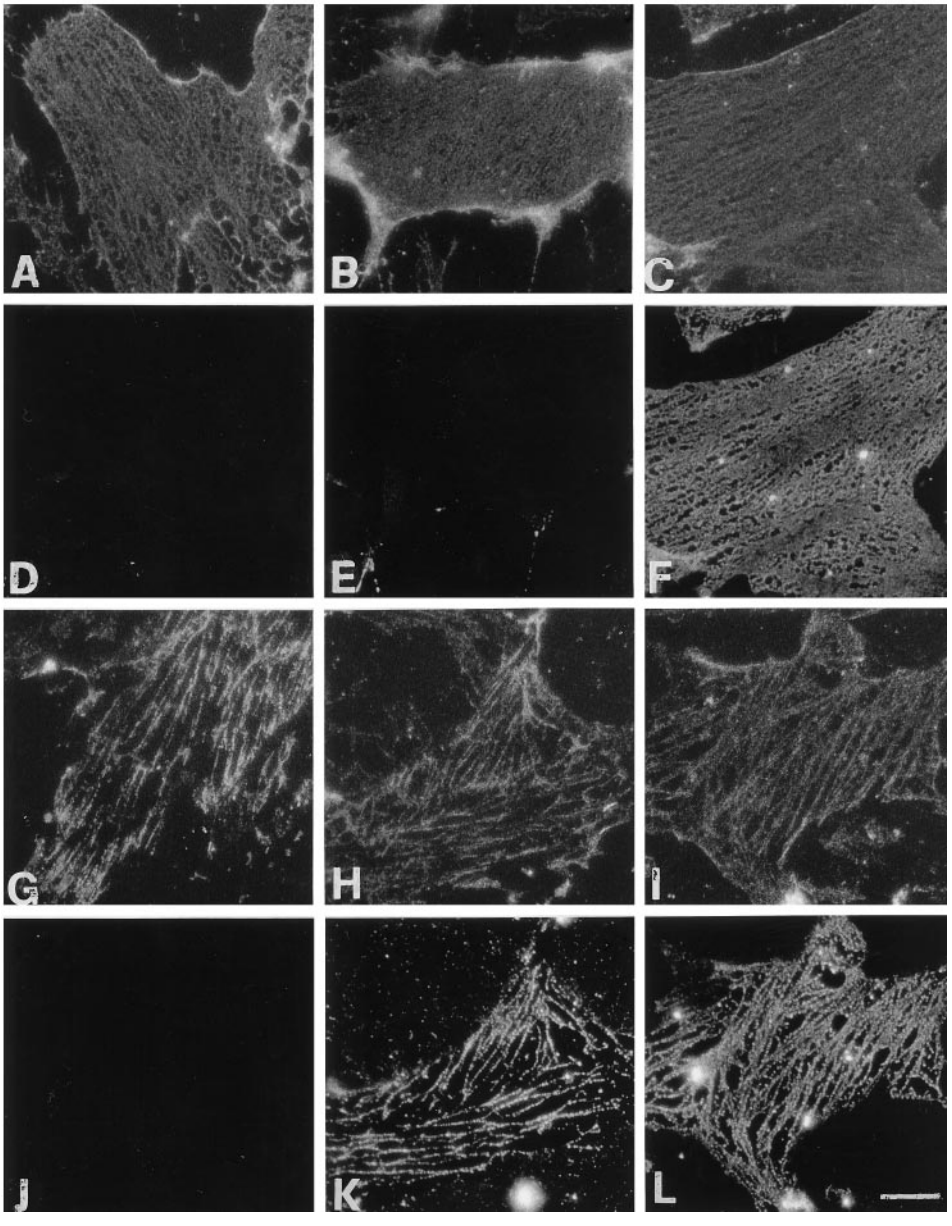

Figure 9. Reconstitution of the binding of α-actinin to VPMs in the absence of F-actin. VPMs were incubated for 10 min in low-ionic-strength buffer containing 4 μM gelsolin at high [Ca^{2+}] (A–F, I, and L), or low [Ca^{2+}] (G, H, J, and K). The samples were further incubated at 37°C in a buffer with high [Ca^{2+}] (A–F, I, and L), or low [Ca^{2+}] (G, H, J, and K). (C, F, H, I, K, and L) The second incubation was in the presence of purified α-actinin, as detailed in MATERIALS AND METHODS. Membranes were then washed and fixed for immunofluorescence. For low [Ca^{2+}]-induced α-actinin redistribution (I and L), after the second incubation at 37°C for 10 min with α-actinin in high [Ca^{2+}]-containing buffer, the samples were immediately transferred to low [Ca^{2+}]-containing buffer and incubated for 10 min at 37°C to induce integrin redistribution along ECM fibrils before fixation. (A–C and G–I) Integrin distribution, by using the β1-cyto polyclonal antibody. (E, F, and J–L) Localization of α-actinin with the specific mAb. (D) F-actin localization with TRITC-phalloidin. Same fields are shown in A and D, B and E, C and F, G and J, H and K, and I and L. Bar, 10 μm.

involved in the regulation of integrin function, which may in turn influence cell behavior (Grzesiak *et al.*, 1992). According to this model, we found that endogenous β1 integrins rapidly redistribute within VPMs incubated at different [Ca^{2+}] levels in the presence of 2.5 mM Mg^{2+}. In particular, high [Ca^{2+}] causes dispersion of integrins out of focal adhesions, whereas β1 integrins rapidly concentrate along ECM fibrils at low [Ca^{2+}] (see scheme of Figure 11A). The mechanisms by which divalent cations modulate integrin activity in intact cells are not clear yet. Although from our analysis we cannot conclude whether the effects induced by Ca^{2+} are due to the action of this ion extracellularly and/or intracellularly, one interesting finding is that redistribution in the presence of low [Ca^{2+}] requires the presence of the cytopalsmic domain of the β1 subunit. Interestingly, Haas and Plow (1996) have demonstrated the formation of a ternary complex between the cytoplasmic domains of αIIb and β3 and a cation, which may constitute a functional intracellular domain and may open the way to the exploration of the regulation of integrin function by intracellular divalent ions.

Integrin distribution cannot be altered by changing the [Ca^{2+}] in the medium of intact CEFs (Cattelino and de Curtis, unpublished results). This observation suggests

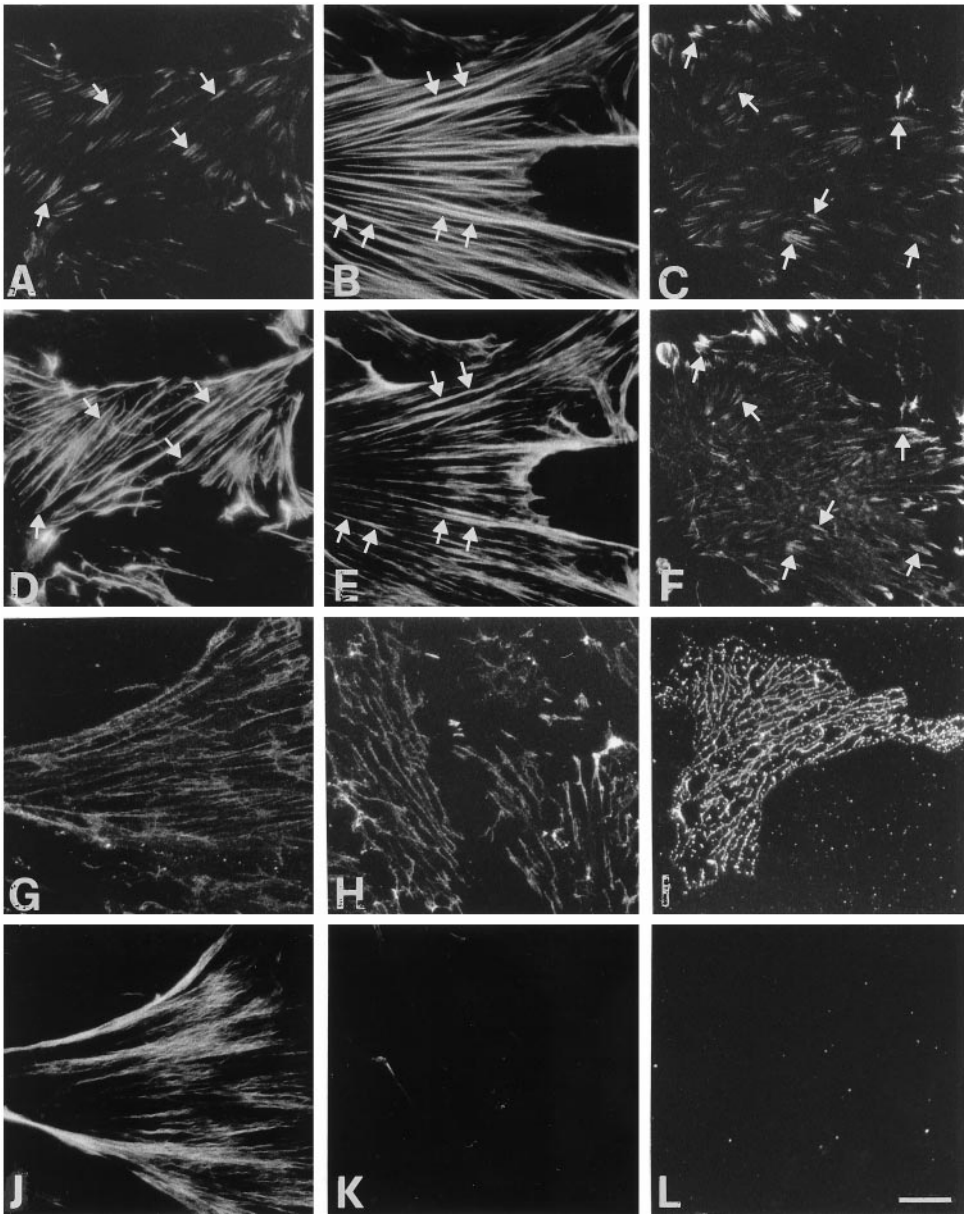

Figure 10. Accumulation of exogenous actin at focal adhesion sites is not affected by inhibitors of actin polymerization. VPMs were incubated for 2 min at 37°C in LCB with 1 μM Rh-actin either immediately after preparation (A–F) or after 15 min incubation at 37°C in LCB (G, H, J, and K). (I and L) VPMs were incubated with exogenous α-actinin (as described in the legend of Figure 9L) before incubation for 2 min at 37°C with Rh-actin in LCB. (C, F, H, I, K, and L) 100 nM cytochalasin D and 100 μg/ml DNase I were present during the incubation with Rh-actin. After fixation, the samples were processed for immunofluorescence with the anti-vinculin mAb (A and C), the β1-cyto polyclonal antibody (G and H), or the anti-α-actinin polyclonal antibody (I). In B, F-actin was detected with FITC-phalloidin, whereas in D, E, F, J, K, and L, bound Rh-actin is shown. Arrows in A, D, C, and F point to sites where Rh-actin colocalizes with vinculin in focal adhesions, whereas arrows in B and E indicate colocalization of Rh-actin with stress fibers. The same field is shown in A and D, B and E, C and F, G and J, H and K, and I and L. Bar, 10 μm.

that cellular events could modulate the affinity of β1 receptors for the ligand. On the other hand, Stuiver *et al.* (1996) have shown that β3 integrin distribution in intact MG-63 osteosarcoma cells can be regulated by the type of extracellular divalent ion, implicating different mechanisms for different receptors and/or cell types.

To correlate [Ca^{2+}]-induced integrin redistribution with changes in receptor activity, we have used function-modulating mAbs. It has been recently shown that two distinct populations of β1 receptors are present on the surface of CEFs, which may correspond to two different functional states of the receptors (Cruz *et al.*, 1997). By using the function-blocking mAb CSAT (Neff *et al.*, 1982) and the β1-stimulating mAb TASC (Neugebauer and Reichardt, 1991), we have been able to confirm these data on untreated VPMs from CEFs. In addition, the inability of TASC to recognize the receptors on VPMs treated at high [Ca^{2+}] is an indication of the low-affinity state of the diffuse β1 receptors, whereas TASC-positive receptors concentrated along ECM fibrils at low [Ca^{2+}] correspond to high-affinity receptors. Interestingly, the stimulatory mAb TASC is able to induce clustering of diffuse receptors along ECM fibrils even in the presence of high [Ca^{2+}] Cattelino and de Curtis, unpublished results). Because morphological analysis did not reveal any evident

[Ca^{2+}]-mediated reorganization of the ECM, our data suggest that the pattern of distribution of the high-affinity integrins is a consequence of the activation of the receptors induced by low [Ca^{2+}] and depends on the organization of the available ECM. According to this hypothesis, at low [Ca^{2+}] high-affinity (TASC-positive) β1 receptors were diffuse on substrates uniformly coated with ligand (Cattelino and de Curtis, unpublished results).

Although the diffuse, low-affinity β1 integrins could be concentrated along ECM fibrils by lowering the [Ca^{2+}] in the buffer, low [Ca^{2+}] somehow locks the receptors in the high-affinity state (see scheme of Figure 11A). Diffusion of the receptors could only be achieved by incubation of VPMs with the function-blocking mAb CSAT. One possible explanation for this observation comes from studies pointing at the interplay between distinct cation binding sites in the regulation of ligand binding (Smith *et al.*, 1994; Hu *et al.*, 1996). Mould *et al.* (1995) found that although high [Ca^{2+}] can displace Mg^{2+} from the integrin, low [Ca^{2+}] greatly increased the apparent affinity of Mg^{2+} for its binding site, suggesting the existence of a distinct high-affinity Ca^{2+}-binding site. This may lead to more efficient ligand binding by the receptor, which may not be reversed by subsequently increasing the [Ca^{2+}].

Intriguingly, VPMs remain spread on the substrate even after incubation for several hours at high [Ca^{2+}], suggesting that adhesion may be mediated by low-affinity binding of β1 integrins to the ECM or by other receptors. Similarly, serum-starved Swiss 3T3 cells remain spread and adherent even when no detectable focal contacts are present (Nobes and Hall, 1994), and it has been recently shown that adhesion of serum-starved cells is still RGD dependent (Barry *et al.*, 1997). The finding that integrin β3 and β5 subunits are present in much lower amounts in CEFs compared with the β1 subunit (Bossy and Reichardt, 1990) suggests that β1 receptors are the major integrin players in VPM adhesion to endogenous ECM. Moreover, analysis of the distribution of the β3 subunit in untreated VPMs showed a diffuse, weak signal, which was not affected by incubation at 37°C at different [Ca^{2+}] levels (Cattelino and de Curtis, unpublished results). These observations exclude β3 integrins as likely major players in the adhesion of VPMs. On the other hand, given the inability of the function-blocking CSAT mAb to detach VPMs from the substrate (Cattelino and de Curtis, unpublished data), the role of other, non-integrin receptors in the adhesion of VPMs to ECM cannot be excluded.

An interesting finding of this study is the uncoupling of integrin redistribution from actin stress fibers and focal adhesions under cell-free conditions. Contractility is important for actin stress fibers and focal adhesion formation (Chrzanowska-Wodnicka and Burridge, 1996), and stress fibers are essential for the maintenance of focal adhesions in intact cells (Domnina *et al.*, 1982). Incubation of VPMs at 37°C leads to a drastic loss of components from the focal contacts of a major fraction of VPMs, indicating that a detectable presence of these proteins at the membrane is not required for integrin redistribution under cell-free conditions. Uncoupling of [Ca^{2+}]-induced integrin localization from the actin cytoskeleton was shown by the fact that disassembly of stress fibers with gelsolin did not affect [Ca^{2+}]-mediated receptor redistribution at 37°C and by the finding that disassembly of stress fibers by gelsolin at 0°C did not perturb receptor localization into focal contact areas. In this direction, Pavalko and Burridge (1991) have shown that disassembly of stress fibers by microinjection of α-actinin fragments into cells only partially affected focal adhesions. On the other hand, it is known that gelsolin-induced stress fiber disassembly in living fibroblasts causes rounding up of cells (Cooper *et al.*, 1987).

We find that reversion of high [Ca^{2+}]-induced diffusion of integrins by low [Ca^{2+}] leads to recruitment of the receptors in elongated ECM fibrillar structures, which are clearly distinguishable from the focal adhesions found in control VPMs (Figure 5, compare, for example, A with C). As depicted in Figure 11A, in intact CEFs and in untreated VPMs, β1 integrins clustered into focal adhesions colocalize with the tip of stress fibers on one face of the membrane and often with part of an ECM fibril on the other face of the membrane (for example, see Figure 2, A and B). In contrast, low [Ca^{2+}]-induced, elongated integrin clusters evidently colocalize with ECM fibrils (see Figure 2, E and F), whereas ultrastructural analysis shows that colocalization of β1 receptors with stress fibers is not evident under these conditions. These findings suggest that coupling between actin stress fibers and ligand-driven localization of high-affinity receptors is required for the recruitment of β1 integrins into focal adhesions of intact cells.

Ex novo reconstitution of adhesive complexes from purified components is made difficult by the fact that focal adhesion assembly occurs at the plasma membrane where clusters of receptors are necessary, and both extracellular ligands and the intracellular milieu are required. Another major finding of this study is the reconstitution and modulation by [Ca^{2+}] of the distribution of a partially reconstituted receptor complex in actin-depleted VPMs. Reconstitution of the binding of focal adhesion proteins to permeabilized or partially disrupted cells has been reported (Avnur *et al.*, 1983; Ball *et al.*, 1986). In our system we can test the binding of purified components to VPMs in which β1 receptors affinity for the ligand can be modulated. The use of purified α-actinin, an actin-binding protein known to interact in vitro with the cytoplasmic tail of integrins (Otey *et al.*, 1990), resulted in decoration of

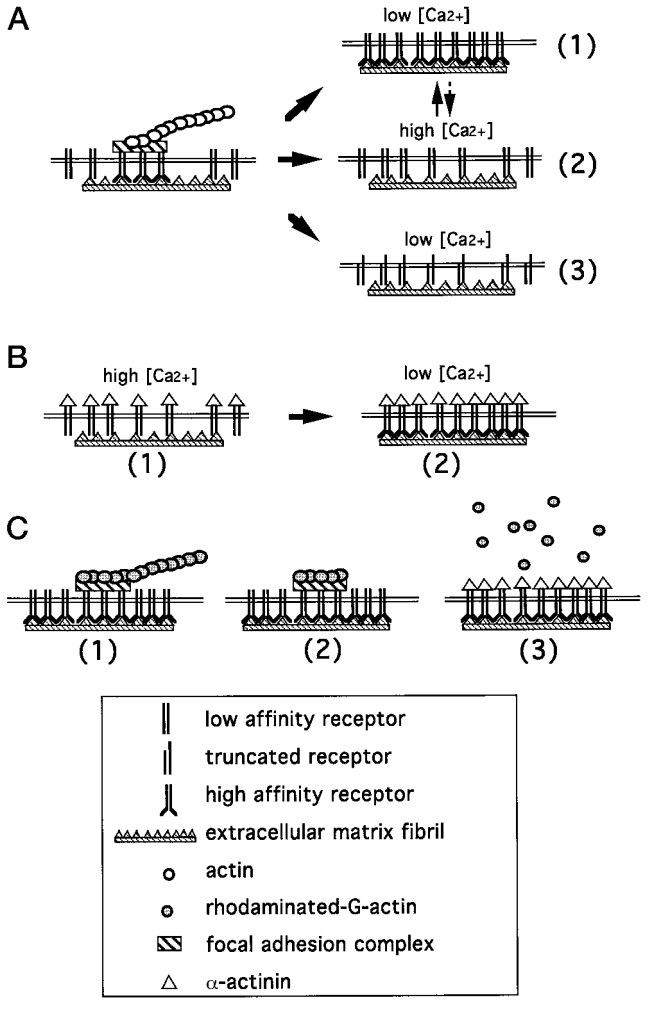

Figure 11. Summary of the results presented in this study: schematic representation of models from the results obtained in this study (also see DISCUSSION). (A) Left drawing, on the ventral surface of intact cells and in untreated VPMs, β1 integrins are found both concentrated in focal adhesions (high-affinity receptors) and diffuse (low-affinity receptors). In focal adhesions, integrins colocalize with focal adhesion components and with the tip of actin stress fibers, whereas they only partially colocalize with ECM fibrils. (1) Low [Ca^{2+}] with 2.5 mM Mg^{2+} induces β1 integrin clustering along ECM fibrils, which may be caused by an increase in receptor affinity for the ligands. (2) High [Ca^{2+}] with 2.5 mM Mg^{2+} causes diffusion of β1 integrins out of focal adhesions, and this may be due to a decrease in the affinity of the receptors for the ligands. [Ca^{2+}]-induced redistribution of β1 receptors does not require actin stress fibers or detectable amounts of focal adhesion proteins. Moreover, high [Ca^{2+}]-induced receptor diffusion can be quasi-reversed by incubation at low [Ca^{2+}] (solid arrow), whereas low [Ca^{2+}]-induced receptor clustering along ECM fibrils can only be affected by incubation in the presence of receptor function-blocking antibodies (dotted arrow). (3) Low [Ca^{2+}] is unable to induce clustering along ECM of integrins with a deleted β1 cytoplasmic domain. In this respect, the finding that activating β1 antibodies are able to induce redistribution of the truncated receptors suggests that low [Ca^{2+}] is not sufficient to activate the truncated integrins and that low [Ca^{2+}]-mediated activation of the wild-type receptor acts via mechanisms requiring the cytoplasmic domain of the β1 subunit. (B) Exogenous actin stress fibers on VPMs, confirming published observations (Geiger, 1981). The novel finding from our study is that after disruption of stress fibers by gelsolin, we can still observe binding of exogenous α-actinin to VPMs in conditions of both high and low affinity of the receptors for the ligands. The pattern of distribution of exogenous α-actinin overlaps with that of β1 integrins and is equally dependent on the [Ca^{2+}] (Figure 11B). The low [Ca^{2+}]-induced codistribution of prebound, exogenous α-actinin with endogenous β1 receptors strongly indicates that they are part of the same complex (Figure 11B). These results suggest that molecular interactions that are believed to be important for focal adhesion assembly can be reconstituted in a cell-free system, and that modulation of the partially reconstituted receptor complex is feasible in this experimental setup.

Reconstitution experiments with VPMs have also evidenced a special property of focal adhesion complexes in recruiting exogenous actin under conditions in which actin polymerization is inhibited (diagram of Figure 11C, 1 and 2). The concentration of cytochalasin D used should indeed block the barbed ends of the actin filaments present (Cooper, 1987). On the other hand, the amount of DNase I used should block all pointed ends and may also bind to the exogenous G-actin. If this is true, exogenous G-actin recruitment to preformed actin filaments should be prevented. Our finding indicates that the actin filament ends are still available at focal adhesions, suggesting that these sites must protect the actin filaments from cytochalsin D and DNase I. One alternative explanation could be that focal adhesions contain proteins that may specifically recruit available G-actin monomers or dimers, by interacting to available sites on the actin molecule. Such a complex may regulate filament growth and may have the ability to nucleate actin polymerization under appropriate conditions. The finding that the reconstituted integrin–α-actinin complex fails to support recruitment of exogenous G-actin to the cell membrane (Figure 11C, 3) is not surprising, considering that α-actinin is a bundling protein, able to interact with F-actin. On the other hand, this result would support the hypothesis that exogenous actin is recruited as monomers in the focal adhesions of VPMs, and that focal adhesions represent preferential actin nucleation sites on the membrane.

Figure 11 (cont). α-actinin bound to actin-depleted VPMs colocalizes with β1 integrins at high [Ca^{2+}] (1) and redistributes with integrins along ECM fibrils at low [Ca^{2+}] (2). (C) Exogenous actin added to VPMs polymerizes along stress fibers (1). Under conditions that prevent actin polymerization (in the presence of cytochalasin D and DNase I), recruitment along stress fibers is prevented, but exogenous actin is still specifically recruited to focal adhesions (2). In reconstitution studies, a complex between activated integrins and α-actinin is not sufficient to recruit exogenous actin on VPMs when actin polymerization is prevented (3).

Altogether, our results show that modulation of integrin affinity in the presence of the extracellular ligands is not sufficient to organize focal contacts, although it is sufficient to induce integrin clustering along ECM fibrils. The uncoupling between integrin clustering and formation of focal adhesions even in the presence of stress fibers could be due to lack or inactivation of important cytoplasmic factors in our experimental conditions (Chen *et al.*, 1994; O'Toole *et al.*, 1994). On the other hand, the identification of experimental conditions allowing specific recruitment of actin into focal adhesions can be used to further explore the molecular machinery connecting adhesive receptors to actin organization by using the described cell-free system.

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