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Differential distribution of two cytoplasmic variants of the α6β1 integrin laminin receptor in the ventral plasma membrane of embryonic fibroblasts

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SUMMARY

The integrin α6β1 is a receptor involved in the adhesion of several cell types to laminin. By using function-blocking antibodies, we have shown that α6β1 is a functional laminin receptor in chick embryo fibroblasts. We also found that these cells express two variants of the α6 subunit, α6A and α6B, characterized by different cytoplasmic domains. By using indirect immunofluorescence with isoform-specific polyclonal antibodies, we showed that the two isoforms of the α6 subunit distribute differently on the ventral plasma membrane of these cells cultured on laminin-coated substrates. In fact, while the α6A subunit was found codistributing with vinculin in focal contacts, the α6B subunit showed a homogeneously distributed punctate pattern. This difference was particularly evident when preparations of ventral plasma membranes were used for the immunolocalization. Furthermore, when cells were cultured on fibronectin, a substrate not recognized by the α6β1 laminin receptor, the distribution of the two α6 isoforms was similar to that observed on laminin, with α6A still colocalizing with vinculin in focal adhesions. Our results indicate that two forms of the α6β1 laminin receptor coexpressed in the same cells show distinctive distributions, and suggest that receptor occupancy by laminin is not essential for the accumulation of the α6Aβ1 integrin in adhesion plaques.

Key words: integrin, fibroblast, focal adhesion

INTRODUCTION

Integrin cytoplasmic domains have been attracting increased interest during the last years because they seem to be involved in transmembrane signaling and have been shown to interact with the cytoskeleton during the processes of cell adhesion and spreading on extracellular matrix (see Sastry and Horwitz, 1993, for a review). Recently, variants with alternative cytoplasmic domains generated by differential RNA processing have been described for the α3, α6 and α7 integrin subunits (Tamura et al., 1991; Hogervost et al., 1991; Collo et al., 1993; Song et al., 1993; Ziober et al., 1993), but their functional significance remains to be established.

The integrin α6β1 plays an important role in mediating cellular adhesion to laminin in a variety of cell types (Sonnenberg et al., 1988, 1990; Hall et al., 1990; Kramer et al., 1990; Shaw et al., 1990; Shimizu et al., 1990; Cooper et al., 1991; de Curtis et al., 1991; Elices et al., 1991). This integrin receptor is specific for different isoforms of laminin, and cannot bind to any other identified extracellular matrix (ECM) constituent (Sonnenberg et al., 1988; Delwel et al., 1993). By using PCR analysis or antibodies specific for the two alternative cytoplasmic domains of the α6 subunit, it has been found that the two different α6 isoforms have a distinct and developmentally regulated distribution in various tissues (Tamura et al., 1991; Cooper et al., 1991; de Curtis and Reichardt, 1993; Hogervorst et al., 1993), and that different cell types can express both or only one of the two isoforms. Recent results indicate that the α6 cytoplasmic domain is essential for binding of the α6β1 receptor to laminin (Shaw and Mercurio, 1993). Furthermore, the cytoplasmic domains of different integrin α subunits play different roles in post ligand binding events (Chan et al., 1992), and in the regulation of ligand binding affinity (O'Toole et al., 1991, 1994). Therefore, the existence of different cytoplasmic variants for the α6 subunits suggests that they may play distinctive roles in the signaling of the laminin receptor during the process of cell adhesion, spreading, migration and neurite outgrowth. Recent results obtained by using cell lines transfected independently with each of the two α6 isoforms do not show dramatic differences in their ability to adhere to different laminin isoforms, and in their ability to be regulated by phorbol esters in a transfected macrophage cell line (Delwel et al., 1993; Shaw et al., 1993). One explanation could be that more subtle differences may exist in post-binding events or in the modulation of receptor function mediated by the cytoplasmic variants, as indicated by a recent study (Shaw and Mercurio, 1994).

In previous work we have shown that retinal neurons express both the α6A and α6B variants, and that the two isoforms have different distribution patterns in the developing chick embryo retina (de Curtis and Reichardt, 1993). More recently, we have found that the two isoforms of the α6β1 laminin receptor extracted from cultured retinal neurons show different sedimentation behaviours when separated on sucrose gradients,

indicating different biochemical properties of these two isoforms (de Curtis and Gatti, 1994). In the present paper we have characterized the adhesive properties of chicken embryo fibroblasts (CEFs) on laminin, and we have shown that α6β1 is an important laminin receptor for these cells. Biochemical and immunocytochemical characterization of the laminin receptor in these cells showed that both α6A and α6B are expressed by CEFs, and that the two isoforms show a dramatic difference in the pattern of distribution on the ventral portion of the plasma membrane. In fact, α6A codistributed with vinculin in focal adhesions, while α6B showed a homogeneously distributed punctate pattern and was not concentrated in focal adhesions. The differential distribution of the two isoforms of the laminin receptor was independent of the substrate on which the cells were cultured, and did not change between short and long culture periods on substrates coated with purified ECM components.

MATERIALS AND METHODS

Reagents and solutions

Chicken eggs were purchased from Incubatoio La Lunga (Besozzo Bardello, Italy). Laminin was purified from Engelbreth-Holm Swarm sarcoma as published (Timpl et al., 1979). Human fibronectin was from Collaborative Research (Bedford, MA). Arg-Gly-Asp-Ser (RGDS) synthetic peptide was from Sigma Chemical Co. (St Louis, MO). Nitrocellulose filters were from Schleicher & Schuell Inc. (Dassel, Germany). Protein A-Sepharose CL-4B and CNBr-Sepharose CL-4B were from Pharmacia LKB Biotechnology Inc. (Piscataway, NY). ^{125}I-Protein A and [^{3}H]glucosamine were from Amersham (Arlington Height, IL).

Protein determination was performed according to Bradford (1976) using a Bio-Rad kit (Bio-Rad Laboratories, Richmond, CA).

Cell culture and metabolic labeling

Chicken embryo fibroblasts (CEFs) were isolated from 10-day-old embryos and cultured at 37°C, 5% CO_2 in DMEM containing 5% fetal calf serum (FCS), 100 U/ml penicillin and streptomycin, 2 mM glutamine. CEFs up to the eighth passage were used for experiments. For metabolic labeling, CEFs were incubated overnight with 100 μCi/ml of [^{3}H]glucosamine in glucose-free medium, supplemented with 0.2 g/l of glucose, 1% FCS, 10 mM sodium pyruvate, 100 U/ml penicillin and streptomycin, 2 mM glutamine.

Antibodies

Two different polyclonal antibodies raised against the same 35 amino acid-long peptide of the cytoplasmic spliced variant α6B were used: α6-cytoB, which has been previously characterized (de Curtis and Reichardt, 1993), and α6-cytoB$_2$, which was produced in rabbit by immunization with the peptide complexed to soybean trypsin inhibitor. Both antibodies were affinity purified on a peptide-CNBr-Sepharose CL-4B column. The production, purification and use of the polyclonal antibody α6-cytoA (de Curtis et al., 1991), of the polyclonal antibody α6-EX against the amino-terminal portion of the chicken α6 subunit (de Curtis and Reichardt, 1993), of the polyclonal antibodies β1-cyto and α5-cyto raised against peptides from the cytoplasmic domains of the β1 and α5 integrin subunits (Tomaselli et al., 1988), and of the anti-laminin polyclonal antibody JW2 (Lander et al., 1983) have been previously described. The monoclonal antibody CSAT against the chick integrin β1 subunit (Neff et al., 1982) was a generous gift from Dr A. F. Horwitz (University of Illinois, Urbana, Illinois), and the monoclonal antibody 16G3 (Nagai et al., 1991), which binds to human fibronectin, was a generous gift from Dr K. M. Yamada (National Institute of Health, Bethesda, MD). The monoclonal antibody against vinculin was purchased from Sigma.

Cell attachment assays

For these, 96-well Linbro/Titer plates (Flow Laboratories, Inc., McLean, VA) were coated overnight at room temperature with 10 μg/ml (or with the indicated concentration) of laminin in TBS or fibronectin in PBS. Coated and uncoated wells were incubated for 3 hours at 37°C with 1% BSA in PBS and washed twice with PBS. CEFs were detached from a 100 mm diameter dish with 0.05% trypsin and 0.02% EDTA, washed twice in culture medium without serum, and plated at a concentration of 30-60,000 cells/well in the absence of serum. When indicated, CEFs were incubated 30 minutes before plating with 100 μg/ml of CSAT, or 0.5 mg/ml of the α6-EX affinity purified antibody. In the assays done in the presence of the anti-fibronectin mAb 16G3 or the anti-laminin polyclonal antibody JW2, ligand-coated wells or coverslips (see below) were incubated overnight with 100 μg/ml of the specific IgG before addition of the cells, and mantained in the wells during the incubation. To look at the effect of the RGDS peptide on cell adhesion, the peptide was directly added to the cells at the moment of plating, at the indicated concentration. After culture for the indicated time, non-adherent cells were removed and wells were processed for cell attachment assay and quantitated as described previously (de Curtis and Reichardt, 1993). In brief, unattached cells were removed by brisk addition of warm medium followed by gentle vacuum suction. The cells were fixed with 3% paraformaldehyde, stained with Crystal Violet (0.5% in 20% methanol), washed with water, solubilized with 1% SDS, and A_{540} was measured in each well. The percentage of inhibition of cell attachment on laminin or fibronectin was calculated as follows:

$$\%\ \text{inhibition} = 1 - \frac{A_{540}\ (\text{treated cells}) - A_{540}\ (\text{BSA})}{A_{540}\ (\text{untreated cells}) - A_{540}\ (\text{BSA})} \times 100\,.$$

In all experiments, adhesion to BSA-coated substrates was negligible. When coverslips were used, they were rinsed twice with PBS to remove non-adherent cells and immediately photographed in phase contrast using an inverted microscope.

Cell extraction, immunoprecipitation, SDS-PAGE and immunoblotting

Confluent CEFs from each 100 mm diameter culture dish were rinsed twice with ice-cold TBS, solubilized with 0.5 ml of lysis buffer (TBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml each of antipain, chymostatin, leupeptin and pepstatin) containing 1% Triton X-100, followed by end-over-end mixing for 30 minutes at 4°C. Insoluble material was removed after centrifugation for 10 minutes at 11,000 ***g*** in a refrigerated microfuge. Aliquots of cell lysate containing 0.5-1 mg protein were incubated 4 hours at 4°C with the specific antibody; 5 μl of antiserum, 10 μl of preimmune serum, or 15 μg of affinity purified antibody were used for each immunoprecipitation. Where indicated, the lysate was boiled for 5 minutes in the presence of 0.5% SDS, and diluted to 0.1% SDS with lysis buffer, before addition of the α6-EX antibody; 25 μl of Protein A-Sepharose beads were added to each immunoprecipitate and incubated for 45 minutes at 4°C. The beads were then washed two or three times with 1 ml of lysis buffer containing 0.2% Triton X-100.

For metabolically labeled cells, similar amounts of TCA-precipitable ^{3}H cpm and 5 μl of immune serum were used for each immunoprecipitation. Immunoprecipitates were washed 8 times with 1 ml of lysis buffer containing 0.2% Triton X-100, once with the same buffer containing 0.5 M NaCl, and once with 20 mM Tris-HCl, pH 7.5.

The immunoprecipitates were then analyzed by SDS-PAGE on 6% acrylamide gels, according to the method of Laemmli (1970). Gels loaded with radioactive immunoprecipitates were dried and exposed to preflashed Hyperfilm-MP films (Amersham).

Western blot methods were as described (de Curtis et al., 1991). α6-EX serum (1:400) was used as primary antibody and incubated for

2 hours at room temperature. For the detection of the primary antibody 0.2 μCi/ml of ^{125}I-Protein A (Amersham, Aarlington Heights, IL) were used and the filters were exposed to Amersham Hyperfilm-MP.

Cell culture on coverslips and preparation of ventral plasma membranes

Glass coverslips were cleaned by boiling in 0.1 M HCl, washing with 70% ethanol, rinsing with double distilled water, and drying in air. Cleaned coverslips were coated with dimethylchlorosilane by briefly dipping them in a 2% solution of dimethylchlorosilane in trichloroethane (BDH Laboratory supplies, England). Coverslips were dried, rinsed with water and sterilized before coating with purified ECM glycoproteins. Treated coverslips were coated with laminin (20 or 100 μg/ml, as indicated) or fibronectin (20 μg/ml) overnight at 4°C, and non-specific binding was blocked by incubation of the coverslips with 1% BSA in PBS, for 3 hours at 37°C. After rinsing twice with PBS, CEFs were cultured in serum-free medium for the indicated times. In each experiment, silane-coated coverslips coated with BSA only were used to assess non-specific binding to the glass, which was always negligible.

For experiments in the presence of cycloheximide, CEFs were cultured for 2 hours with 20 μg/ml of cycloheximide in serum-free medium before trypsinization, and trypsinization was stopped by adding 1 mg/ml soybean trypsin inhibitor. Cells plated on coverslips were subsequently cultured for 1½ hours in serum-free medium with 20 μg/ml of cycloheximide before fixation.

For the preparation of ventral plasma membranes (VPMs) a modification of the lysis-squirting technique was utilized (Nermut et al., 1991). Cells cultured on coverslips were washed twice with ice-cold water. After 1 minute, cells were squirted over by using a jet of ice-cold water from a water bottle, and immediately fixed with 3% paraformaldehyde.

Immunofluorescence

Cells cultured on coverslips or VPMs preparations thereof were fixed with 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with 0.2% gelatine in PBS before staining. When VPMs had to be incubated with the α6-EX antibody, 0.2% SDS was used instead of 0.2% Triton X-100. Cells were then incubated for 1 hour at room temperature with 10 μg/ml β1-cyto IgG, 25 μg/ml affinity purified α6-cytoA IgG, 20 μg/ml affinity purified anti-α6-cytoB-2 IgG, 20 μg/ml affinity purified α6-EX IgG, or FITC-conjugated phalloidin (Sigma). In all cases, cells were coincubated with a mAb against vinculin (Sigma). Cells were then incubated for 30 minutes with FITC-conjugated sheep anti-rabbit IgG together with TRITC-conjugated sheep anti-mouse IgG (Boehringer, Mannheim, Germany), and observed using a Zeiss-Axiophot microscope.

Crosslinking experiments

A procedure similar to the one described by Enomoto-Iwamoto et al. (1993) was used to study the association of integrins with the extracellular matrix proteins coating the substrate. CEFs cultured on laminin- or fibronectin-coated coverslips for 2 hours were washed 3 times with PBS, and then incubated for 10 minutes at room temperature with 0.4 mM BS[3] in PBS, 2 mM PMSF (Pierce, Rockford, Illinois). Crosslinking was stopped by 2 minutes incubation with 10 mM Tris-HCl, pH 7.5, 2 mM PMSF; cells were washed 4 times with PBS and extracted 5 minutes with RIPA buffer (0.1% SDS, 0.1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM PMSF). After 3 washes with PBS, cells were fixed with paraformaldehyde and used for immunofluorescence, as described.

RESULTS

α6β1 is a laminin receptor for cultured CEFs

To characterize the interactions of CEFs with laminin, we first cultured the cells for 1½ hours in serum-free conditions on laminin-coated plastic at different substrate concentrations, using fibronectin for comparison. Fig. 1A shows the concentration-dependent increase of cell adhesion to the substrate, which reached a plateau around 10 μg/ml of protein coating concentration for both laminin and fibronectin. The results showed that adhesion of CEFs to fibronectin was significantly higher than to laminin. In fact, under these conditions, about

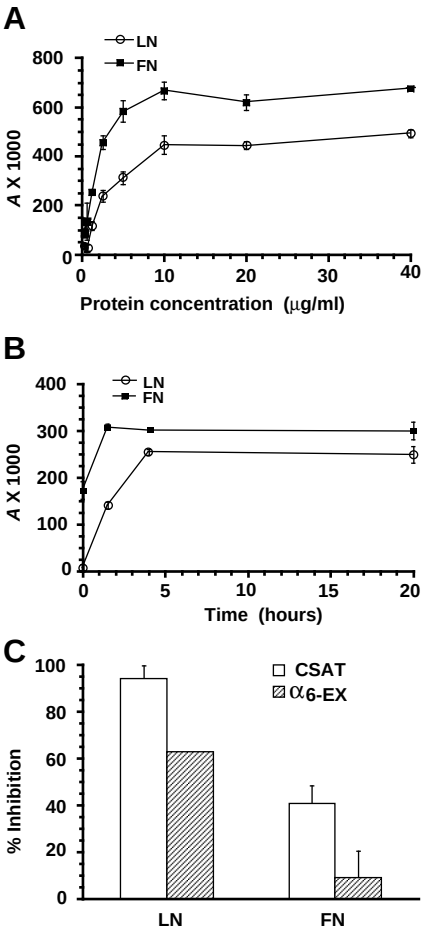


Fig. 1. Characterization of adhesion of CEFs to laminin and fibronectin. (A) Dependence of CEFs adhesion on substrate concentration. Cells were plated on wells coated with increasing concentrations (0.3 to 40 μg/ml) of laminin (LN, ○) or fibronectin (FN, ■), and allowed to adhere for 90 minutes. Cell adhesion was quantified as described in Materials and Methods, and expressed as *A* ×1,000. About 60,000 cells/well were used in this experiment. (B) Time course of CEFs adhesion on laminin and on fibronectin. CEFs were cultured on wells coated with laminin (LN, ○) or fibronectin (FN, ■) (20 μg/ml each) for the time indicated, and the adhesion to each substrate was measured as described in Materials and Methods. Each point is the mean of triplicate samples. About 30,000 cells/well were used in this experiment. (C) Inhibition of adhesion to laminin and fibronectin by anti-integrin function-blocking antibodies. CEFs were preincubated with the anti-β1 monoclonal antibody CSAT (100 μg/ml, □) or with the polyclonal antibody α6-EX (500 μg/ml, ▨) and plated on wells coated with laminin (LN) or fibronectin (FN). After 90 minutes culture, attachment assays were carried out as described in Materials and Methods. Each sample was in triplicate (CSAT) or duplicate (α6-EX). Bars indicate the s.d.

50% of the cells added remained attached to laminin-coated wells, and about 80% of the cells added were bound to fibronectin-coated wells (not shown). We also tested for specific binding to purified laminin or fibronectin after culturing CEFs in serum-free conditions for up to 20 hours (Fig. 1B). At all time points adhesion to laminin was lower compared to fibronectin. Binding of cells to fibronectin at time=0 (Fig. 1B) was due to the high adhesivity of CEFs to fibronectin even when plated for a few minutes at room temperature before the washings (see Materials and Methods).

To identify receptors involved in the adhesion of CEFs to laminin, the function-blocking anti-chick-β1 mAb CSAT and the polyclonal antibody α6-EX raised against the amino-terminal portion of the extracellular domain of the chick α6 integrin subunit were used in cell attachment assays. Both antibodies interfered heavily with adhesion of CEFs to laminin (Fig. 1C): CSAT inhibited adhesion almost completely, while α6-EX inhibited about 60% of CEFs adhesion. No significant inhibition by the α6-EX antibody of adhesion to fibronectin was detected, showing that the inhibition was specific for laminin, while the CSAT antibody only inhibited 40% of CEFs adhesion to fibronectin. In cell attachment assays in the presence of lower concentrations of fibronectin, CSAT was able to inhibit to a somewhat higher extent cell adhesion (up to about 50%), but was never able to abolish it completely (not shown), probably due to the presence of other non-β1 fibronectin receptors in these cells. The incomplete inhibition of adhesion to laminin by the α6-EX antibody could be due to the presence of other laminin receptors in these cells, or to the low efficiency of this antibody in recognizing the native form of the laminin receptor on the cell surface. In support of this hypothesis, we found that the α6-EX antibody was significantly more efficient in immunoprecipitating the denatured, mature α6 polypeptide than the non-denatured mature α6 (not shown).

CEFs express both cytoplasmic variants of the α6β1 laminin receptor

For the biochemical characterization of the α6β1 laminin receptor, Triton X-100 extracts from CEFs were immunoprecipitated with polyclonal antibodies raised against different portion of the laminin receptors. The use of cytoplasmic variant specific antibodies, α6-cytoA and α6-cytoB, allowed us to show that both isoforms of the laminin receptor were present in CEFs, as shown in Fig. 2a, where the lower band of about 130 kDa represents the mature, processed form of the α6 polypeptides (arrowheads), while the upper band, of about 150 kDa, represents the immature, non-cleaved form (arrows), as also indicated by the fact that a monoclonal antibody against the extracellular portion of the α6 subunit is also recognizing 2 bands with the same M_r in immunoblots (not shown).

The α6A isoform was clearly more abundant than α6B. We believe that these results reflect a real difference in the levels of expression of the two isoforms, and not just a difference in the efficiency of immunoprecipitation of the antibodies; in fact, it was possible to deplete the α6B subunit from chick tissue extracts expressing high levels of the α6B polypeptide with the α6-cytoB antibody (not shown). The affinity purified α6-cytoB$_2$ antibody was also efficient in immunoprecipitating the α6B polypeptide (Fig. 2a, lanes 4 and 5), and gave the best results in immunofluorescence experiments. Both α6A and

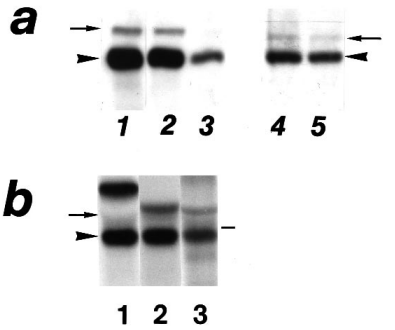


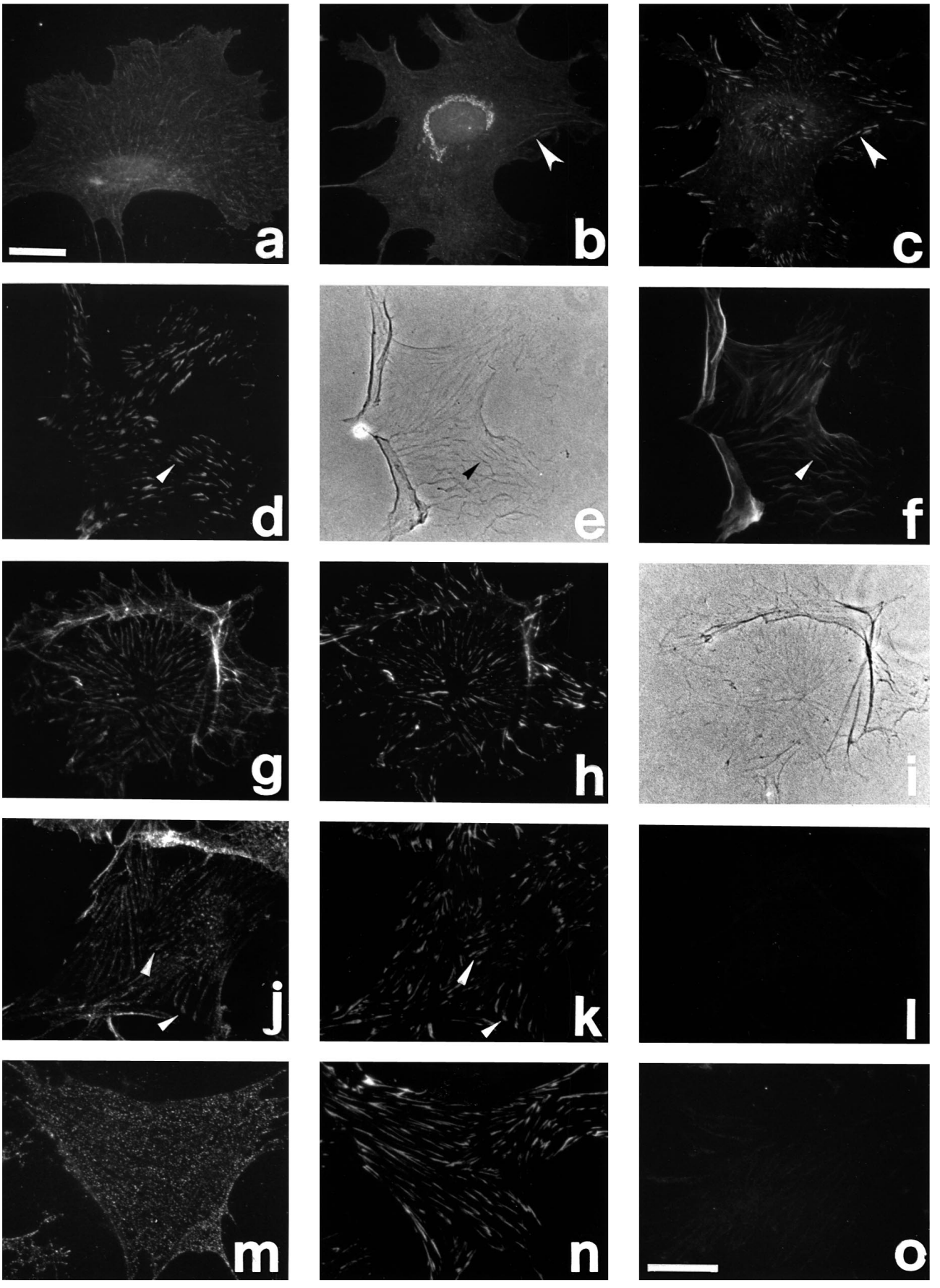
Fig. 2. Expression of α6Aβ1 and α6Bβ1 in CEFs. (a) Extracts from CEFs were obtained as described in Materials and Methods, and the immunuoprecipitations were carried out using the following antibodies: α6-EX (lane 1), α6-cytoA (lane 2), α6-cytoB (lanes 3 and 4), or α6-cyto-B$_2$ (lane 5). In lane 1, proteins in the cell extract were denatured by boiling with SDS before immunoprecipitation. The immunoprecipitates were analysed by western blot with the α6-EX antibody, after SDS-PAGE under reducing conditions. The arrowheads indicate the mature, processed α6 polypeptides, while the arrows indicate the immature form. (b) Immunoprecipitates from lysates of metabolically labeled CEFs using the following antibodies: α5-cyto (lane 1); α6-cytoA (lane 2); α6-cytoB$_2$ (lane 3). Immunoprecipitates were separated by SDS-PAGE under non-reducing conditions. The arrow shows the band corresponding to the α6 polypeptide, while the arrowhead indicates the coprecipitating β1 polypeptide. The molecular mass standard of 116 kDa is indicated on the right.

α6B could be specifically coprecipitated with the β1 integrin subunit (Fig. 2b), and they were both clearly different in molecular mass from the α5 subunit (160 kDa, Fig 2b, lane 1) that was immunoprecipitated with the α5-cyto antibody.

Differential distribution of the α6 subunit isoforms in CEFs cultured on laminin

Before looking at the subcellular distribution of the two different cytoplasmic variants of the α6 laminin receptor subunit, we made a particular effort to find conditions in which adhesion of CEFs to purified ECM glycoproteins was specific. When acid washed coverslips were treated with silane before coating with purified ECM components, non-specific adhesion to control coverslips, coated only with BSA, was negligible

Fig. 3. Distribution of integrin subunits in permeabilized CEFs and in VPMs. CEFs were cultured on laminin-coated coverslips for 20 hours in serum-free medium. Intact cells (a-c) or VPMs prepared from cells grown on coverslips coated with 100 μg/ml (d-f), or 20 μg/ml (g-o) laminin were fixed, permeabilized with Triton X-100, and stained for immunofluorescence as described in Materials and Methods. The primary antibodies used were: β1-cyto (a,g); α6-cytoA (b,j); preimmune serum for α6-cytoA diluted 1:250 (l); α6-cytoB$_2$ (m); preimmune serum for α6-cytoB$_2$ diluted 1:200 (o); anti-vinculin mAb (c,d,h,k,n); FITC-phalloidin (f). The same cell is shown in b and c; the same VPM is shown in d,e,f; in g,h,i; in j,k; in m,n. First antibodies were revealed by FITC anti-rabbit IgG and TRITC anti-mouse IgG. Arrowheads in b and c, and in j and k show colocalization of vinculin with integrin α6A subunit in focal adhesions. Arrowheads in d,e and f show overlap of vinculin with phalloidin staining and with dark filamentous structures visible by phase contrast in VPMs. Bars, 20 μm (bar in a applies to a-c; bar in o applies to d-o).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both after short or 20 hours culture in serum-free medium (not shown). Specific adhesion of CEFs was also confirmed by the virtually complete inhibition of cell attachment and spreading by the JW2 antibody after 20 hours culture on laminin-coated coverslips, while no effect was observed on laminin by the anti-fibronectin 16G3 mAb (not shown). Specific inhibition of cell adhesion by anti-laminin JW2 antibody was also observed after shorter culture periods (not shown). On the other hand, adhesion to fibronectin was significantly inhibited by the RGD peptide and by the mAb 16G3, but not by the JW2 anti-laminin antibody (not shown).

Once the conditions for specific adhesion of CEFs to laminin had been determined, we looked at the localization of integrin subunits by indirect immunofluorescence on adherent and spread cells after 20 hours culture on laminin. Both affinity purified α6-cytoA and α6-cytoB$_2$ antibodies specifically stained 100% of the cells, and the pattern of distribution of the two antigens was substantially different. The α6-cytoA showed a strong perinuclear staining corresponding to a typical Golgi staining (Fig. 3b). This was confirmed by the colocalization of the α6-cytoA perinuclear staining with the staining for the lectin WGA, a Golgi marker (not shown). The α6-cytoA antibody also showed a diffuse surface staining, and staining of peripheral focal adhesions, identified by the colocalization with the focal adhesion specific marker vinculin (Fig. 3b and c, arrowheads). In comparison, α6-cytoB$_2$ showed a punctate, homogeneously distributed surface staining, and did not show any evident accumulation in focal adhesions in permeabilized CEFs (not shown). A clear colocalization with focal adhesions together with a diffuse surface staining was observed with the β1-cyto antibody against the cytoplasmic portion of the β1 subunit (Fig. 3a).

To be able to look more clearly at the distribution of these integrin subunits on the portion of the plasma membrane in direct contact with the laminin- or fibronectin-coated substrates, we prepared VPMs after a brief hypotonic treatment of the cells, as described in Materials and Methods. These structures were visible by phase microscopy thanks to the presence of dense fibrils which largely overlapped with the distribution of actin filaments and focal adhesions, as shown after staining with FITC-phalloidin and anti-vinculin antibody, respectively (Fig. 3d-f). Furthermore, the colocalization of the β1 integrin subunit with vinculin in focal adhesions was much clearer in the VPMs (Fig. 3g,h) compared to intact permeabilized cells (Fig. 3a). This is probably due to the elimination in VPM preparations of the fluorescent signal derived from the distribution of the integrin subunits on the dorsal plasma membrane, and from intracellular staining. Also the differences between the distribution of the α6A and α6B polypeptides appeared more striking, as the former antigen appeared to overlap with vinculin throughout the VPMs (Fig. 3j,k), while the homogeneous, punctate distribution of α6B and its lack of evident accumulation with vinculin in focal contacts was confirmed (Fig. 3m,n). Similar results were obtained by using both affinity purified α6-cytoB and α6-cytoB$_2$ antibodies, but the last one gave a stronger signal. When VPMs were stained with the α6-EX antibody, which is able to recognize the extracellular portion of both α6 isoforms, the staining was clearly present in focal adhesions, as shown by the codistribution with vinculin, but also in areas of the ventral plasma membrane where vinculin is not evident, and where the distribution of α6 is characterized by a punctate staining (Fig. 4).

Comparison of the distribution of the α6A and α6B isoforms in CEFs cultured on laminin or fibronectin

Receptor occupancy by the extracellular ligands is considered essential for integrin receptor localization to focal adhesions. To look if this was the case also for the α6A subunit in CEFs, we compared the distribution of the two α6 isoforms in preparations of VPMs from cells cultured for 20 hours on laminin or fibronectin in serum-free conditions. Colocalization of α6A with vinculin in focal contacts seemed to correlate with increased laminin density on the substrate, as colocalization was more striking on coverslips coated with 100 μg/ml laminin compared to 20 μg/ml laminin (compare Fig. 5a,c, with Fig. 3j,k). This also correlated with stronger attachment of the cells to the substrate, from where they were less easily detached during the procedures for VPMs preparation. Distribution of α6B did not appear detectably affected at increased laminin density (compare Fig. 5b,d, with 3m,n).

Specificity of adhesion to fibronectin was assessed compared to non-adhesive control substrate (not shown). CEFs on fibronectin tended to be more spread than on laminin, and to detach less easily during the procedures for the preparation of VPMs. Under these culture conditions, the α6A polypeptide codistributed clearly with vinculin in focal contacts (Fig. 5e,g),

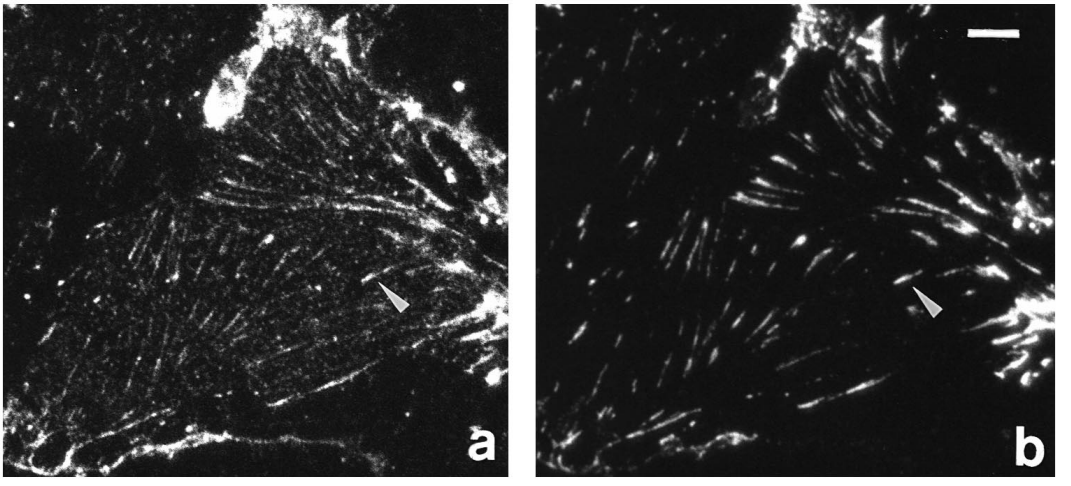

Fig. 4. Distribution of α6 and vinculin in VPMs plated on LN. VPMs were prepared from CEFs cultured 20 hours in serum-free conditions on LN-coated coverslips (100 μg/ml). After fixation, VPMs were double stained with α6-EX antibody (a) and anti-vinculin antibody (b). Arrowheads show colocalization of α6 with vinculin in focal adhesions. Areas among focal adhesions, devoid of vinculin staining, show a punctate staining for α6.

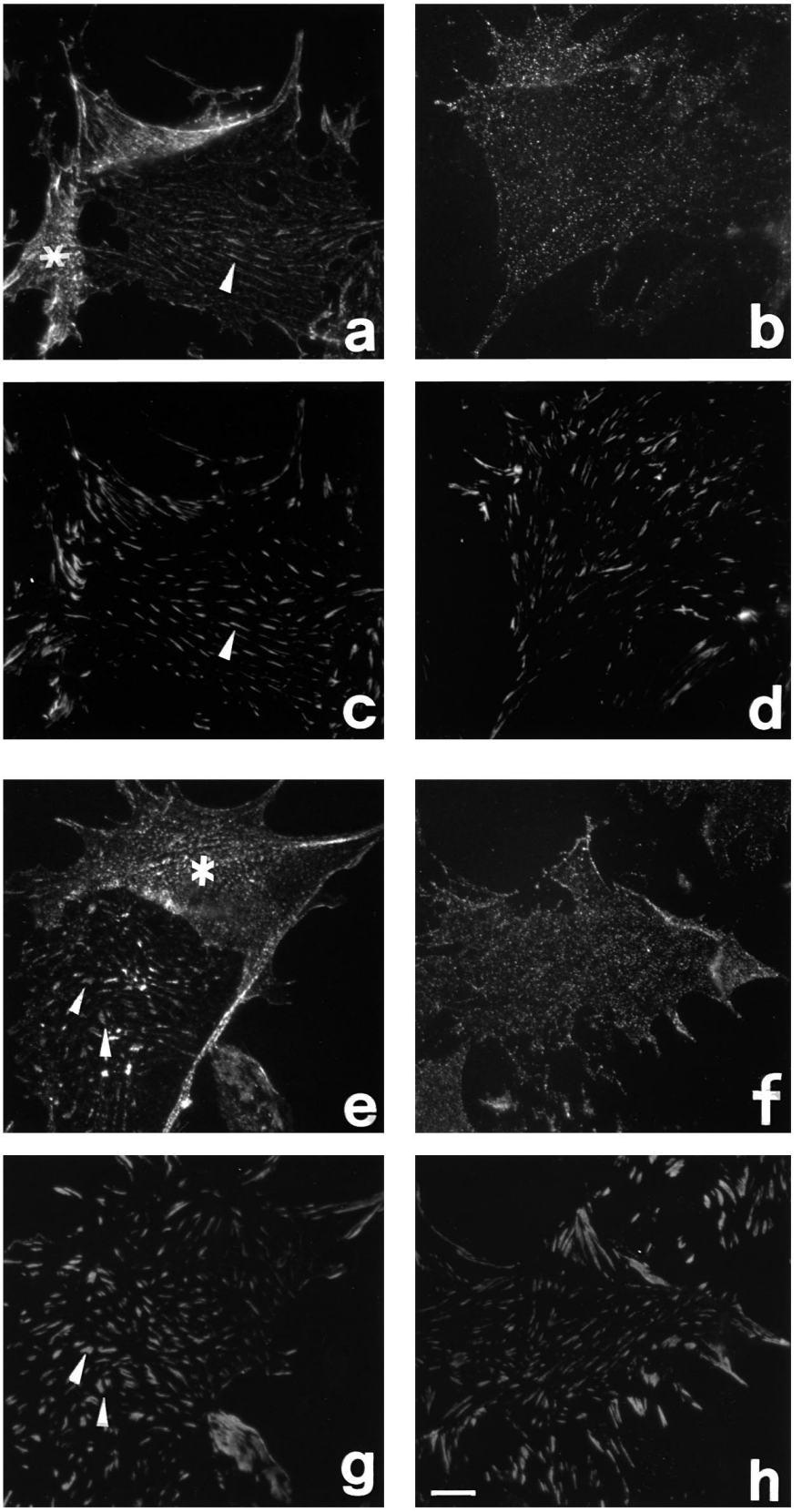

Fig. 5. Distribution of α6 cytoplasmic variants in VPMs on laminin and fibronectin. VPMs were prepared from CEFs cultured 20 hours in serum-free conditions on laminin-coated coverslips (100 μg/ml, a-d) or on fibronectin-coated coverslips (20 μg/ml, e-h). After fixation, VPMs were double stained with α6-cytoA (a,e), α6-cytoB$_2$ (b,f), and anti-vinculin (c,d,g and h) antibodies. The same cell is shown in a and c, b and d, e and g, and f and h. Arrowheads show colocalization of the α6A subunit with vinculin in focal adhesions. The asterisks in a and e show areas where the dorsal plasma membrane is still present. Bar, 10 μm.

while the α6B subunit showed the same homogeneously distributed punctate pattern as in cells attached to laminin (Fig. 5f,h). These data suggested that the localization of the α6Aβ1 laminin receptor in focal adhesions was independent from the presence of the specific ligand on the substrate. It is worth noting that the same pattern of distribution of the two α6

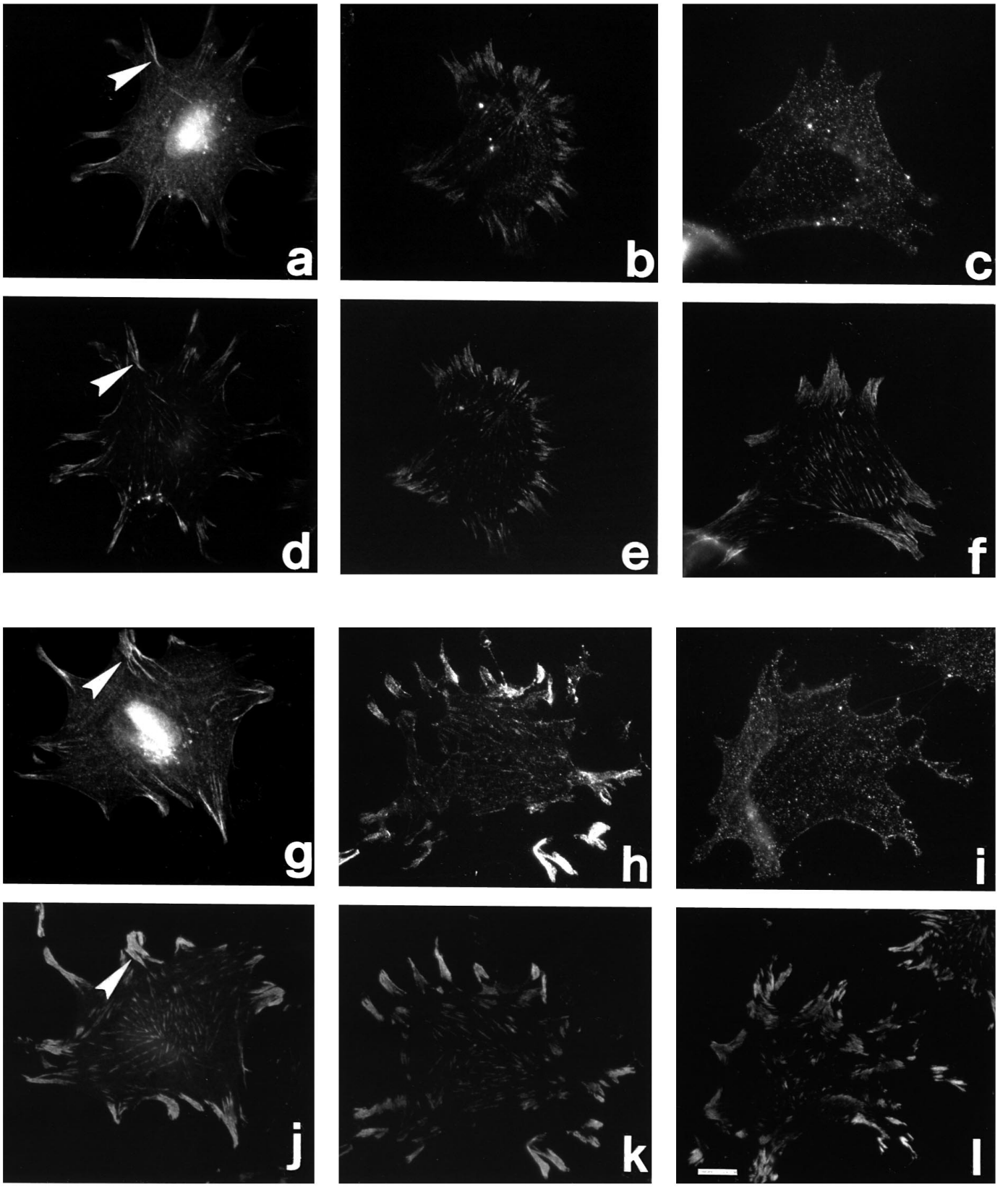

Fig. 6. Distribution of integrin subunits in cells and VPMs after short-term culture of CEFs on laminin or fibronectin. Cells were cultured for $1\frac{1}{2}$ hours on laminin-coated coverslips (a-f), or on fibronectin-coated coverslips (g-l). Intact cells (a,d,g,j) or VPMs (b,c,e,f,h,i,k,l) were fixed, permeabilized, and stained for immunofluorescence as described in Materials and Methods. Each sample was double stained with anti-integrin subunit antibodies (a-c, and g-i) or with anti-vinculin mAb (d-f, and j-l, respectively). Anti-integrin antibodies were: α6-cytoA (a,b,g,h), and α6-cytoB_2 (c,i). Arrowheads show codistribution of α6A with vinculin in focal adhesions. Bar, 10 μm.

isoforms was also observed in CEFs cultured on collagen IV (not shown).

Differential distribution of the α6A and α6B isoforms in short term cultures of CEFs on laminin or fibronectin

We also analyzed the distribution of the α6A and α6B after short-term (1½ hours) cultures of CEFs on laminin or fibronectin in serum-free conditions. After 1½ hours, the distribution of vinculin in the VPMs showed concentration of focal contacts at the periphery of the cells, which were probably still in the process of spreading. In this situation, colocalization of α6A with peripheral focal adhesions was striking both in intact cells and in VPMs (Fig. 6a,d and b,e, respectively). The same was true for cells plated on fibronectin (Fig. 6g,j and h,k), which after 1½ hours showed more extended areas with strong vinculin staining at the cell borders compared to cells on laminin. The homogeneously distributed punctate staining of α6B on the VPMs was similar to that observed after 20 hours culture, with the absence of evident accumulation in focal adhesions, and was independent of the ECM component coating the substrate (Fig. 6c,f and i,l). Colocalization of α6A with vinculin in focal adhesions was still observed in cells which had been preincubated with cycloheximide for 2 hours before plating, and then cultured for 1½ hours on fibronectin with cycloheximide (Fig. 7a,b). These data again suggest that the pattern of distribution of the α6A and α6B isoforms on the VPMs was independent of the presence of the specific ligand on the substrate. In agreement with published results, the localization of α5 to focal adhesions was not detectable by the use of the available α5-cyto antibody, probably due to blocking of the cytoplasmic domain in intact cells (Enomoto-Iwamoto et

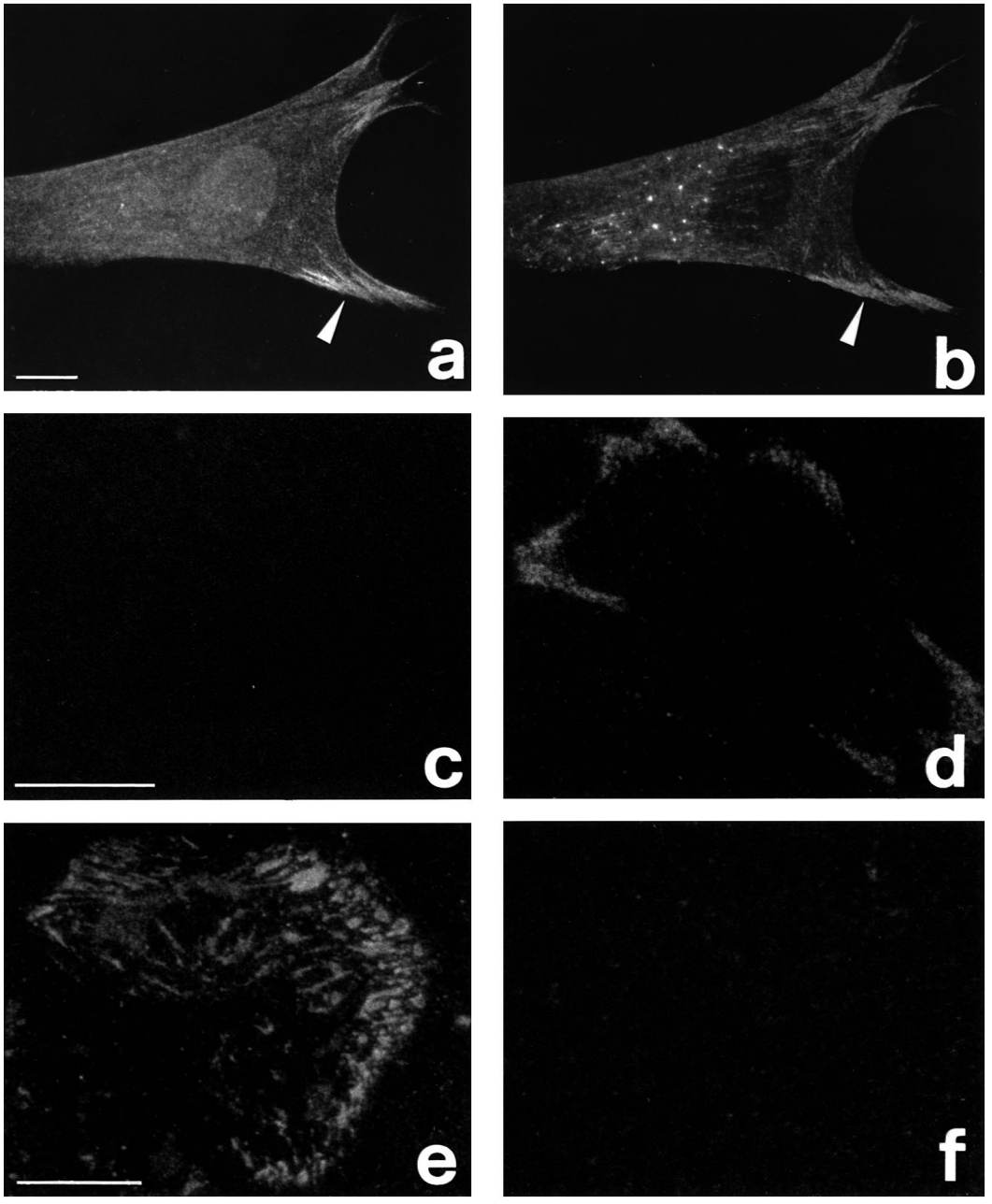

Fig. 7. Distribution of integrin α subunits on fibronectin and laminin after short-term culture. Staining for immunofluorescence was performed on CEFs cultured for 1½ hours (a and b) or for 2 hours (c-f) on fibronectin (a,b,c,e) or laminin (d,f). In a and b the same cell is shown that was cultured in the presence of cycloheximide, as described in Materials and Methods. In c-f cells were crosslinked with BS^3 and then extracted with RIPA buffer before fixation. Antibodies were: α6-cytoA (a,c,d); α5-cyto (e,f); anti-vinculin (b). Arrowheads show codistribution of α6-cytoA and vinculin in focal adhesions (a,b). Bars, 10 μm.

al., 1993) and VPM preparations. On the other hand, association of α5 with focal adhesions could be shown after ionic detergent extraction of cells cross-linked to the substrate. Cross-linkers have been used to identify close interactions between integrin receptors and their ligands (Enomoto-Iwamoto et al., 1993). If CEFs were cross-linked to the extracellular matrix proteins coating the substrate and extracted with RIPA buffer before fixation, the prints of focal adhesion sites remaining on the substrate could be detected only by using antibodies against the receptor subunit specific for the extracellular component present on the substrate. In fact, under these conditions we found that the α6A polypeptide could be detected in prints of focal contacts on laminin (Fig. 7d), but not on fibronectin (Fig. 7c), while α5 could be detected on fibronectin (Fig. 7e) but not on laminin (Fig. 7f), showing that integrins not involved in binding to the substrate are extracted by the RIPA buffer.

DISCUSSION

A number of studies have shown that the β-subunit cytoplasmic tail is responsible for the association of integrin receptors with the cytoskeleton (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; La Flamme et al., 1992; Bauer et al., 1993). Many integrin receptors localize to focal adhesion sites only upon binding their ligand, while unoccupied receptors show a diffuse distribution on the cell surface (Singer et al., 1988; Dejana et al., 1988; Fath et al., 1989). Two recent studies have shown that the cytoplasmic domains of the α1 and αIIb subunits play a role in the ligand-dependent integrin localization to focal adhesions (Briesewitz et al., 1993; Ylänne et al., 1993). In both cases removal of the cytoplasmic domains of the α subunit resulted in ligand-independent localization of the mutant receptors to focal adhesions.

To further investigate the role of different isoforms of receptors characterized by distinct α cytoplasmic domains, we have analyzed the distribution of the α6 cytoplasmic variants in CEFs adherent to different purified ECM components. In this paper we have shown that α6β1 is an important laminin receptor for CEFs, and that these cells express both cytoplasmic variants of the α6β1 laminin receptor. Our results show that these receptor isoforms distribute differently within the ventral membrane of CEFs, with α6A codistributing with vinculin in focal adhesions, while α6B is homogeneously distributed in a punctate pattern. Furthermore, our data show that this difference in the distribution of the two isoforms is maintained when CEFs are cultured on fibronectin, an extracellular component not recognized by the integrin α6β1 laminin receptor.

Dependence of adhesion of CEFs to laminin by integrins was shown by using the CSAT mAb against chicken β1 subunit, which strongly inhibited adhesion to laminin, as already shown for other cell types (Horwitz et al., 1985), and the polyclonal antibody α6-EX raised against a fusion protein corresponding to a large portion of the extracellular domain of chick α6 (de Curtis and Reichardt, 1993). In the presence of this antibody, CEF adhesion was inhibited by about 60%. The incomplete inhibition could be due to the presence of other β1-laminin receptors in these cells, or to the low efficiency of the α6-EX antibody in recognizing the native form of the receptor. Inhibition of cell adhesion on fibronectin by CSAT was also partial, probably due to the presence of β3-type fibronectin receptors in these cells (Hynes et al., 1989), while no significant inhibition was observed on fibronectin by the α6-EX antibody.

To look at the subcellular distribution of the two α6 isoforms, we used a procedure for the preparation of VPMs which allowed a much clearer view of the ventral surface of cells seeded on different ECM substrates compared to intact permeabilized cells. By using the anti-peptide antibodies α6-cytoA and α6-cytoB_2 specific for the two cytoplasmic variants of the α6 subunit, a striking difference in the pattern of distribution of the two isoforms on the ventral surface of cells cultured on laminin was observed. In fact, a significant fraction of the α6Aβ1 receptor colocalized with vinculin in focal adhesions, even though there was still a fraction distributing in areas in which vinculin was not evident. On the contrary, the α6B subunit was homogeneously distributed in a punctate pattern, suggesting that the α6Bβ1 receptor could be present in aggregates without accumulating in focal adhesions. Specificity of the staining with the affinity purified antibodies against the two α6 isoforms was indicated by the fact that the staining observed was absent when the respective preimmune sera were used (Fig. 3). Furthermore, staining for α6B was similar when two different antibodies raised against the same α6-cytoB peptide were used. Moreover, the use of the α6-EX antibody against the extracellular domain of the receptor in immunofluorescence experiments confirmed the observation that the α6 subunit can be present both in focal adhesions, and in a diffuse punctate pattern on the ventral surface of CEFs.

The distribution of α6B in CEFs is similar to the distribution of α1β1 on the dorsal and ventral surface of astrocytes plated on different substrates, including collagen and laminin, for which α1β1 is a functional receptor in these cells, even if not accumulated in focal adhesions (Tawil et al., 1993). On the other hand, the same receptor localizes in focal adhesions in fibroblasts. It should be noted that another laminin receptor for astrocytes, α6β1, was localized in focal adhesions in these cells. The differential distribution of the two laminin receptors α1β1 and α6β1 on the surface of astrocytes is comparable to the differential distribution of the two isoforms of the α6β1 laminin receptor on the surface of CEFs. As for α1, α6B can also show a punctate distribution, as in CEFs, or accumulate in focal adhesions, as in human OVCAR-4 cells (Hogervorst et al., 1993). In these cells both α6A and α6B isoforms were found codistributing with vinculin in focal adhesions on laminin. These diverse results on the distribution of the two α6 isoforms in different cell types could be explained by hypothesizing that localization to focal adhesions is regulated by competition of different integrin receptors for these sites. In CEFs, the predominant expression of the α6Aβ1 isoform could explain the detection of only the α6A subunit in focal adhesions of CEFs.

Alternatively, these results may be explained by assuming that the distribution of the same isoform in different cell types can be modulated by the interaction with distinct cellular environments, and that the distribution of each α6 isoform in the same cellular environment can be modulated by the interaction with different molecules. This latter idea is supported by our recent observation that the isoforms of the α6β1 receptor

extracted from embryonic neural retinal cells have distinct biochemical properties (de Curtis and Gatti, 1994). Furthermore, as shown for α1β1 (Tawil et al., 1993) and for αvβ1 (Zhang et al., 1993), the finding that α6Bβ1 does not accumulate in focal adhesions does not rule out the possibility that this receptor is functional in CEFs.

Another interesting finding from this study is that the pattern of distribution of the two isoforms of the laminin receptor were not affected in VPMs of CEFs adherent to fibronectin, a substrate not recognized by α6β1. In fact, α6A clearly colocalized with vinculin in focal adhesions also in CEFs plated on fibronectin. This was true also in experiments in which short time culture on fibronectin in the presence of cycloheximide was used to reduce the possibility that laminin synthesis and secretion may be responsible for α6A localization to focal adhesions. Under these conditions extensive colocalization of α6A with vinculin was observed at adhesion sites at the periphery of the cells. Another indication that α6A localization to focal adhesions is not due to deposition of endogenous laminin on the substrate comes from cross-linking experiments. Enomoto-Iwamoto et al. (1993) have used this method to evaluate involvement of integrin receptors in substrate adhesion. These authors found that in NIH 3T3 cells α6 could be cross-linked to the substrate only if laminin was present, whereas α5 was cross-linked only when fibronectin was present, showing a direct involvement of these receptors with the two respective extracellular matrix ligands. In agreement with these results, we found that in CEFs α6A could be cross-linked only on laminin, while α5 was cross-linked only on fibronectin, indicating that α6A localization to focal adhesions on fibronectin does not depend on binding of this receptor to the substrate. These results are apparently in contrast with the current view according to which localization to focal adhesions only occurs if receptor occupancy by the ligand has occurred. The correlation between ligand occupancy and localization of the receptor to focal adhesions may not be valid for all integrins, due to differences in the properties of distinct α cytoplasmic tails. In this respect, it has also to be considered that the use of VPMs has allowed a more detailed analysis of the distribution of integrin subunits on the ventral cell surface compared to what can be obtained by using intact cells.

Our data, together with a number of studies on different integrin subunits, indicate that the cytoplasmic portion of distinct α subunits is involved in the regulation of integrin localization, dependent both on the structure of the cytoplasmic domain and the intracellular environment.

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