The distribution of distinct integrins in focal contacts is determined by the substratum composition

KARL R. FATH[1], CORA-JEAN S. EDGELL[2] and KEITH BURRIDGE[1]

[1]*Department of Cell Biology and Anatomy* and [2]*Pathology Department, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA*

Summary

The distribution of two integrins, the fibronectin receptor and the vitronectin receptor, has been explored in an endothelium-derived cell line plated onto various substrata. On a fibronectin substratum, in the presence of serum, these cells develop focal contacts that contain the fibronectin receptor, whereas the vitronectin receptor is diffusely distributed over the cell surface. Conversely, cells plated onto vitronectin-coated coverslips concentrate only the vitronectin receptor within focal contacts. The accumulation of the vitronectin receptor within focal contacts also occurs when the cells are plated on uncoated coverslips but in the presence of serum. Therefore, we conclude that under normal culture conditions (i.e. in serum-containing media), the vitronectin receptor is the predominant form of integrin involved in substratum adhesion. This conclusion is supported by experiments in which cells were cultured on fibronectin-coated coverslips in the presence of serum. Initially these cells developed focal contacts containing only the fibronectin receptor. Within several hours, however, there was a progressive replacement of focal contacts containing the fibronectin receptor by focal contacts expressing the vitronectin receptor. After approximately 12 h in culture, most cells contained focal contacts expressing only the vitronectin receptor.

Focal contacts containing either the fibronectin or vitronectin receptor were both associated with the termini of stress fibres and contained the proteins talin and vinculin. These observations lead us to propose that the cell does not discriminate between these different integrins when assembling the cytoskeletal components at the cytoplasmic face of focal contacts.

Key words: extracellular matrix, fibronectin receptor, talin, vitronectin receptor.

Introduction

Many cultured cells form specialized adhesions to the underlying substratum, known as focal contacts, adhesion plaques or focal adhesions (reviewed by Burridge, 1986; Burridge *et al.* 1988; Woods & Couchman, 1988). At the focal contact, the extracellular face of the plasma membrane is separated from the substratum by 10–15 nm (Izzard & Lochner, 1976), and the cytoplasmic face anchors actin microfilament bundles (stress fibres). The formation of focal contacts requires the adsorption of extracellular matrix (ECM) proteins such as fibronectin (FN) or vitronectin (VN) to the substratum. Although substratum-bound FN promotes the formation of focal contacts (Couchman *et al.* 1982; Woods *et al.* 1986), cells in normal culture conditions clear the FN from the adhesions as a result of their traction on the substratum (Avnur & Geiger, 1981; Grinnell, 1986). The cleared FN is assembled into fibrils, which move on the cell surface towards the nucleus (Avnur & Geiger, 1981). This clearance requires serum or other proteins in the media because cells plated in serum-free (or low serum concentrations) media do not remove FN from focal contacts (Grinnell, 1986).

In contrast to FN, the relationship of VN (serum spreading factor) to focal contact formation has been studied in less detail. This serum component promotes cell adhesion and adsorbs very tightly to glass (Hayman *et al.* 1985*b*). When cells are grown on glass coverslips in serum-containing media, VN coats the coverslips but, unlike FN, it is not cleared by cells (Baetscher *et al.* 1986; Neyfakh *et al.* 1983). Although VN promotes adhesion, it is evenly spread on the substratum and is not concentrated beneath focal contacts. Detection of VN beneath focal contacts by immunofluorescence microscopy requires permeabilization or detachment of the cells due to its inaccessibility to antibodies (Baetscher *et al.* 1986; Neyfakh *et al.* 1983). Because it is not cleared from focal contacts, we have suggested that VN may be more important than FN for the formation of focal

contacts by many cells grown in the presence of serum (Burridge *et al.* 1988).

The integrin family of cell surface receptors for ECM proteins, such as FN and VN, has recently received considerable attention (reviewed by Buck & Horwitz, 1987*a*; Hynes, 1987; Ruoslahti & Pierschbacher, 1987). Integrins are concentrated within focal contacts (Chen *et al.* 1985; Damsky *et al.* 1985; Giancotti *et al.* 1986; Kelly *et al.* 1987; Marcantonio & Hynes, 1988) and provide a potential transmembrane link between the ECM and cytoskeletal proteins such as talin (Horwitz *et al.* 1986). The binding of integrins to their ECM ligands is disrupted by some specific antibodies (Brown & Juliano, 1985; Chen *et al.* 1985; Cheresh, 1987; Greve & Gottlieb, 1982; Neff *et al.* 1982) and peptides (Hayman *et al.* 1985*a*; Pierschbacher *et al.* 1983; Yamada & Kennedy, 1987). These agents also disrupt focal contacts, indicating the importance of the integrin ECM receptors in formation and maintenance of these adhesions.

Whereas avian cells apparently contain an integrin that binds to both FN and VN (Buck & Horwitz, 1987*b*), mammalian cells express distinct integrins for these two ECM components (see, e.g., Hynes, 1987; Ruoslahti & Pierschbacher, 1987). Antibodies specific for the FN receptor (FNR) or VN receptor (VNR) permit the exploration of the role of these two receptors in focal contact formation. Singer and coworkers (1988) have recently investigated the distribution of VNR and FNR in human fibroblasts and melanoma cells cultured on different substrata. When these cells were plated in the presence of serum, both receptors were initially codistributed in the focal contacts. However, with time there was a redistribution of the FNR to sites of adhesion to FN-containing bundles, while the VNR remained in the focal contacts.

In this paper we have explored the behaviour of the FNR and VNR in a human endothelium-derived cell line plated on different substrata. In contrast with the results of Singer *et al.* (1988), we find that in the presence of serum only the VNR is concentrated in the focal contacts. We also show that on FN-coated coverslips, initially the FNR is prominent in focal contacts whereas the VNR is absent. Within a few hours, however, there is a progressive development of new adhesions expressing the VNR and a decrease in adhesions containing the FNR, even though FN remains extensively spread on the substratum. In contrast, on VN-coated coverslips over a period of 37 h only the VNR was detected in focal contacts. In addition, we show that focal contacts expressing either the FNR or the VNR are both able to support stress fibres and contain typical focal contact proteins such as talin and vinculin.

After completing this work we received a copy of a manuscript describing the distribution of the VNR and FNR in endothelial cells plated on different ECM substrata (Dejana *et al.* 1988).

Materials and methods

Cell culture

The cells used were a human endothelium-derived line called EA hy 926 (EA) (Edgell *et al.* 1983), which expressed von Willebrand Factor as detected by immunofluorescence (data not shown). Cells were normally grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum (FCS) and supplemented with penicillin and streptomycin. Serum-free cultures were established by washing suspended cells at least twice in serum-free media before dispersing onto coverslips.

Antibodies and proteins

The rabbit polyclonal antibody (N681) directed against talin was prepared by repeated immunization with purified human platelet talin, using both native protein as well as material eluted from preparative SDS–polyacrylamide gels. The antiserum has been used to stain the focal contacts of many cell types and was found to stain a single high molecular weight band on Western blots of these cells (our unpublished observations). Before using the antisera to study the distribution of talin in EA cells, we characterized its immuno-specificity by immunoblotting (Fig. 1). The rabbit polyclonal antibody recognizing fibronectin was a generous gift from L. B. Chen (Chen *et al.* 1976). The monoclonal antibody LM609, which recognizes the VN receptor α/β complex, was a generous gift from Dr David Cheresh (Cheresh, 1987). The polyclonal antibody raised against the hamster FN receptor was kindly provided by Dr R. Juliano (Brown & Juliano, 1986).

Glass coverslips were coated with 40 $\mu g\,ml^{-1}$ purified human plasma fibronectin (New York Blood Center) in PBS or 40 $\mu g\,ml^{-1}$ purified human vitronectin (Calbiochem) in PBS for 1–3 h at 37°C. The coated coverslips were rinsed in PBS, then transferred to the cell culture media.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

SDS–PAGE was performed in slab gels using the buffer system of Laemmli (1970). Gels contained 10% acrylamide and 0·13% bisacrylamide and were immunoblotted as described (Tidball *et al.* 1986).

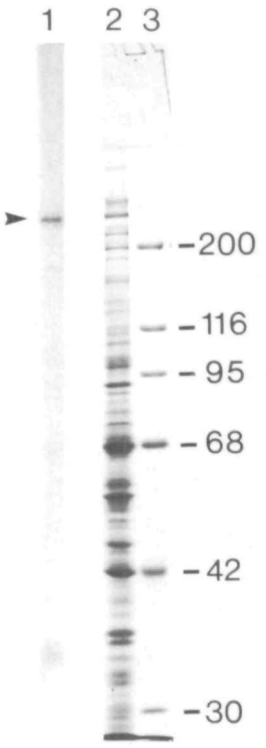

Fig. 1. Talin antibody characterization. Lane 1: a Western blot autoradiograph of total EA hy 926 proteins electrophoresed on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose. The blot was probed with rabbit polyclonal antiserum directed against mammalian platelet talin (see Materials and methods) followed by a ^{125}I-labelled antibody recognizing rabbit IgG. The immunoblot shows a single reactive band (arrowhead) with a molecular weight of about 230 000, corresponding to talin. Lane 2: a Coomassie Blue-stained gel of an equivalent sample to that in lane 1. Lane 3: molecular weight markers ($\times 10^{-3}$): myosin heavy chain (200), β-galactosidase (116), phosphorylase *b* (95), bovine serum albumin (68), ovalbumin (42), and carbonic anhydrase (30).

Indirect immunofluorescence

Cells on glass coverslips were rinsed with PBS and fixed in 3·7% formaldehyde in PBS for 10 min at room temperature. The cells were permeabilized with 0·2% Triton X-100 in Tris-buffered saline (TBS: 50 mM-Tris·HCl, 150 mM-NaCl, 0·1% NaN_3, pH 7·6) for 3 min. Cells were stained with the primary antibody diluted in TBS for 1 h at 37°C, rinsed in TBS and stained with a 1/50 dilution of the appropriate secondary antibody. The secondary antibodies used were: FITC–goat anti-mouse IgG (H&L chain specific; Cappel), affinity-purified and cross-species adsorbed RITC–donkey anti-rabbit IgG (Chemicon), and FITC–donkey anti-goat IgG (H&L chain specific; Jackson Immunoresearch lab). For double-label studies, cells were simultaneously stained with a mixture of the primary antibodies, rinsed, and simultaneously stained with a mixture of the fluorescent secondary antibodies. Polymerized actin was stained with 6·6 μM-rhodamine–phalloidin (Molecular Probes). Note that the EA cells contained a pronounced perinuclear mound, which appeared bright when stained indirectly with all antibodies. The coverslips were viewed on a Zeiss IM 35 microscope equipped with epifluorescence optics. Fluorescence micrographs were photographed at EI 1600 on T-Max 400 and developed with T-Max developer (Kodak).

Results

Accumulation of VNR and FNR in focal contacts

Cells of a human endothelium-derived line (EA) were plated in serum-free medium on glass coverslips that had been coated with purified human FN. The cells spread and accumulated FNR in their focal contacts by 1 h as detected by immunofluorescence (Fig. 2A,C). These adhesions also contained talin (Fig. 2B) and vinculin (not shown), and were associated with the termini of actin-containing stress fibres (Fig. 2D). The stress fibres in these cells were often less pronounced when compared with those in fibroblasts. In many cells there were circumferential bundles of actin filaments, which did not terminate in focal contacts.

Similarly, when EA cells were grown on substrata coated with purified human VN in serum-free medium, the cells formed focal contacts containing the VNR (Fig. 3A,C) as well as talin (Fig. 3B) and vinculin (not shown), and these adhesions were associated with the ends of stress fibres (Fig. 3D). The VNR adhesions were often more round or comma-shaped than the thinner FNR adhesions that developed on FN (cf. Fig. 2 with Fig. 3).

FNR and VNR clustering at focal contacts is specified by the substratum

To determine the relationship of the FNR and VNR to the substratum, EA cells were plated on either purified FN or VN in serum-free media and double-labelled for VNR and FNR. On FN substrata, the FNR accumulated in focal contacts but the VNR was diffusely distributed (Fig. 4A,B). Conversely, when the cells were plated on

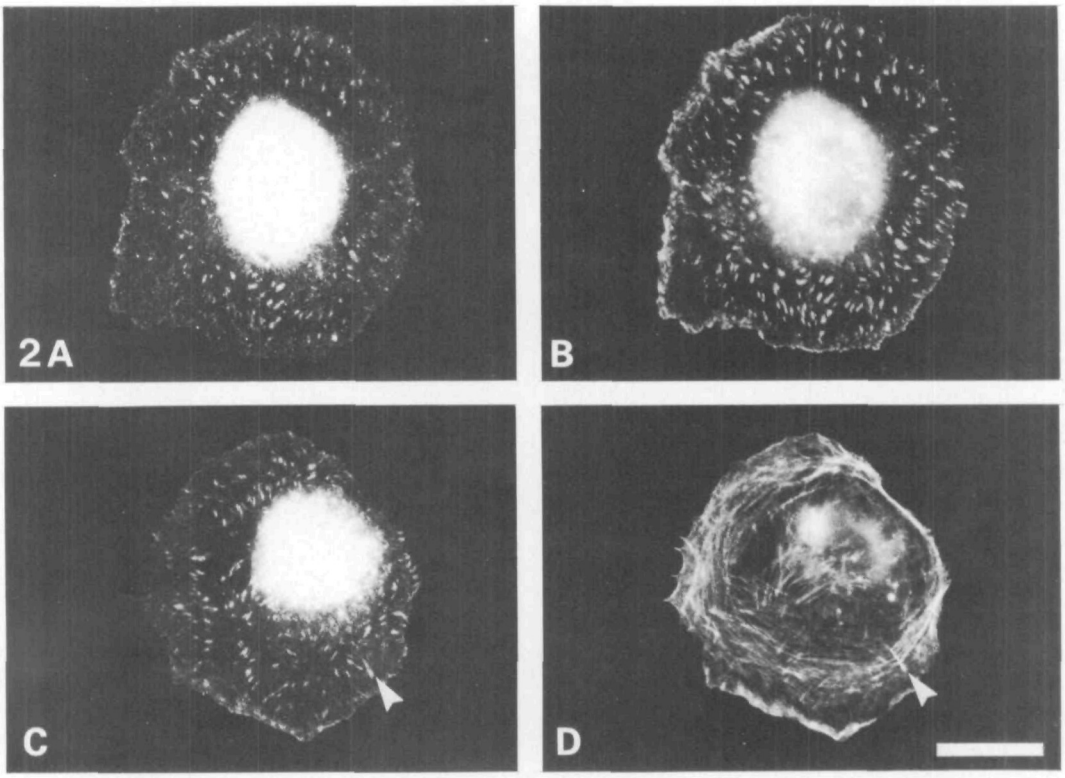

Fig. 2. Localization of FNR in cell adhesions to FN-coated substrata. EA cells were cultured for 1 h on FN-coated coverslips in serum-free medium, then double-labelled for FNR and talin (A,B) or FNR and actin (C,D). On FN substrata the FNR (A) is concentrated in focal contacts that also contain talin (B). These FNR adhesions (C) can also support stress fibre terminations (arrowheads in C,D) as seen by rhodamine–phalloidin staining. Note that EA cells contain many circumferential actin-filament bundles. Bar, 20 μm.

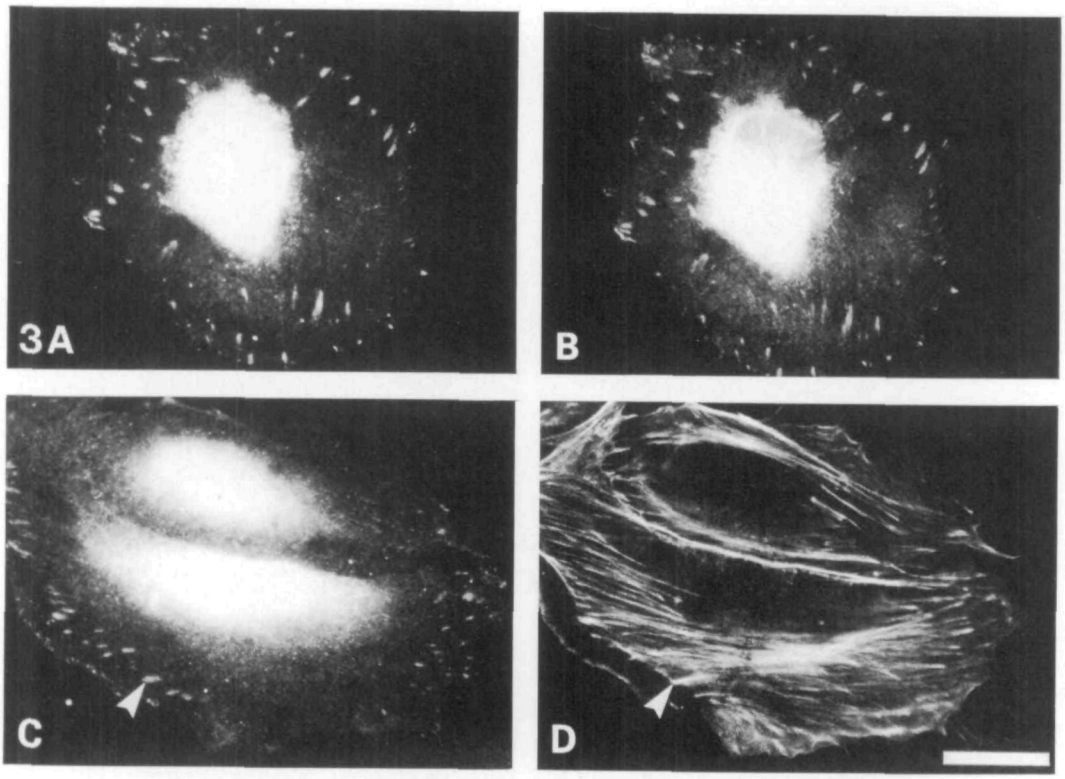

Fig. 3. Localization of VNR in focal contacts on VN-coated substrata. EA cells were cultured for 1 h on VN-coated coverslips in serum-free medium, then double-labelled for VNR and talin (A,B) or VNR and actin (C,D). Note the extensive co-localization of VNR (A) and talin (B) in focal contacts. The VNR-containing focal contacts (C) were also associated with the ends of stress fibres stained with rhodamine–phalloidin (D). The arrowheads in C and D indicate a stress fibre that terminates in a VNR-containing focal contact. Bar, 20 μm.

coverslips coated with purified VN, the VNR was concentrated in the focal contacts but the FNR was diffuse (Fig. 4C,D). These results suggest that accumulation of the specific integrins in focal contacts depends on the composition of the substratum.

EA cells plated in serum-containing media form VNR focal contacts

Having shown that the EA cells can assemble focal contacts containing either the FNR or VNR when plated on purified substrata, we sought to determine which receptor would be assembled into contacts when the cells were plated in normal cell culture conditions with serum present. In these experiments, EA cells were plated on uncoated glass coverslips in medium supplemented with serum (10% FCS). As shown in Fig. 4 (E,F), cells adhering to serum-coated coverslips formed VNR adhesions that lacked the FNR. The FNR was diffusely distributed throughout the cell. These adhesions also accumulated talin and vinculin, and were associated with stress fibre termini (data not shown). These results suggest that in normal culture conditions (i.e. in medium supplemented with serum), the VNR is the predominant integrin in substratum adhesions formed by these cells.

In the presence of serum, EA cells on FN substrata switch from FNR to VNR in their focal contacts

The above results, combined with the finding that cells can clear FN adsorbed to coverslips (our unpublished results; Avnur & Geiger, 1981; Grinnell, 1986), suggested that the EA cells might prefer serum VN over substratum-attached FN when forming focal contacts. We tested this hypothesis by plating cells on FN-coated substrata in serum-containing medium. EA cells were plated on purified human FN in media containing 10% FCS and stained for FNR and VNR receptors following 1, 3·5, 6, 12, 24, 37 h in culture. As shown in Fig. 5 (A,B), the cells initially formed focal contacts similar to those on FN in serum-free media. These adhesions accumulated the FNR but not the VNR, which was diffusely distributed in these cells. After several hours, however, there was a major reorganization of the focal contacts (Fig. 5C–F). Focal contacts containing VNR and generally lacking the FNR began forming at the cell periphery. Some FNR adhesions remained in many cells, but these were usually more centrally located. Moreover, the FNR adhesions tended to become more fibrillar (see below). After 12 h, a majority of cells contained only VNR adhesions and the FNR was diffuse. This complete switch in the integrins expressed in focal contacts was detected in some cells as early as 6 h after plating (Fig. 5G,H).

In order to determine if the fibrillar pattern of the FNR that develops with time in culture was a result of association with FN fibrils, EA cells were plated on FN substrata and double-labelled for FN and FNR. In

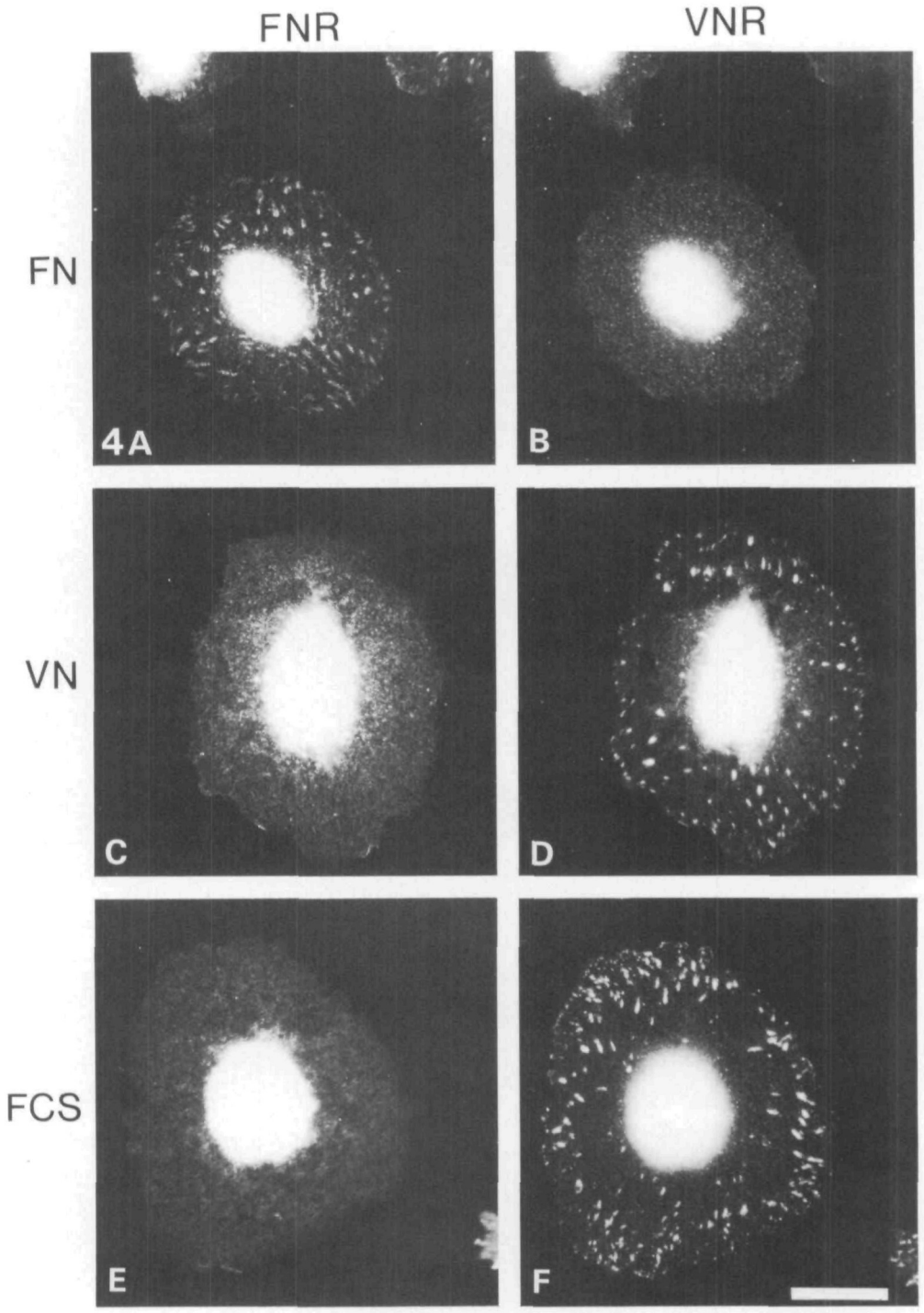

Fig. 4. Double-labelling of VNR and FNR distribution on different substrata. EA cells were plated in serum-free media on glass coverslips that had previously been coated with purified fibronectin (A,B), vitronectin (C,D), or on uncoated coverslips in media containing 10% foetal calf serum (E,F). The cells were cultured for 1 h, then fixed and double-labelled for VNR and FNR. On FN substrata the FNR (A) but not VNR (B) is concentrated in focal contacts. Conversely, on purified VN substrata the VNR is concentrated in focal contacts (D) while the FNR is diffuse (C). Cells plated on previously uncoated coverslips in the presence of serum contain VNR (F) but not FNR (E) in focal contacts. Bar, 20 μm.

comparison with many fibroblastic cells, the EA cells did not clear FN extensively from the substratum, although they did assemble small bundles of FN on the ventral surface. The FNR coincided with these FN fibrils (e.g. arrows in Fig. 6A,B). There was also another staining pattern in which the FNR was concentrated in focal contacts that failed to stain along their entire length for FN (see arrows in Fig. 6C,D). The apparent absence of FN from these areas may be due to clearing of the FN, although it is surprising that the FNR remains at these sites without FN. Alternatively, FN may be present at these regions but inaccessible to the FN antibody because

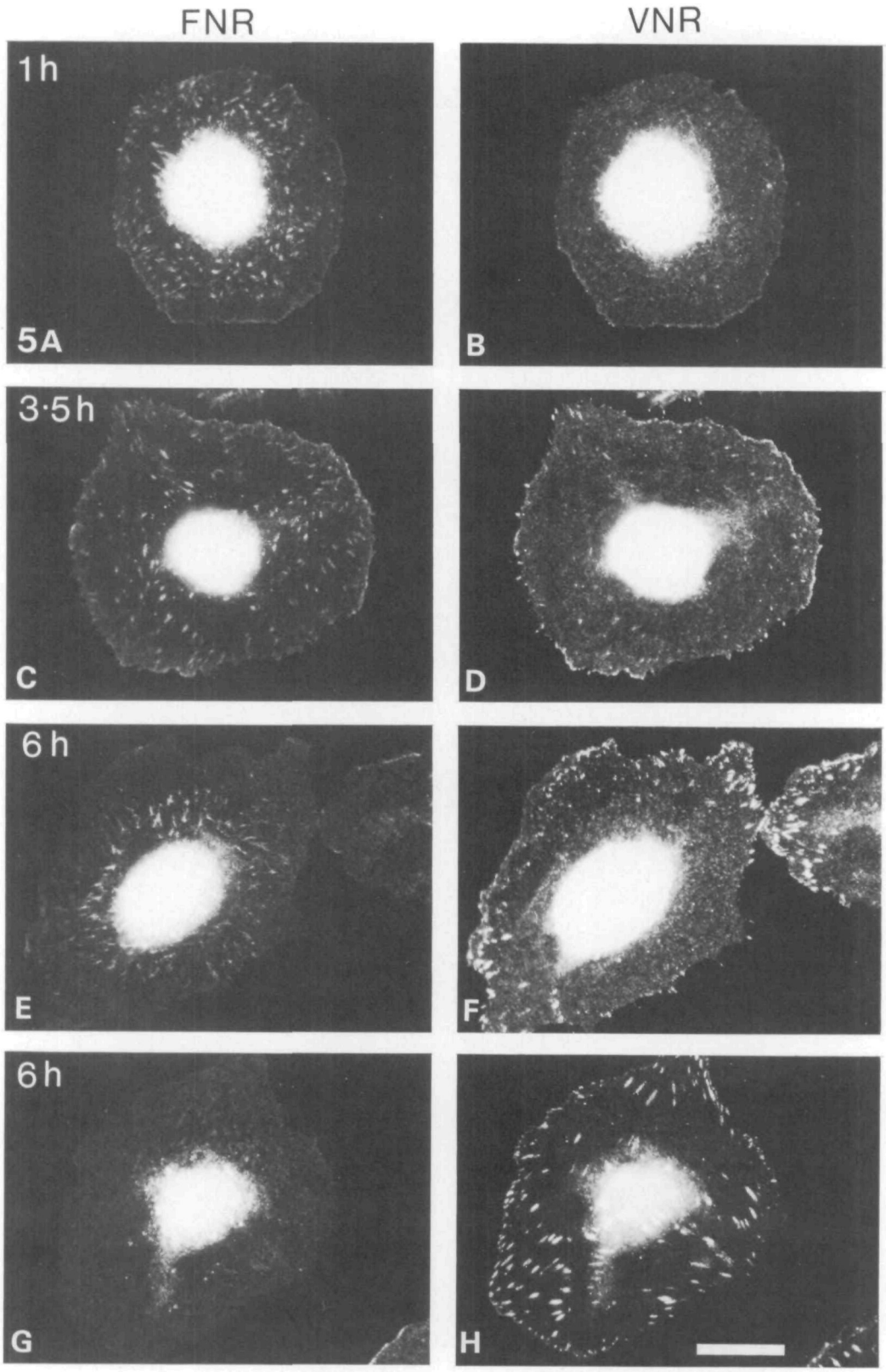

Fig. 5. Cells plated in 10% FCS on FN-coated coverslips switch from FNR- to VNR-containing adhesions. EA cells were seeded on coverslips coated with FN in serum-containing media and double-labelled for FNR and VNR at 1 h (A,B), 3·5 h (C,D) or 6 h (E–H) after plating. At 1 h the FNR (A) but not VNR (B) was concentrated in focal contacts. By 3–4 h the FNR (C) was often located in central, fibrillar adhesions, while the VNR (D) began to accumulate in peripheral contacts. The fibrillar FNR distribution is more pronounced at 6 h (E) and the VNR is predominant in peripheral focal contacts (F). Many cells by 6 h, and more markedly by 12 h (not shown), contain only VNR adhesions (H) whereas the FNR (G) is diffusely distributed. Bar, 20 μm.

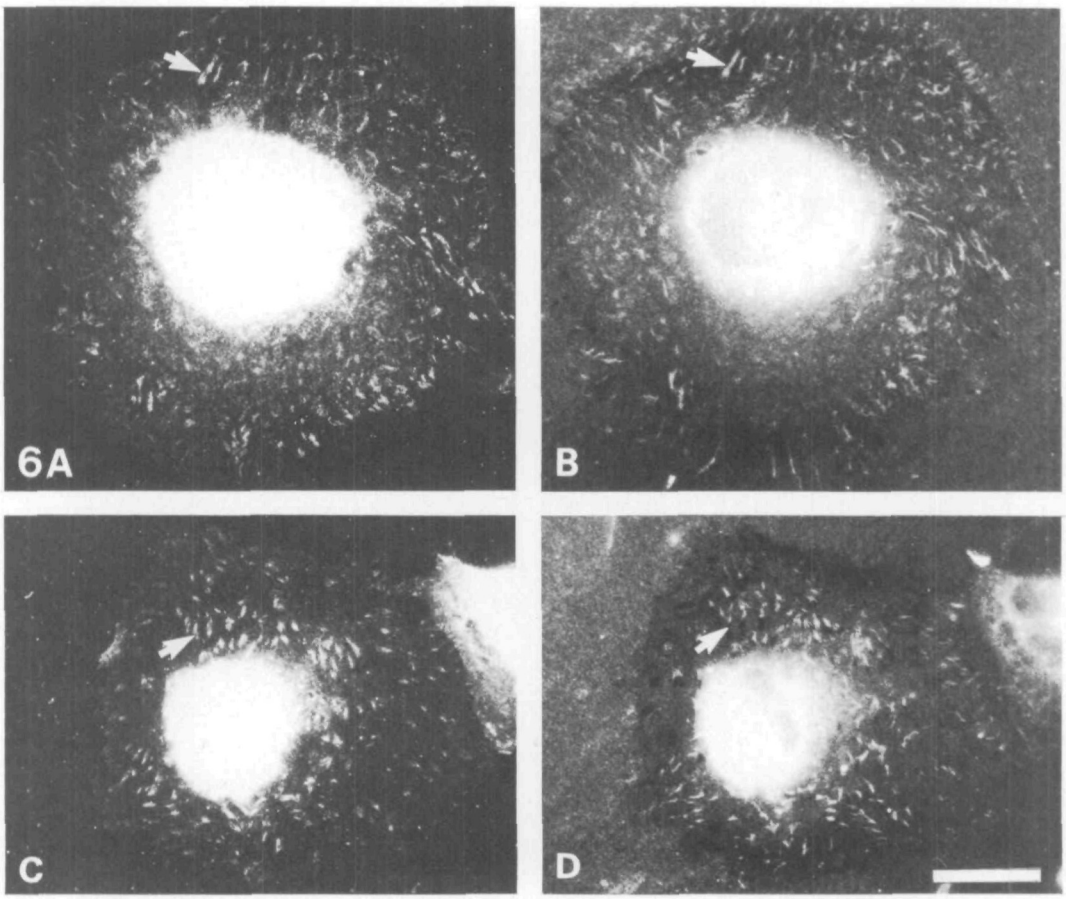

Fig. 6. Association of FNR with FN fibrils. EA cells were cultured on FN-coated substrata in medium containing 10% FCS for 3·5 h, then double-labelled for FNR (A,C) or for fibronectin (B,D). In A,B the arrows mark an accumulation of FNR (A) that co-localizes with FN fibrils (B). In C,D the arrows indicate the association of FNR (C) with focal contacts that do not stain with the FN antibody along their entire length (D). Bar, 20 μm.

of the tight substratum adhesion.

The above results showed that EA cells plated on FN can switch from FNR- to VNR-containing adhesions when supplied with media containing soluble VN. We wanted to determine if cells on VN substrata develop focal contacts containing FNR when grown in the presence of serum. EA cells were plated in media containing 10% FCS on glass coverslips that had been coated with purified human vitronectin. The cells formed adhesions throughout the entire ventral cell surface containing VNR, but the FNR was diffuse. Only the VNR was observed in cells examined at 1, 3·5, 6, 12, 24, 37 h after plating (data not shown).

Discussion

In this study we have examined the behaviour of two members of the integrin family of ECM receptors, the fibronectin receptor and the vitronectin receptor, and their relationship to focal contacts formed on different substrata by an endothelium-derived cell type. Our first conclusion is that both receptors can be concentrated in focal contacts depending on the composition of the substratum: cells plated on a VN-coated substratum contain the VNR but no FNR in their focal contacts, whereas cells plated on FN initially contain only the FNR in their focal contacts. Most cells grown in tissue culture are plated in the presence of serum and not on specially prepared substrata. Because both FN and VN are present in serum and both promote adhesion, we wanted to determine which integrin would be present in the focal contacts of cells plated in serum. Under these conditions, we found that the EA cells only expressed the VNR in their focal contacts. It should be noted that FN was present in this serum and was detected on the substratum by immunofluorescence microscopy (data not shown). This absence of the FNR from focal contacts formed in the presence of serum differs from the recent finding of Singer *et al.* (1988), who found that human fibroblasts and melanoma cells express both receptors in their focal contacts when plated in serum. However, they found that with time the FNR became redistributed away from focal contacts as bundles of FN were assembled, so that mature focal contacts expressed only the VNR. Both studies lead to the same conclusion regarding mature focal contacts formed in the presence of serum: these adhesions predominantly involve the VNR. This conclusion is consistent with previous observations of the ECM components. In the presence of serum, FN tends to be cleared from

focal contacts (Avnur & Geiger, 1981) whereas VN remains adsorbed to the substratum (Baetscher *et al.* 1986; Neyfakh *et al.* 1983). Moreover, FN is generally absent from contacts of cells grown in serum, suggesting that some other ECM component is responsible for formation of these adhesions.

Because the VNR is concentrated in focal contacts formed by cells in the presence of serum, we wanted to determine what occurred when cells were plated on FN-coated substrata, but in the presence of serum. That is to say, would the cells prefer the adsorbed FN or the soluble VN when forming new adhesions? The results of these experiments were quite striking. The focal contacts that formed first were entirely of the FNR type, but with time new focal contacts developed that contained the VNR. These new contacts were first detected at the cell periphery, the region where focal contacts have been shown to develop (Bershadsky *et al.* 1985; DePasquale & Izzard, 1987; Izzard & Lochner, 1980). After approximately 6 h in culture, some cell adhesions contained only the VNR, although many cells exhibited focal contacts containing either the VNR or the FNR. Only occasionally did we observe single focal contacts containing a mixture of both receptors. In cells showing two types of focal contacts, generally the VNR adhesions were peripheral while the FNR adhesions were more perinuclear. These latter focal contacts were often more linear and elongated, due in part to the formation of small bundles of FN on the ventral surface. Compared with many cells, however, these EA cells do not clear a substantial amount of the adsorbed FN and only assemble the FN into small fibrils. These cells secrete little, if any, FN (data not illustrated). Some cells retained the FNR associated with the FN-containing fibrils for prolonged periods (37 h was the longest time point examined), but from 12 h onwards most cells had focal contacts that contained only the VNR. Examining the ECM components on the substratum revealed that both FN from the initial coating as well as VN adsorbed from the serum were present. This leads us to conclude that, for these cells in the presence of serum, VN is the preferred ECM substratum and the VNR is the integrin normally concentrated in focal contacts.

A major objective in this research area has been to determine how stress fibres are attached to the plasma membrane at the focal contact. The integrins may have a major role as transmembrane receptors linking the extracellular ECM to the intracellular cytoskeleton. A potential interaction between integrins and the cytoskeletal protein talin has been demonstrated *in vitro* using the avian form of integrin and talin (Horwitz *et al.* 1986). We have suggested that focal contacts containing different integrins can support the same cytoskeletal proteins (Burridge *et al.* 1988). Our data are consistent with this idea. Focal contacts containing either the FNR or the VNR support stress fibres and concentrate typical focal contact proteins such as talin and vinculin. Sequence analysis of various cloned integrins, including the mammalian FNR and VNR, reveals considerable homology in the cytoplasmic domains of these receptors, particularly for the β chains (Argraves *et al.* 1987; Fitzgerald *et al.* 1987). This observation, together with the finding that either the FNR or VNR can support an equivalent repertoire of cytoskeletal proteins, leads us to suggest that the cell does not discriminate between VN or FN (and possibly other ECM components) when assembling stress fibres at focal contacts. Different integrins will be recruited to the focal contacts, depending on the ECM component adsorbed to the substratum, but because of their similar cytoplasmic domains these different integrins will, in turn, recruit the same set of cytoskeletal proteins at the cytoplasmic face of the focal contact.

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