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Identification of a Role of the Vitronectin Receptor and Protein Kinase C in the Induction of Endothelial Cell Vascular Formation

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Abstract When cultured on a basement membrane substratum, endothelial cells undergo a rapid series of morphological and functional changes which result in the formation of histotypic tube-like structures, a process which mimics in vivo angiogenesis. Since this process is probably dependent on several cell adhesion and cell signaling phenomena, we examined the roles of integrins and protein kinase C in endothelial cell cord formation. Polyclonal antisera directed against the entire vitronectin ($\alpha_v\beta_3$) and fibronectin ($\alpha_5\beta_1$) receptors inhibited cord formation. Subunit-specific monoclonal antibodies to α_v, β_3, and β_1 integrin subunits inhibited cord formation, while monoclonal antibodies to α_5 did not, which implicated the vitronectin receptor, and not the fibronectin receptor, in vascular formation Protein kinase C inhibitors inhibited cord formation, while phorbol 12-myristate 13-acetate (PMA) caused endothelial cells to form longer cords. Since the vitronectin receptor has been shown to be phosphorylated in an in vitro system by protein kinase C, the possible functional link between the vitronectin receptor and protein kinase C during cellular morphogenesis was examined. The vitronectin receptor was more highly phosphorylated in cord-forming endothelial cells on basement membrane than in monolayer cells on vitronectin Furthermore, this phosphorylation was inhibited by protein kinase C inhibitors, and PMA was required to induce vitronectin receptor phosphorylation in endothelial cells cultured on vitronectin. Colocalization studies were also performed using antisera to the vitronectin receptor and antibodies to protein kinase C. Although no strict colocalization was found, protein kinase C was localized in the cytoskeleton of endothelial cells initially plated on basement membrane or on vitronectin, and it translocated to the plasma membrane of C-shaped cord-forming cells on basement membrane. Thus, both the vitronectin receptor and protein kinase C play a role in in vitro cord formation.

Key words: angiogenesis, basement membrane, integrins, phosphorylation, cord formation

The vitronectin receptor (VNr) is a member of the integrin family of extracellular matrix receptors and consists of an α_v subunit, most commonly associated with the β_3 subunit, although it may also form complexes with β_1 and β_5 subunits [for review see Horton, 1990]. The VNr has binding affinities for a number of cell adhesion proteins, including vitronectin, laminin, fibronectin, von Willebrand's factor, fibrinogen, and thrombospondin [Horton, 1990]. It is expressed predominantly in placenta [Pytela et al., 1985], osteoclasts [Horton et al., 1985], human neuroblastoma cells [Gray and Dedhar, 1988], vascular endothelium [Athanasou et al., 1990], and cultured endothelial cells [Fitzgerald et al., 1985; Charo et al., 1986; Plow et al., 1986]. The receptor may function in the coagulation cascade and with complement factors [Horton, 1990]; it may also be important for osteoclast function [Chambers et al., 1986] and for the mediation of phagocytosis of cells undergoing apoptosis [Savill et al., 1990].

Integrin receptors have been shown to be phosphorylated in several cell systems. For example, the phosphorylation of the fibronectin receptor may regulate its function during cellular differentiation [Dahl and Graebel, 1989]. The $\alpha_6\beta_1$ laminin binding integrin exhibited phorbol 12-myristate 13-acetate (PMA)-induced phosphorylation of the α_6 subunit, which may facili-

Abbreviations used IL-1β, interleukin-1β, PKC, protein kinase C, PMA, phorbol 12-myristate 13-acetate, TNF-α, tumor necrosis factor-α, VNr, vitronectin receptor

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tate its binding to cytoskeletal elements [Shaw et al., 1990]. The common β_2 subunit of the leukocyte adhesion receptors (CD11/CD18 family) also becomes phosphorylated when cells are treated with PMA [Hara and Fu, 1986]. The phosphorylation of the CD11/CD18 family may by constitutive or stimulus-induced on the CD11 and CD18 chains, respectively, in neutrophils [Buyon et al., 1990] and monocytes [Chatila et al., 1989]. The IIIa (β_3) subunit of the platelet glycoprotein GPIIb/IIIa is phosphorylated on threonine during platelet activation with thrombin or PMA [Parise et al., 1990]. Moreover, a VNr with the subunit composition $\alpha_v\beta_s$ has been identified on osteosarcoma cells, of which the β_s subunit was shown to be phosphorylated on serine when the cells were treated with PMA [Freed et al., 1989]. This phosphorylation was protein kinase C (PKC)-dependent, as the β_s subunit was an in vitro substrate for PKC [Freed et al., 1989]. However, the functional significance of the phosphorylation of these integrins is still unclear.

Endothelial cells have been shown to possess several integrin receptors [reviewed in van Mourik et al., 1990; Albelda and Buck, 1990; Giltay and van Mourik, 1988]. Endothelial cell integrins include the types I and IV collagen binding integrin ($\alpha_1\beta_1$) [Kramer and Marks, 1989; Kramer et al., 1990], the collagen and laminin receptor ($\alpha_2\beta_1$) [Languino et al., 1989; Albelda et al., 1989], the fibronectin/laminin/collagen receptor ($\alpha_3\beta_1$) [Albelda et al., 1989], and the fibronectin receptor ($\alpha_5\beta_1$) [Dejana et al., 1988]. In addition, endothelial cells have been shown to express the VNr $\alpha_v\beta_3$ [Dejana et al., 1988; Albelda et al., 1989].

Although human umbilical vein endothelial cells (HUVECs) will form capillary-like structures after several weeks in culture on plastic or gelatin [Kubota et al., 1988; Maciag, 1990], they will form these structures within 5 h when plated on basement membrane [Grant et al., 1989]. We have examined the formation of these capillary-like structures after 18–20 h of culture on basement membrane. Because it was not determined in our work whether these structures possess lumens, they are referred to here as cords. Cord formation may be dependent upon several cell-surface extracellular matrix binding proteins. This study was designed to determine the role of the VNr and its possible subunits, as well as the potential role of PKC in the induction of cord formation in endothelial cells in vitro. Our data suggest that the VNr and PKC play important roles in the induction of endothelial cell cord formation in vitro.

MATERIALS AND METHODS

HUVEC Cell Culture and Cytokine Upregulation

HUVECs (passage 4–10) were obtained from Clonetics (San Diego, CA) and cultured in endothelial growth medium-umbilical vein (EGM-UV) containing 10% fetal bovine serum (ICN/Flow Laboratories, McLean, VA), epidermal growth factor (10 ng/ml), hydrocortisone (1 μg/ml), bovine brain extract (4 ml/l), and heparin (all four from Clonetics). Gelatin-coated 100 mm dishes were routinely used for passaging HUVECs. Cells were maintained in a 37°C incubator with 10% CO_2. For plating onto vitronectin (Telios Pharmaceuticals, San Diego, CA), the vitronectin was diluted in sterile water to 50 μg/ml per well in a 6-well plate.

Concentrations of kinase modulators and cytokines were determined in separate assays and as described in Molony and Armstrong [1991]. PMA (5 μg/ml) (Sigma Chemical Co., St. Louis, MO), Calphostin C (10^{-7} M) (Kamiya Biomedical Co., Thousand Oaks, CA), 4 α-phorbol (10^{-7} M) (Calbiochem, La Jolla, CA), staurosporine (0.013 M) (Upstate Biotechnology, Inc., Lake Placid, NY), tumor necrosis factor-α (TNF-α; 10 U/ml) (Genzyme Corporation, Cambridge, MA), and interleukin-1β (IL-1β; 100 U/ml) (Genzyme) were added to cells diluted in media at the time of plating.

Immunolocalization Assays

HUVECs were plated onto glass coverslips (Carolina Biological Supply Co., Burlington, NC) coated with extracellular matrix substrata and allowed to attach 5–8 h at 37°C. Cells on coverslips were then washed in phosphate-buffered saline (PBS) and fixed in 3.7% formaldehyde for 10 min. Coverslips were washed in Tris-buffered saline (TBS; 0.005 M Tris-HCl; 0.03 M NaCl; 0.001% sodium azide) and cells were permeabilized in 0.2% Triton X-100 in PBS for 2–3 min, followed by washing in PBS. Primary antisera to the VNr (Telios) were diluted 1:100 and incubated on coverslips 1 h at 37°C, followed by washing in PBS. Antibodies to PKC (Mab 1.9) recognize all of the isozymes of PKC [Mochly-Rosen and Koshland, 1987] and were from Gibco (Grand Island, NY). Fluorescein-conjugated secondary antibodies (Cappell, Malvern, PA) were

diluted 1:500 and incubated with cells on coverslips 1 h at 37°C. Coverslips were then washed in TBS, then water, and were mounted onto slides with gelvatol. Microscopy and photography were performed with a Nikon Diaphot inverted photomicroscope with T-Max 400 film.

Cord Formation Assay

The design of the cord formation assay was based on Grant et al. [1989]. A basement membrane matrix "Matrigel" (Collaborative Research, Inc., Bedford, MA), derived from the Englebreth-Holm-Swarm mouse tumor-secreted extracellular matrix, was diluted 1:5 in sterile water and allowed to polymerize at 37°C for 1 h to overnight. Excess liquid was pipetted off prior to plating of the cells. HUVECs were seeded onto basement membrane-coated plates at 3.5×10^4 cells per well in a 48-well plate. For the inhibition studies, the following antisera and antibodies were used at a 1:50 to 1:100 dilution (100 and 50 μg/ml, respectively): polyclonal antisera to the VNr (Chemicon International, Inc., Temecula, CA, or Telios Pharmaceuticals, San Diego, CA) and fibronectin receptor (FNr) (Telios), monoclonal antibodies to the α_v subunit of the VNr (Telios) [Freed et al., 1989; Vogel et al., 1990], the β_3 subunit (AMAC, Inc., Westbrook, ME), and the β_1 subunit (AMAC), and the adhesion blocking antibody to the α_5 subunit (Telios) [Wayner et al., 1988]. Each assay was repeated three times with six replicates of each treatment.

Digital Analysis of Cord Formation

Cord formation assays were quantitated using a digitizer tablet with the SigmaScan program (Jandel Scientific, Corte Madera, CA). Photographs of each treatment of each cord formation assay were taken on 35 mm film and prints were made. The lengths of the cords in each of the prints were then measured. The program automatically calculated the average lengths and standard errors for each of the treatments. These values were then corrected for actual micrometers (μm) of the cords.

Cell Viability Assays

Cell viability assays were performed as previously described in Plumb et al. [1989]. The water-soluble dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced only by viable cells to a purple formazan product. After the cord formation assays were performed and photographed, 0.2 mg/ml MTT was added to the cells in media and incubated for 3 h at 37°C and 10% CO_2. The medium was removed and the metabolized product was solubilized in 400 μl dimethylsulfoxide and 50 μl Sorensen's glycine buffer (0.1 M glycine; 0.1 M NaCl, pH 10.5) [Plumb et al., 1989]. The absorbance was read at 570 nm in a microplate reader.

Phosphate Labeling of Cells

HUVECs were plated overnight on the matrices of interest, followed by incubation of the adherent cells with phosphate-free Dulbecco's Modified Eagle's Media (Irvine Scientific, Santa Ana, CA) in the presence of 10% dialyzed fetal calf serum for 30 min. [^{32}P]orthophosphate was then added to the cells at 1 mCi per ml together with the cytokines or the PKC modulators at the above concentrations. After 18 h, cells were harvested in RIPA buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.25% deoxycholate; 1 mM PMSF; 100 U/ml aprotinin; 50 μg/ml leupeptin; 10 μg/ml α2-macroglobulin; 1 mM Na_3VO_4; 0.2 mM EGTA) and lysates were subjected to immunoprecipitation as described below. This experiment was repeated five times.

Immunoprecipitation Assays

HUVECs were lysed in RIPA buffer (see above) and incubated with VNr antisera (Telios or Chemicon) diluted 1:100 for 1 h at 4°C. Then 25 μl of a 1:1 slurry of Protein A Sepharose beads previously washed in TBS and blocked with BSA was added to lysates and incubated at 4°C with end-over-end rocking. Protein A beads with the antigen-antibody complexes were then washed extensively in RIPA buffer. Antigen-antibody complexes were removed from the Protein A beads under reducing conditions and boiling for 2 min. The beads were pelleted by centrifugation and the samples were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970]. Molecular weight markers were from Bio-Rad Laboratories (Richmond, CA) and represented the following molecular weights: 200,000, 116,000, 92,500, 67,000, 45,000, 31,000, 21,500, and 14,000 D. Immunoprecipitated proteins were visualized by Coomassie blue staining and exposure to hyperfilm autoradiography film (Amersham Corp., Arlington Heights, IL).

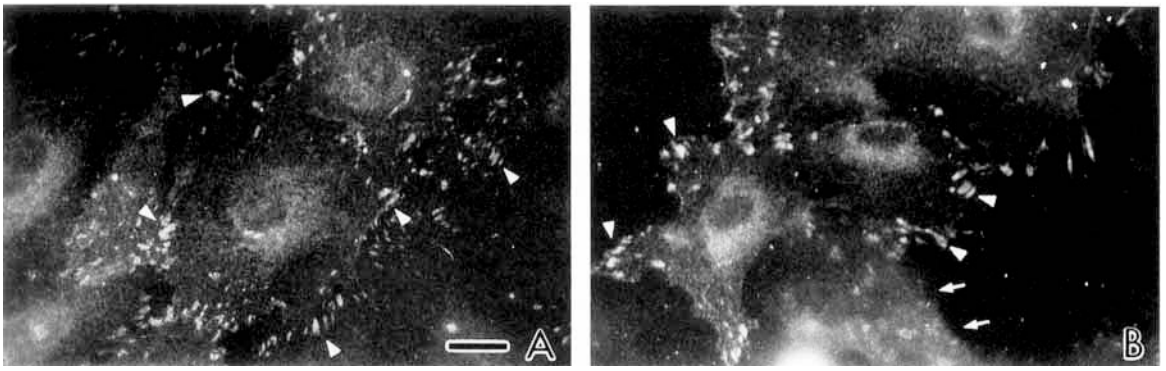

Fig. 1. Immunolocalization of the VNr in HUVECs plated on vitronectin- and basement membrane-coated glass coverslips for 4 h. HUVECs were labeled with rabbit anti-human VNr antisera, followed by washing and incubation with fluorescein-conjugated goat anti-rabbit IgG. **(A)** HUVECs plated on vitronectin; **(B)** HUVECs plated on basement membrane. Arrowheads indicate some of the adhesion plaques with VNr localization. Arrows indicate basement membrane material into which endothelial cells are spreading. Bar = 10 μm.

RESULTS

Immunolocalization of the VNr

In order to affirm that the VNr is present in endothelial cells cultured on basement membrane, and to visualize that localization within the cells, immunolocalization assays were performed (Fig. 1). The VNr, which is usually found in focal contacts when cells are grown on vitronectin (Fig. 1A, arrowheads), was also found in focal contacts when the cells were cultured on basement membrane at the early stages of cord formation (Fig. 1B, arrowheads). Note that the cells on vitronectin appear more spread out in morphology (Fig. 1A), while those on basement membrane appear to be invading the basement membrane (Fig. 1B, fluorescent material at arrows).

Inhibition of Cord Formation by Integrin Antisera and Antibodies

To assess the roles of the VNr and FNr in endothelial cell cord formation, antisera directed against each receptor complex were used (Fig. 2). Note that in untreated cells, or cells in the presence of normal rabbit serum, a cord network formed (Fig. 2A,B, respectively). Antisera to the entire $\alpha_v\beta_3$ molecule (Fig. 2C), and to the entire $\alpha_5\beta_1$ receptor complex (Fig. 2D), inhibited cord formation as seen by the lack of an anastomotic network. To further define this inhibition at the subunit level, antibodies directed against the α_v, β_3, α_5, and β_1 subunits were used (Fig. 2E,G,F,H, respectively). The antibodies to the α_v and β_1 subunits inhibited cord formation most effectively (Fig. 2E,H, respectively), while antibodies to the β_3 subunit inhibited somewhat (Figure 2G). However, antibodies to the α_5 integrin subunit did not inhibit cord formation, as seen by the formation of a full network of cords (Fig. 2F). Furthermore, antibodies to PKC and tubulin (both directed against intracellular antigens, and thus considered controls) had no effect on in vitro cord formation (data not shown).

To further quantitate the inhibition of cord formation observed in Figure 2, photographs were taken from six replicates of each treatment from three separate experiments, and the length of each cord was measured using a digitizing tablet and averaged for each treatment (Fig. 3). Untreated cells (NT) formed cords with an average length of 34 μm, as did cells in the presence of normal rabbit serum (NRS) (Fig. 3). Note that antisera to both the VNr and FNr complexes decreased the average length of the cords to about 18–19 μm (Fig. 3). The VNr subunit-specific antibodies to α_v and β_3 both resulted in similar lengths of 18 and 20 μm, respectively (Fig. 3). Addition of the β_1 antibodies resulted in shorter cords, with average lengths of 15 μm (Fig. 3). However, antibodies to the FNr α_5 subunit resulted in lengths of cords which were similar to controls at 34 μm, which correlates with the finding in Figure 2 in which no inhibition of cord formation was seen with this antibody (Fig. 3). Note that a decrease in length from 34 to 15 μm represents about a 50% inhibition of cord formation, while 18 to 10 μm long cords are about 45% shorter than controls (Fig. 3).

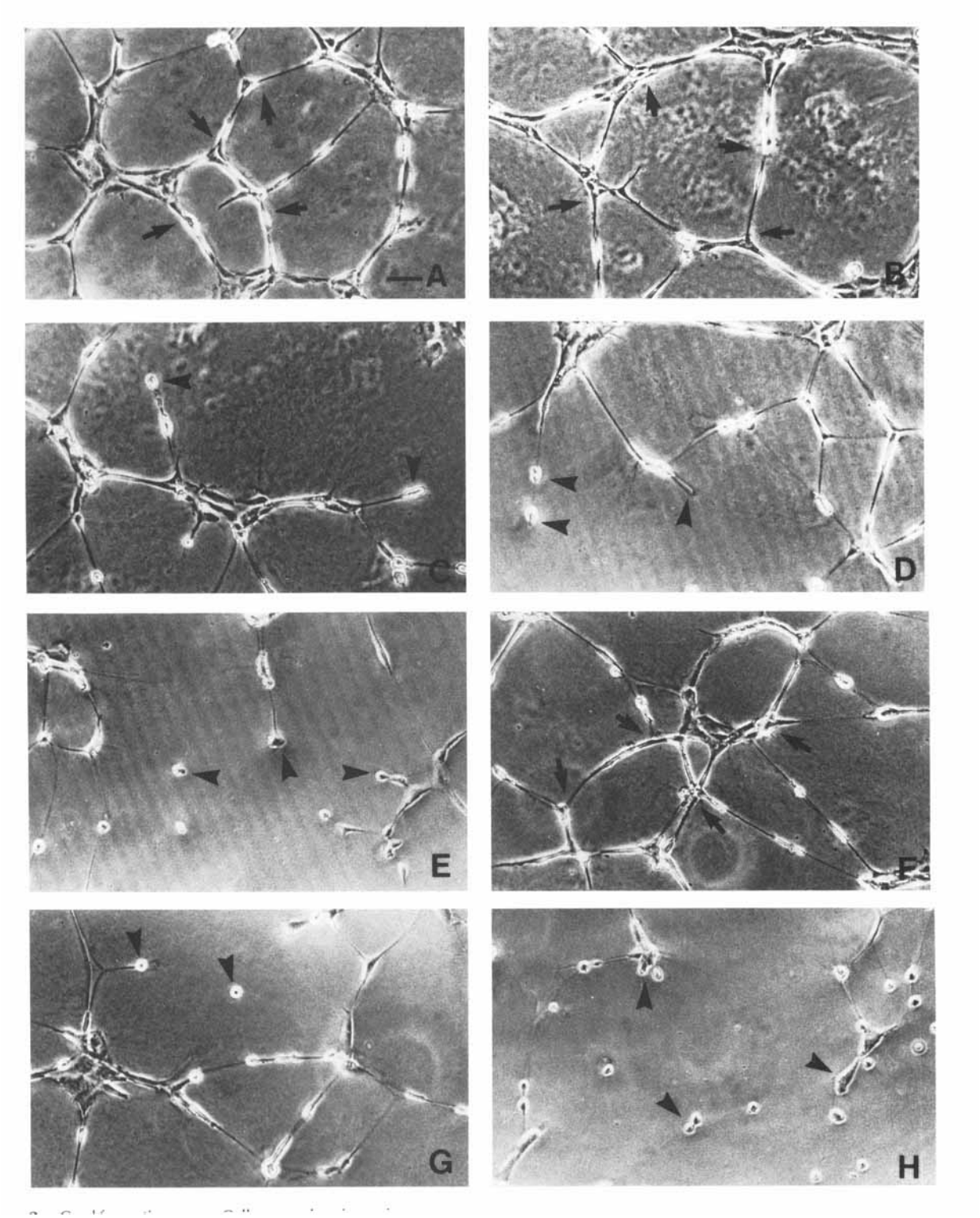

Fig. 2. Cord formation assay Cells were plated onto basement membrane-coated surfaces and allowed to attach and form cords for 18 h in the presence of various antibodies or antisera, all diluted 1 50 in media **(A)** No antibodies or antisera, **(B)** normal rabbit serum **(C)** antisera to the VNr, **(D)** antisera to the FNr **(E)** antibody to α_v, **(F)** antibody to α_5, **(G)** antibody to β_3, **(H)** antibody to β_1 integrin subunit Note that cells in A, B, and F have formed an anastomotic network of cords, while cells in C, D, and G were partially inhibited from forming cords Cells in E and H were slightly more inhibited than those in C, D, and G Arrows in A, B, and F indicate cells which have formed an anastomotic network without inhibition Arrowheads in C–E, G, and H indicate cells which have been inhibited from making the necessary connections to form a network Each cord formation assay was performed three times with six replicates of each treatment Bar = 40 μm

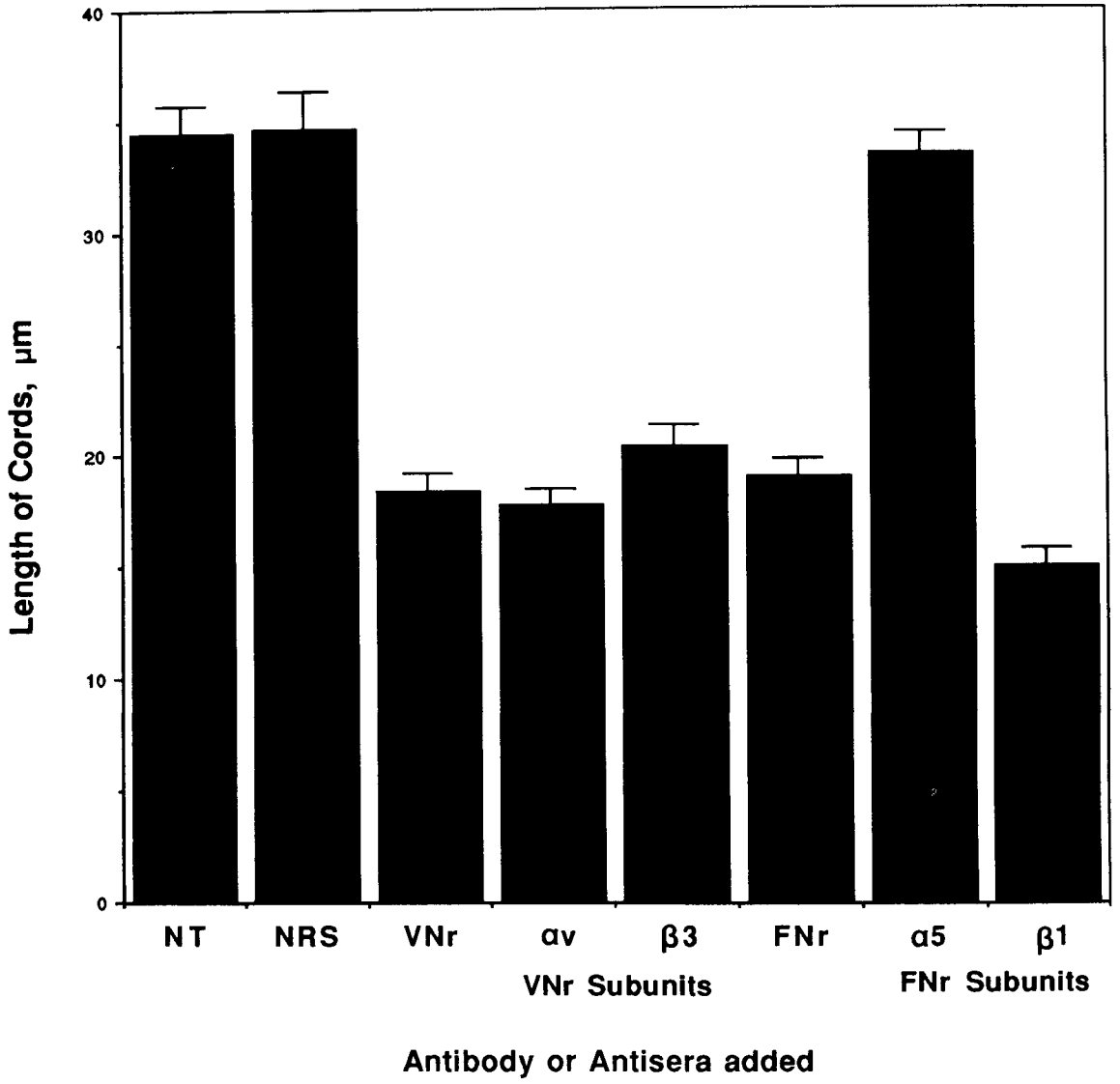

Fig. 3. Quantitation of cord formation assay Photographs of each replicate of each treatment from Figure 2 were taken and lengths of the cords were measured using a digitizing tablet on the Jandel SigmaScan program NT, not treated, NRS, normal rabbit serum, VNr, antisera to the vitronectin receptor, α_v, antibody to the α_v integrin subunit, β_3, antibody to the β_3 integrin subunit, FNr, antisera to the fibronectin receptor, α_5, antibody to the α_5 integrin subunit, β_1, antibody to the β_1 integrin subunit Note that the lengths of cells in the presence of normal rabbit serum and the α_5 subunit antibody were similar to the controls, while antisera to the VNr, FNr, and antibodies to the α_v and β_3 subunits decreased cord length by about 45% Antibodies to the β_1 subunit decreased cord lengths by about 50% Three different experiments, including six replicates each, were digitized, and their values were averaged to obtain the lengths for the histogram

Effects of PKC Modulators on In Vitro Cord Formation

The cord formation assay was also performed in the presence of PKC activators and inhibitors (Fig. 4). HUVECs in the presence of the control phorbol ester 4α-phorbol had a similar appearance to the untreated cells (Fig. 4C,A, respectively). These cells formed anastomotic networks of cords, which are indicated by arrows (Fig. 4A–C). PMA appeared to cause a lengthening of the cords (Fig. 4B). PDBu had an effect similar to PMA (data not shown). The PKC inhibitor Calphostin C at 0.1 µM appeared to inhibit cord formation (Fig. 4D), as implied by the absence of a complete cord network and by the presence of rounded-up cells (Fig. 4D, arrowheads). Staurosporine at 0.013 M also inhibited to the same degree as Calphostin C (data not shown). It is important to note that this inhibition is not due to toxicity as shown by a cell viability assay (see

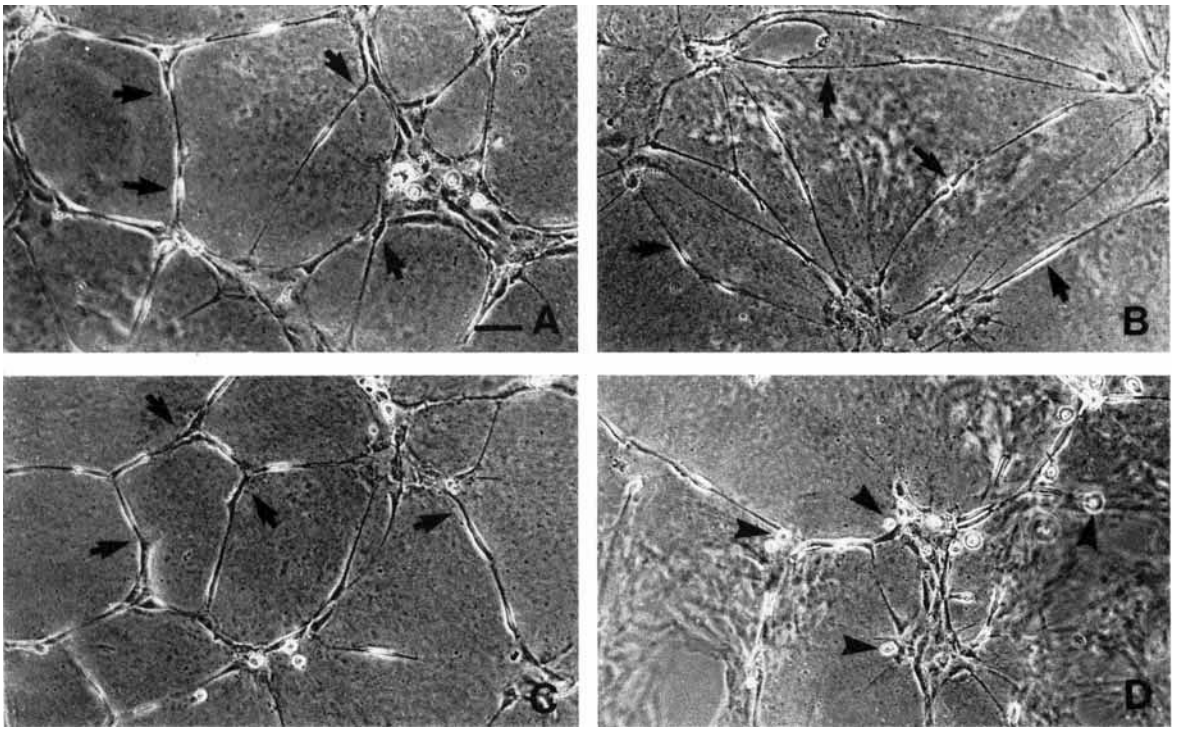

Fig. 4. Cord formation assay. HUVECs were tested for inhibition of cord formation in the presence of PKC inhibitors and stimulators. **(A)** Untreated cells; **(B)** PMA-treated cells; **(C)** 4 α-phorbol-treated cells; **(D)** Calphostin C-treated cells. Note that the cords in the PMA-treated wells appear longer than in controls (B vs. A). Untreated and 4 α-phorbol-treated cells appeared similar in the amount of cord formation (A and C, respectively), and Calphostin C appeared to perturb cord formation in that a complete network is absent and many cells appear rounded and not elongated as in the controls (D and A, respectively). Arrows in A and C indicate cells which have formed cords in an anastomotic network. Arrowheads in D indicate cells which were inhibited from forming a network. Bar = 40 μm.

Materials and Methods) performed after the cord formation assays. Cell viability was not decreased by any of the treatments used at the indicated concentrations.

The lengths of the cords in Figure 4 were analyzed and quantitated in the same manner as described for Figure 3 (Fig. 5). Untreated cells (NT) in this series of experiments typically formed cords with average lengths of 34 μm, as was also the case in Figure 3. The control 4 α-phorbol-treated cells also formed cords of about 34 μm (Fig. 5). Addition of PMA increased the average lengths of the cords to 47 μm (Fig. 5). The addition of Calphostin C resulted in a decrease in average cords length to 15 μm (Fig. 5), which was 50% shorter than the cords found in controls.

Phosphorylation of the VNr

Since the VNr and PKC appear to be mediators of in vitro cord formation, combined with the data of Freed et al. [1989] which indicated that PKC phosphorylates the VNr β_3 subunit in vitro, the phosphorylation of the VNr was examined in endothelial cells on plated vitronectin or on basement membrane in the presence of various modulators of PKC (Fig. 6). It is important to note that this autoradiograph was overexposed to bring up any band which may be present in the endothelial cells cultured on vitronectin and that normal rabbit serum controls did not have the 105,000 band (data not shown), which appears smeared due to the overexposure of the autoradiograph to the gel (Fig. 6). In untreated cells on basement membrane, the level of phosphorylation of the VNr was increased (Fig. 6A, lane 1) over that found in untreated endothelial cells on vitronectin (Fig. 6B, lane 1). The addition of PMA or 4 α-phorbol to endothelial cells on basement membrane had little effect on this already high level of phosphorylation (Fig. 6A, lanes 2 and 3). However, Calphostin C eliminated this phosphorylation completely on both matrices (Fig. 6A,B, lane 4). In order to see

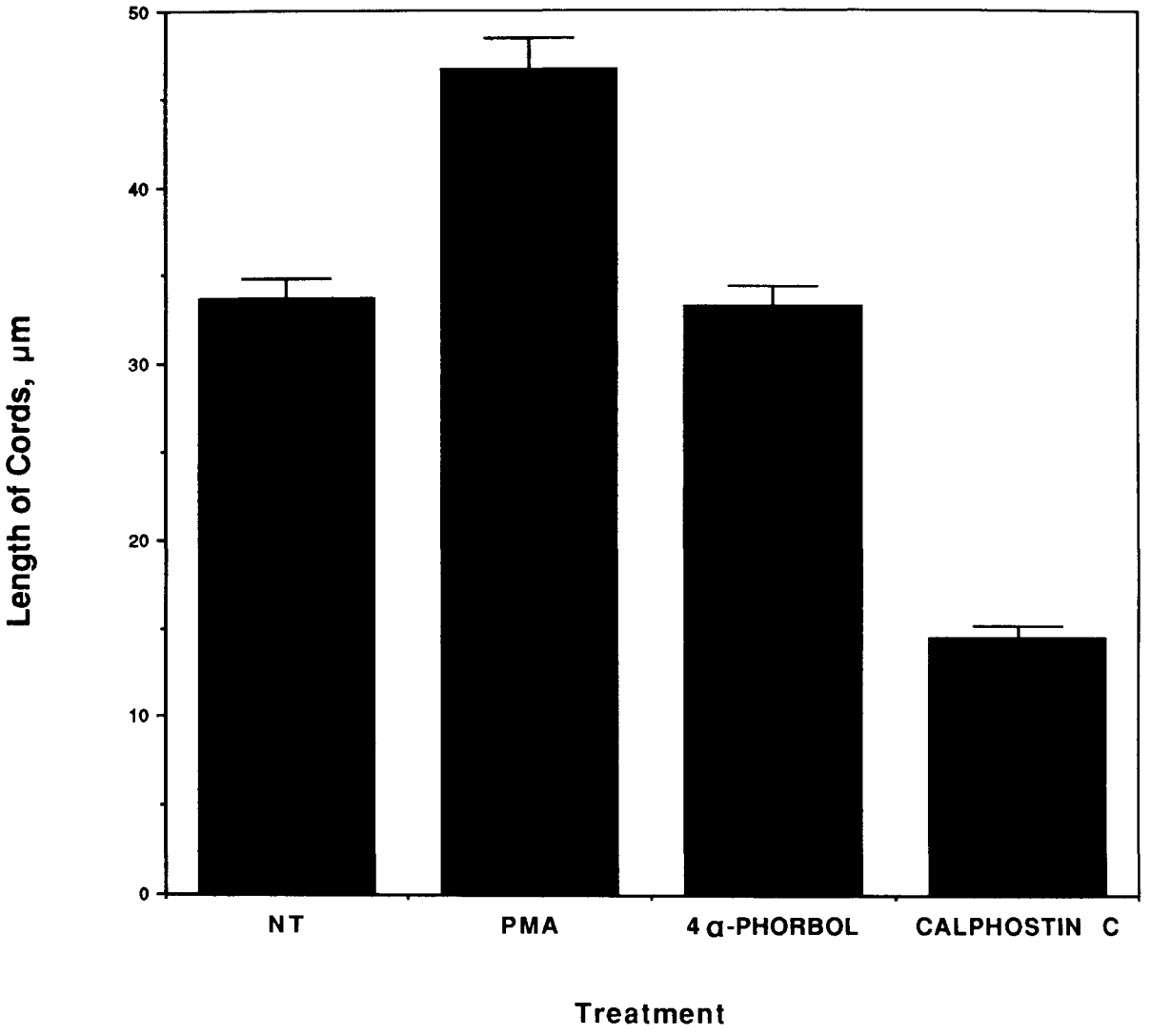

Fig. 5. Quantitation of cord formation. The lengths of the cords were measured as in Figure 3. NT, not treated; PMA, phorbol 12-myristate 13-acetate-treated. Note that untreated and 4 α-phorbol-treated cells had similar lengths (34 μm), while PMA actually increased the lengths of the cords form 34 to 47 μm. Calphostin C decreased the lengths of the cords by about 50%.

any phosphorylation of the VNr on vitronectin, the cells were treated with PMA (Fig. 6B, lane 3). The addition of PMA to endothelial cells on gelatin (Fig. 6B, lane 3) brought the levels of VNr phosphorylation up to those seen in untreated cells on basement membrane (Fig. 6A, lane 3). When TNF-α and IL-1β were added to endothelial cells on basement membrane, VNr phosphorylation levels were similar to those seen in untreated cells (Fig. 6A, lanes 5 and 6 compared with lane 1). When endothelial cells were cultured on vitronectin, IL-1β increased receptor phosphorylation, but to a lesser degree than PMA (Fig. 6B, lane 6 compared with lane 3).

Immunolocalization of the VNr and PKC

Since both the VNr and PKC appear to be important for cord formation, combined with the result that a PKC inhibitor inhibits the phosphorylation of the VNr in endothelial cells on basement membrane, double immunolocalization of the VNr and PKC was performed (Fig. 7). The antibodies to PKC have specificity for all of the isozymes of PKC [Mochly-Rosen and Koshland, 1987]. Untreated cells were cultured on vitronectin or basement membrane-coated coverslips for 5, 8, or 18 h, followed by double-labeling with the VNr and PKC antisera and

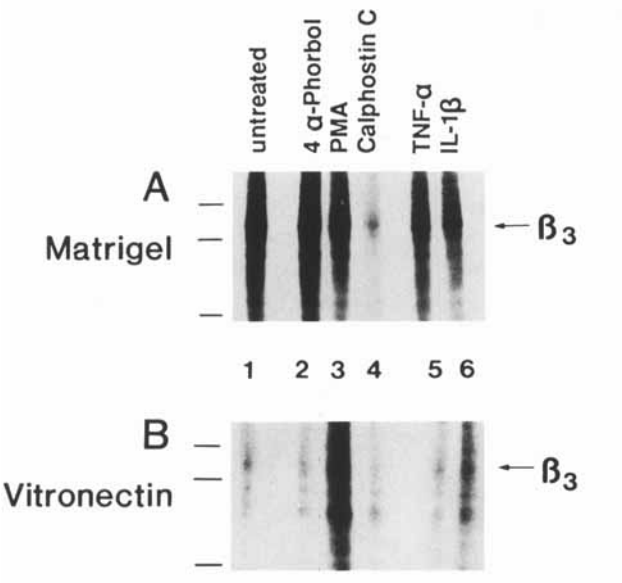

Fig. 6. Phosphorylation of the VNr. Cells were labeled with [^{32}P], followed by lysis in RIPA buffer (see Materials and Methods) and immunoprecipitation with the VNr antisera and SDS-PAGE. **(A)** Cells cultured on basement membrane (Matrigel); **(B)** cells cultured on vitronectin. **(Lane 1)** Untreated cells; **(lane 2)** 4 α-phorbol-treated cells; **(lane 3)** PMA-treated cells; **(lane 4)** Calphostin C-treated cells; **(lane 5)** TNF-α-treated cells; **(lane 6)** IL-1β-treated cells. Lines at left indicate molecular weight markers (Bio-Rad): 116,000, 92,500, and 67,000. The phosphorylated 105,000 β_3 band is indicated at right. The autoradiograph was intentionally overexposed to obtain the few bands found in B. This figure is representative of one of five times this experiment was repeated.

antibodies (Fig. 7). In the cells cultured on vitronectin and on basement membrane for 5 h and not yet forming cords, PKC appeared to be localized with the cytoskeleton (Fig. 7A,C). However, in the cells which were cultured in basement membrane for 8 h that were beginning to form cords, PKC appeared to translocate to the plasma membrane, thus indicating its activation (Fig. 7C, arrows). Once the cords were formed at 18 h, PKC appeared to extend the length of the cells, possibly associating with the cytoskeleton again (Fig. 7E, arrows). Note that the VNr did not colocalize with PKC at the time points examined (Fig. 7B,D,F). On vitronectin, the receptor appeared in adhesion plaques (Fig. 7B); on cord-forming cells on basement membrane, it was less organized and present throughout the cell (Fig. 7D). In cells which have already formed cords, the VNr appeared again at the adhesion plaques at the tips of the cells in cords (Fig. 7F, arrowheads).

DISCUSSION

The aim of the present study was to investigate potential roles of the VNr and PKC in endothelial cell cord formation in vitro. Antibodies to the VNr inhibited cord formation, as did inhibitors of PKC. Furthermore, these two phenomena may be linked, as the VNr β subunit has been shown to be phosphorylated by PKC in vitro [Freed et al., 1989; Parise et al., 1990]. Our results indicated that the induction of VNr phosphorylation by basement membrane was inhibited by PKC inhibitors and that phosphorylation of the receptor was induced by PMA when endothelial cells were cultured on vitronectin. Although the VNr and PKC did not colocalize in double immunolocalization studies, PKC did translocate to the plasma membrane in cells which were forming cords, further indicating that PKC is activated during that process. These findings are important not only for the understanding of vascular formation, but also for the understanding of cellular differentiation and morphogenesis in the phosphorylation of cell adhesion receptors when cells are placed on an in vivo-like substrate.

Basement membranes contain mostly laminin, type IV collagen, entactin/nidogen, heparan sulfate proteoglycan, and several growth factors [Kleinman et al., 1986]. Endothelial cells may adhere to and spread upon this predominantly laminin-containing matrix by more than one mechanism. For example, bovine aortic endothelial cells possess laminin binding proteins [Yannariello-Brown et al., 1988], and HUVECs may utilize laminin as a substrate in vitro for cell adhesion and cord formation on a basement membrane [Grant et al., 1989]. It is also probable that HUVECs adhere to laminin through the VNr, although other binding mechanisms cannot be ruled out and are likely to exist in addition to the VNr. The adhesion of microvascular endothelial cells to laminin via $\alpha_v\beta_3$ has been studied [Kramer et al., 1990]. These studies examined 1) microvascular endothelial cell binding to laminin and found that it was inhibited by antisera to the VNr α_v subunit, and 2) laminin-Sepharose affinity chromatography of receptor complexes and found that the $\alpha_v\beta_3$ receptor complex bound well to laminin via an RGD-insensitive manner [Kramer et al., 1990]. Thus, endothelial cells may bind laminin through two or

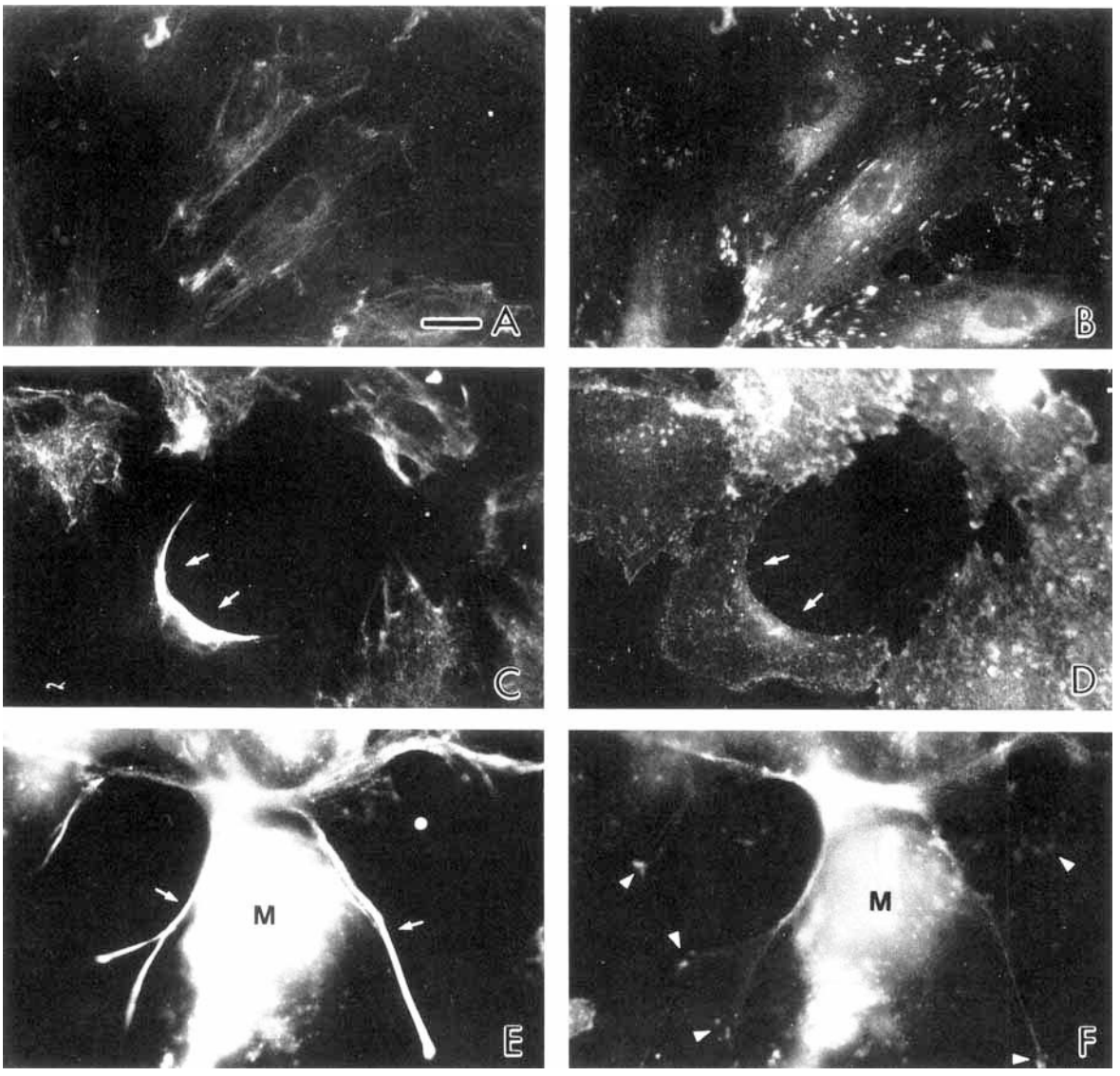

Fig. 7. Immunolocalization of PKC and the VNr in HUVECs. Cells were plated onto vitronectin or basement membrane-coated coverslips for 5 h, followed by fixation and permeabilization. Cells were double-labeled with rabbit polyclonal antisera to the VNr or mouse monoclonal antibodies to PKC, followed by fluorescein-conjugated goat anti-rabbit and rhodamine-conjugated goat anti-mouse IgG. **(A,B)** Cells cultured on vitronectin; **(C–F)** cells cultured on basement membrane. (A,C,E) PKC localization; (B,D,F) VNr localization on the same cells as in A, C, and E. Note that PKC localization is cytoskeletal on cells still in a monolayer (A). In cells forming cords, PKC translocated to the plasma membrane (E, arrows). Note that the VNr appears in plaques before and after cord formation (B and F, respectively), while it is less organized in the cord-forming cells (D). Arrowheads in F indicate VNr localization to adhesion plaques at the tips of cord-forming cells. M, Matrigel (fluorescent material). Bar = 10 μm.

more mechanisms: integrins and non-integrin laminin binding proteins.

The VNr α_v subunit has classically been believed to associate with the β_3 subunit [Horton, 1990]. However, it has also been shown to associate with β_1 [Bodary and McLean, 1990; Dedhar and Gray, 1990; Vogel et al., 1990], β_5 [Ramaswamy and Hemler, 1990; Smith et al., 1990], β_x [Cheresh et al., 1989], and β_s [Freed et al., 1989]. It is possible that the VNr interacts with either the β_1 or β_3 subunit, as antibodies against both inhibited cord formation to a similar extent. This does not rule out the possibility that other subunits are interacting with the β_1 subunit and playing a role in cord formation. Moreover, the adhesion blocking antibody to the α_5

subunit [Wayner et al., 1988] did not inhibit cord formation, further suggesting that the action of β_1 is separate from α_5 and the FNr in this assay.

Since PKC inhibitors inhibited both cord formation and VNr phosphorylation, a role for VNr phosphorylation in cord formation is possible. VNr phosphorylation could play a role in the changes in integrin-cytoskeleton interactions which must occur during cord formation. Integrins have been shown to interact with the cytoskeleton [Burridge et al., 1987]. For example, the FNr was shown to interact with talin [Horwitz et al., 1986] and the β_1 integrin subunit has been shown to interact with α-actinin [Otey et al., 1990]. Furthermore, phosphorylation of integrins has been postulated to regulate their interactions with the cytoskeleton, as well as with extracellular matrix adhesion in a number of cell types [Shaw et al., 1990; Dahl and Graebel, 1989; Buck and Horwitz, 1987; Danilov and Juliano, 1989]. Phosphorylated integrin has a lower binding affinity for fibronectin and talin [Buck and Horwitz, 1987], suggesting altered cell adhesive and intracellular interactions due to its phosphorylation. Regulation of cytoskeletal interactions by phosphorylation has not yet been demonstrated for the VNr, although the β_3 subunit has been shown to colocalize with vinculin and talin [Zambonin-Zallone et al., 1989]. Some phosphoproteins did coprecipitate with the VNr in cells cultured on basement membrane in the phosphorylation studies presented here; these proteins could be cytoskeletal proteins. Perhaps matrix-regulated phosphorylation of the VNr in turn regulates its associations with cytoskeletal proteins involved in cell motility.

In support of a role of PKC in cellular differentiation on basement membrane, PKC is translocated to the plasma membrane of endothelial cells forming cords from a largely cytoskeletal localization in cells grown in a monolayer on vitronectin. Activation of PKC is often accompanied by its translocation to the plasma membrane [Nelsestuen and Bazzi, 1991], presumably to phosphorylate proteins present there. The translocation of PKC from the cytoskeleton to the plasma membrane indicates that it is activated during cord formation. Since it has been shown that PKC activation is involved in basement membrane-induced neural growth cone extension [Bixby, 1989], it may play a role in a number of cellular differentiation events observed in cells on basement membranes, including cord formation. It has also been shown that PKC activators suppress bovine endothelial cell proliferation, and thus PKC activation may promote endothelial cells to cease dividing and begin differentiating into non-growing tubes during angiogenesis [Doctrow and Folkman, 1987]. Recently, this role of PKC in endothelial cell cord formation has been confirmed by another group using inhibitors and activators of PKC [Kinsella et al., 1992]. It is also important to note that the PKC antibodies we used had a broad specificity for PKC isozymes [Mochly-Rosen and Koshland, 1987].

In conclusion, both the VNr and PKC play important roles in endothelial cell cord formation in vitro. Whether phosphorylation of the VNr regulates its associations with intracellular cytoskeletal components or signal transduction pathways are critical questions whose answers may provide insights into basic cellular morphogenesis.

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