Cell Surface Distribution of Fibronectin and Vitronectin Receptors Depends on Substrate Composition and Extracellular Matrix Accumulation

Irwin I. Singer,* Solomon Scott,* Douglas W. Kawka,* Diana M. Kazazis,* James Gailit,‡ and Erkki Ruoslahti‡

*Department of Biochemical and Molecular Pathology, Merck, Sharp, and Dohme Research Laboratories, Rahway, New Jersey 07065; and ‡Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, California 92037

Abstract. We used antibodies against the alpha subunits of the human fibronectin receptor (FNR) and vitronectin receptor (VNR) to localize simultaneously FNR and VNR at major substrate adhesion sites of fibroblasts and melanoma cells with double-label immunofluorescence microscopy. In early (2–6-h) serum-containing cultures, both FNR and VNR coaccumulated in focal contacts detected by interference reflection microscopy. Under higher resolution immunoscanning electron microscopy, FNR and VNR were also observed to be distributed randomly on the dorsal cell surface. As fibronectin-containing extracellular matrix fibers accumulated beneath the cells at 24 h, FNR became concentrated at contacts with these fibers and was no longer detected at focal contacts. VNR was not observed at matrix contacts but remained strikingly localized in focal contacts of the 24-h cells. Since focal contacts represent the sites of strongest cell-to-substrate adhesion, these results suggest that FNR and VNR together play critical roles in the maintenance of stable contacts between the cell and its substrate. In addition, the accumulation of FNR at extracellular matrix contacts implies that this receptor might also function in the process of cellular migration along fibronectin-containing matrix cables.

To define the factors governing accumulation of FNR and VNR at focal contacts, fibroblasts in serum-free media were plated on substrates coated with purified ligands. Fibronectin-coated surfaces fostered accumulation of FNR but not VNR at focal contacts. On vitronectin-coated surfaces, or substrata derivatized with a tridecapeptide containing the cell attachment sequence Arg-Gly-Asp, both FNR and VNR became concentrated at focal contacts. These observations suggest that the availability of ligand is critical to the accumulation of FNR and VNR at focal contacts, and that FNR might also recognize substrate-bound vitronectin.

ADHESION of cultured cells involves a series of complex interactions between the cytoskeleton and the extracellular matrix. During attachment to planar substrata, the lower cell membrane forms several unique types of contacts that are recognizable with interference reflection microscopy (IRM)[1] and immunofluorescence microscopy (IFM). Early adhesion sites include the labile close contacts that exhibit a 30-nm cell-to-substrate space and the more tenacious focal contacts that approach to within 10 nm of the substratum (30, 31). Focal contacts appear black by IRM, show high concentrations of the actin-binding proteins vinculin and talin (7, 19), and are situated at the membrane insertion sites of actin microfilament bundles. More recently, a third type of adhesion site termed the extracellular matrix contact was described (8). It is composed of colinear trans-

membrane associations of actin microfilaments, a fibronectin receptor, and fibronectin-containing extracellular matrix fibers closely apposed to the substratum (9). Electron microscopy has shown that extracellular matrix contacts contain fibronexuses: close transmembrane associations of fibronectin fibers and 5-nm actin microfilaments (42–44). In timecourse studies, focal contact formation preceded the appearance of fibronexuses and extracellular matrix contacts (45), which subsequently accumulated basement membrane heparan sulfate proteoglycan (47).

Fibronectin and vitronectin are two dominant serum glycoproteins that are important for the attachment and spreading of cultured cells (25, 29); both ligands have related but distinct receptors in human cells (39–41). Close relatives of the human fibronectin receptor (FNR) and vitronectin receptor (VNR) include the chicken integrin complex (28, 53), gp IIb/IIIa of platelets (38), the LFA-1, Mac-1, and p150,95 leukocyte surface glycoproteins (33), and the VLA antigens of T lymphocytes (51, 52). Members of this receptor superfam-

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ily, termed the integrins (28), are heterodimeric glycoprotein complexes composed of alpha and beta subunits (33, 38–40, 48). Many of these receptors recognize the sequence Arg-Gly-Asp present in their ligands (28, 35, 36, 38–41). The alpha and beta subunits of integrin receptor complexes form two distinct groups of homologous polypeptides (18, 28, 33, 41, 49, 53). Beta subunits of several integrin receptors can be identical or closely similar, while the alpha subunits appear to be unique for each receptor (21, 28, 41, 48, 51, 52). Thus, FNR shares its beta subunit with four other VLA adhesion receptors (51, 52), and VNR beta subunit is closely related or identical to the IIIa component of gpIIb/IIIa complex (21, 28, 41). Much of the ligand-specific binding information is thought to reside in the alpha subunits of these receptors (5, 33, 41).

Insights into the role of extracellular matrix receptors in cellular adhesion may be obtained by localizing them at the surfaces of cultured cells by IFM. Such studies have been performed on the chicken fibroblast integrin receptor complex that recognizes fibronectin, laminin, collagen IV, and vitronectin (1, 2, 23, 26, 27, 41). By means of mAbs that bind the beta subunit of this complex (5), integrin was localized at the periphery of focal contacts (15) and within extracellular matrix contacts (9, 10). In mammalian fibroblasts, antibodies to FNR were located within rather than around focal contacts (20, 22), and VNR was concentrated in structures resembling focal contacts (11, 13).

To examine the relative roles of mammalian fibronectin and vitronectin receptors in cellular adhesion, we compared surface patterns of FNR and VNR simultaneously on cells grown under various conditions. To avoid detecting related integrin receptors, we used antibodies that specifically recognize the alpha subunits of the human FNRs and VNRs. These receptors were visualized by double-label IFM and immunoscanning electron microscopy, and localized in relation to substrate adhesion sites by IRM. We found that although both receptors appeared randomly distributed on the upper cell membrane, they became coconcentrated at focal contacts at early times in serum-supplemented cultures. At later times when abundant matrix fibers were synthesized, FNRs were depleted from focal contacts and became concentrated at extracellular matrix contacts. In addition, while FNRs and VNRs were colocalized in focal contacts formed on substrata derivatized with Arg-Gly-Asp sequences or coated with vitronectin, they were distributed differentially on fibronectin surfaces.

Materials and Methods

Cells

Human gingival (Gin I) fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and propagated in DME with 10% FBS. M-21 human malanoma cells, a gift from R. Reisfeld (Scripps Clinic and Research Foundation, La Jolla, CA), were cultured in RPMI 1640 containing 10% FBS. These cell lines were subcultivated twice weekly, and were in passages 9–18 for the experiments reported here; they were free of *Mycoplasma* contamination measured with a DNA hybridization assay (Gen-Probe, San Diego, CA). Both cell types demonstrated efficient attachment to fibronectin or vitronectin-coated surfaces in attachment assays performed as described (45).

Antibodies and Proteins

Antisera to FNR and VNR complexes purified from human placentas were
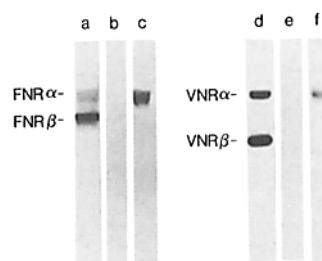
Figure 1. Immunoblotting of affinity-purified antibodies for the alpha subunits of FNR and VNR. FNRs (*a, c,* and *e*) and VNRs (*b, d,* and *f*) were isolated from human placenta (37), their alpha and beta subunits separated by nonreducing SDS-PAGE, and then transferred to nitrocellulose. Individual lanes were incubated with the following antibodies: (*a*) anti-FNR alpha and beta subunits; (*b* and *c*) affinity-purified anti-FNR alpha subunit; (*d*) anti-VNR alpha and beta subunits; (*e* and *f*) affinity-purified anti-VNR alpha subunit. Bound antibodies were detected by peroxidase-conjugated goat anti-rabbit IgG. The anti-FNR and anti-VNR were purified by affinity chromatography on whole receptors. Affinity-purified anti-FNR alpha subunit IgG and anti-VNR alpha subunit IgG were isolated as described in Materials and Methods.

raised in rabbits as previously described (37). The FNR antiserum for our experiments was adsorbed on fibronectin-Sepharose to avoid contamination with antifibronectin IgG. Antibodies specific for portions of the alpha chains of FNR and VNR were affinity purified using insert-coded proteins immobilized on nitrocellulose filters as described (3, 49). Immunoblotting analysis showed that these antibodies were specific for the alpha subunits of FNR and VNR, respectively (Fig. 1). A mouse mAb (mAb 142) that recognizes the alpha subunit of human VNR (11, 12) was provided by D. A. Cheresh (Scripps Clinic and Reserach Foundation, La Jolla, CA). Monospecific rabbit anti-human fibronectin IgG (45, 47) was used to detect fibronectin-containing extracellular matrix fibers. Human fibronectin and vitronectin used for coating culture substrata were purified from plasma by previously described methods (17, 24). ELISAs performed on the vitronectin did not detect any fibronectin contamination. The Arg-Gly-Asp-containing tridecapeptide, Try-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Cys, was synthesized and purified as previously described (45). Affinity-purified fluorescein-conjugated goat anti-rabbit IgG and rhodamine-labeled goat anti-mouse IgG were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). BSA (type III; Sigma Chemical Co., St. Louis, MO) was chromatographed on columns of gelatin-Sepharose and glass beads (24) to remove putative fibronectin or vitronectin contaminants, followed by heat denaturation at 80°C for 10 min.

Light Microscopy

Cells for IFM and IRM study were dispersed in medium with 10% FBS and seeded into multiplate wells (No. 3524; Costar, Cambridge, MA) (2 × 10⁴ cells/well) containing 12-mm-diam glass coverslips as before (46). To explore the effects of purified ligands on the distribution of FNR and VNR, cells were suspended in serum-free media containing 2 mg/ml BSA, 25 mM Hepes, and sometimes 25 µg/ml cycloheximide and 50 µg/ml antifibronectin IgG. These cells were plated on coverslips precoated with 5-µg/ml solutions of either fibronectin, vitronectin, or an Arg-Gly-Asp-containing tridecapeptide as previously described (45). The cultures were fixed with 3.5% paraformaldehyde in 0.1 M sucrose, 0.1 M Na-cacodylate buffer (pH 7.2), and 4.5 mM CaCl₂, permeabilized with 0.1% Triton X-100, and treated with 5 mg/ml NaBH₄ in 0.1 M Tris-HCl buffer (pH 7.2) followed by a solution of nonfat dry milk (32) to reduce the background (45). Single-label IFM staining was performed as previously described (45). In double-labeling experiments, affinity-purified rabbit anti-FNR alpha chain IgG and mouse mAb-recognizing VNR alpha subunit (12) were applied to the coverslips as a clarified mixture (44) to avoid labeling cells with putative complexes of cross reacting antibodies. In blocking experiments, this mixture was preincubated with purified FNR (110 µg/ml) or VNR (170 µg/ml) for 3 h at 37°C, followed by centrifugation at 15,000 g for 15 min at 4°C. A solution containing the affinity-purified anti-rabbit IgG and anti-mouse IgG fluorochrome conjugates was used to detect the primary antibodies. Radial immunodiffusion was used to monitor the species specificity of these immunoconjugates. The stained cultures were studied with a Zeiss Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) equipped for simultaneous dual-label IFM and IRM as previously described (45, 47).

Immunoscanning Electron Microscopy

To study the distribution of FNR and VNR on the dorsal cell surface at higher resolution, coverslip cultures were fixed with formaldehyde and labeled with anti-FNR or anti-VNR IgGs as described above, and then with 5 nm collodial gold goat anti-rabbit IgG (Janssen Pharmaceutica, Piscataway, NJ). Refixation was performed with 2% glutaraldehyde in 0.1 M phosphate buffer followed by silver enhancement as previously described (34). The cultures were then dehydrated with ethanol, incubated in amyl acetate, critical point dried from liquid carbon dioxide, and sputter coated with gold. Scanning electron micrographs were taken at 100–120 kV using a JEOL 200 CX TEMSCAN electron microscope with the stage tilted to 30°.

Results

Localization of Fibronectin and Vitronectin Receptors at Substrate Adhesion Sites

The distributions of FNR and VNR at the cell-to-substrate attachment surface was monitored by IFM with alpha subunit-specific antibodies (Fig. 1) that we shall call FNR and VNR probes. In early (6-h) serum-containing cultures of human gingival fibroblasts, FNR was concentrated at ovoid peripheral plaques that resembled focal contacts (Fig. 2 A, arrowheads). FNR also accumulated at the cell margin and at fibrous structures similar to extracellular matrix cables under the center of the cell (Fig. 2 A, arrows). Likewise, VNR immunostaining was localized at the cell edge, and at focal contact-like structures, but did not exhibit the fibrous pattern observed for FNR beneath the cell center (Fig. 2 B). Gin I fibroblasts grown for 24 h exhibited a striking redistribution of FNR. These cells had synthesized conspicuous arrays of extracellular matrix fibers beneath the attachment surface at the cell center that were brightly positive for FNR (Fig. 2 C) but lacked VNR labeling (Fig. 2 D). On the other hand, FNR staining was no longer found in peripheral foci (Fig. 2 C), although plaque-like VNR labeling was still seen at these sites (Fig. 2 D). Similar patterns of FNR and VNR were observed in human melanoma cultures (Fig. 2, E–H), but the labeling was influenced by the reduced ability of these cells to form extracellular matrix fibers. At 6 h, both FNR (Fig. 2 E) and VNR (Fig. 2 F) were localized in foci along the extended M-21 cell processes; these foci were smaller than those observed in Gin I fibroblasts. Later (24 h), melanoma cells showed FNR staining in small centrally located fibers (Fig. 2 G, arrow), but unlike the fibroblasts, these melanoma cells sometimes retained the plaque-like FNR staining (Fig. 2 G, arrowhead) exhibited in 6-h cells. The focal concentrations of VNR in 24-h M-21 cells were unchanged (Fig. 2 H, arrowheads).

Codistribution of Fibronectin and Vitronectin Receptors at Focal Contacts and Extracellular Matrix Attachment Sites

The patterns obtained with FNR and VNR probes suggested that both receptors were colocalized in structures resembling focal contacts, and that FNR also accumulated at sites similar to extracellular matrix contacts. By performing dual-label IFM with IRM, both FNR and VNR could be studied simultaneously at these two types of contacts identified independently by physical means. Receptor localization at focal contacts and extracellular matrix contacts was verified using transparent overlays as previously described (44).

Under IRM, gingival fibroblasts cultured for 6 h in 10%

FBS (Fig. 3, A–C) exhibited prominent focal contacts just inside the leading edge of the cell (Fig. 3 C, arrowheads) and centripetally located extracellular matrix contacts which appeared light gray to white (Fig. 3 C, arrows). Both FNR (Fig. 3 A, arrowheads) and VNR antibodies (Fig. 3 B, arrowheads) stained completely the focal contacts observed with IRM, whereas only FNR labeling was found at extracellular matrix contact sites (corresponding arrows). When conspicuous extracellular matrix fibers developed at 24 h (Fig. 3, D–F), the peripheral focal contacts (arrowheads in Fig. 3 F) remained positive for the VNR (arrowheads in Fig. 3 E), but exhibited no localized FNR labeling (matching arrowheads in Fig. 3 D). However, at this time, FNR was concentrated in contacts with the extracellular matrix fibers, while VNR did not accumulate at these sites (corresponding arrows in Fig. 3, D–F). Similarly, the M-21 melanoma cells displayed a striking codistribution of FNR and VNR at focal contacts (Fig. 3, G–I, arrowheads) after 6 h. After 24 h, VNR staining remained concentrated at focal contacts (Fig. 3, K and L, arrowheads), but extracellular matrix contacts were VNR negative (matching arrows in Fig. 3, K and L). Fibrous arrays of FNR labeling were found immediately adjacent to but not within focal contacts (corresponding arrowheads in Fig. 3, J and L), and at extracellular matrix contact sites (Fig. 3, J and L, arrows).

### Specificity of Double-Label Immunofluorescence Staining

Blocking experiments were conducted to verify the specificity of the FNR and VNR staining patterns. When the primary mixture of receptor antibodies was preincubated with purified human VNR, the fluorescein staining produced by anti-FNR was observed at focal contacts and extracellular matrix sites (Fig. 4 A), while the rhodamine staining due to anti-VNR was eliminated (Fig. 4 B). Conversely, pretreatment of the primary antibody solution with purified human FNR abolished the FNR labeling pattern (Fig. 4, C and G) while leaving intact the VNR labeling at focal contacts (Fig. 4, D and H; IRM not shown). In a further check of immunospecificity, we monitored the effect of pretreating either FNR or VNR antibodies with irrelevant antigen. As shown in Fig. 4 E, incubating FNR antibodies with purified human VNR did not diminish the FNR imunostaining intensity, while the use of purified FNR blocked the labeling (Fig. 4 F). Similar results were obtained in the converse experiment (data not shown).

Effects of Substrate Composition on Localization of Fibronectin and Vitronectin Receptors

The serum-containing media used in the above experiments contain various adhesive ligands. To elucidate the effects of individual adhesive proteins on the distribution of FNR and VNR, coverslips were coated with fibronectin or vitronectin, and serum-free cultures were established. After short (2-h) culture periods, 77% of the fibroblasts and all of the M-21 cells lacked extracellular fibronectin fibers detectable with IFM (not shown). On fibronectin-coated surfaces, both cell types exhibited striking concentrations of FNR at focal contacts (Fig. 5, A and C, arrowheads), whereas no VNR accumulated at these sites (corresponding arrowheads in Fig. 5, B and D). In contrast, cells cultured on vitronectin sub-
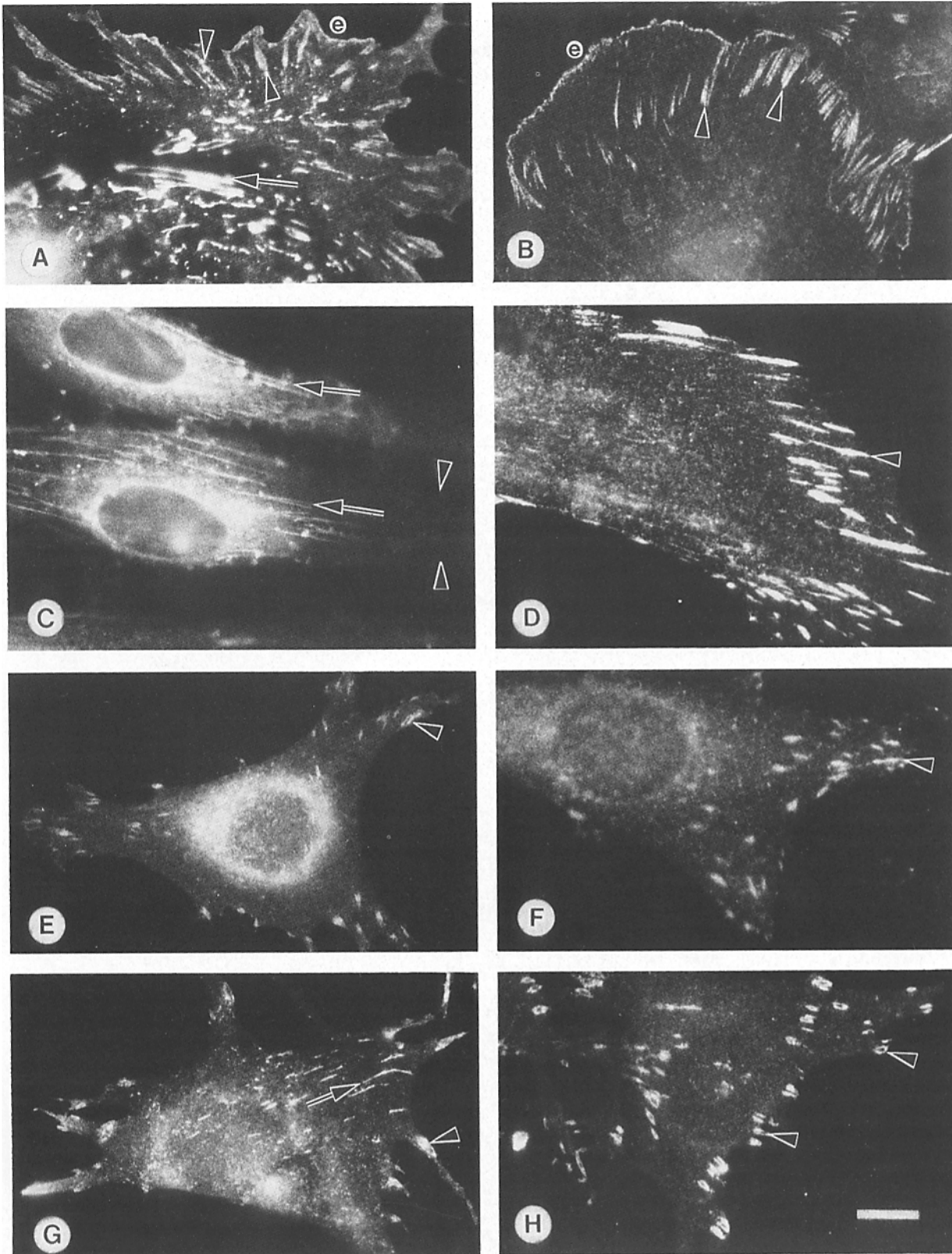
Figure 2. IFM distribution of the FNR and VNR detected with affinity-purified antibodies against the alpha subunits of FNR (*A, C, E* and *G*) and VNR (*B, D, F,* and *H*) in cells cultured with 10% FBS. (*A*) Human Gin I fibroblast cultured for 6 h exhibited peripheral concentrations of FNR that resembled focal contacts (*arrowheads*), and fibrous patterns beneath the center of the cell (*arrow*); there was also labeling in the plasmalemma at the cell edge (*e*). (*B*) Staining of a preparation similar to that in *A* with VNR-specific antibodies showed that VNR was localized in focal contact-like structures (*arrowheads*) and at the cell edge (*e*), but did not exhibit a fibrous pattern at the cell center. (*C*) Linear arrays of FNR staining were concentrated in the substrate-binding membrane at the centers (*arrows*) of Gin I cells grown for 24 h, but this receptor was no longer localized at the cellular periphery (*arrowheads*). (*D*) VNR accumulated in structures resembling focal contacts (*arrowhead*) at the periphery of Gin I fibroblasts cultured for 24 h, but was not concentrated in the central linear arrays observed for FNR. (*E*) Human M-21 melanoma cells exhibited a punctate distribution of FNR (*arrowhead*) after 6 h; (*F*) VNR showed a similar labeling pattern (*arrowhead*) in these cells. (*G*) After 24 h, FNR was localized at both fibrous (*arrow*) and focal contact-like (*arrowhead*) structures in M-21 cells, whereas (*H*) VNR was concentrated only at sites that appeared to be focal contacts (*arrowheads*). Bar, 10 μm.
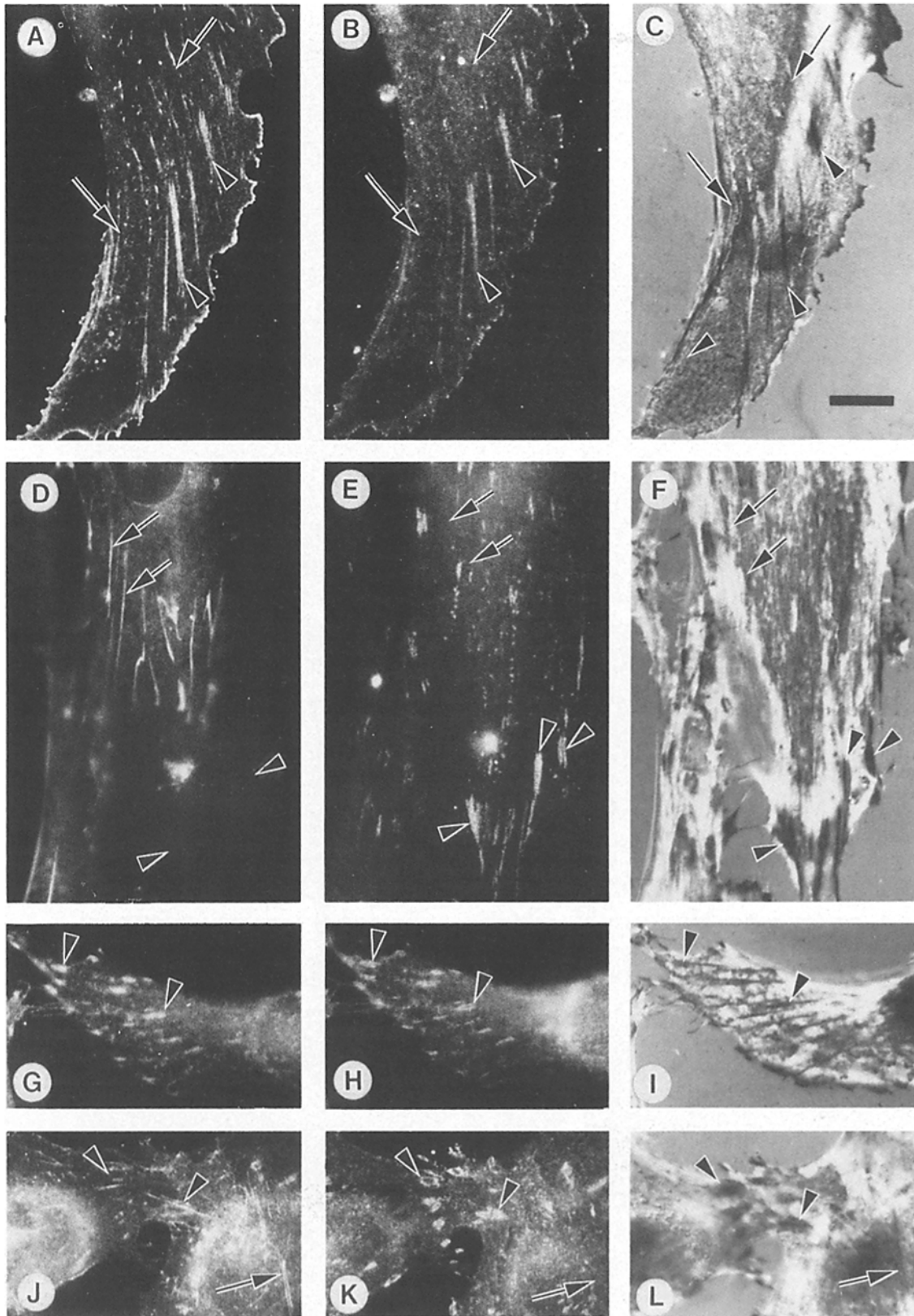
Figure 3. Double-label IFM micrographs depicting the codistribution of FNR (*A, D, G,* and *J*) and VNR (*B, E, H,* and *K*) at substrate contact sites detected with IRM (*C, F, I,* and *L*) in human cells plated in 10% FBS. Cultures were fixed, permeabilized, and double labeled using a mixture of rabbit anti–FNR alpha chain IgG and mouse monoclonal anti–VNR alpha subunit IgG as described in Materials and Methods. After 6 h, elevated concentrations of FNR (*A*) and VNR (*B*) were colocalized at focal contacts (*C, corresponding arrowheads*), while extracellular matrix contacts were positive for FNR but not VNR (*matching arrows*). After 24 h, most focal contacts retained VNR staining (*E, arrowheads*) but lacked concentrated FNR labeling (*D, arrowheads*), whereas high levels of FNR (*D, arrows*) were found in the matrix contacts (*F, corresponding arrows*) that were deficient in VNR (*E*). In melanoma cells (*G–L*), focal contacts (*matching arrowheads*) containing codistributions of FNR (*G*) and VNR (*H*) were seen at 6 h without extracellular matrix contacts, which appeared by 24 h (*J–L*). These fibrous contacts (*L, arrow*) showed localized FNR but not VNR staining (*J* and *K, arrows*). Focal contacts (*L, arrowheads*) with VNR labeling (*K, arrowheads*) were sometimes associated with fibrous arrays of FNR staining (*J, arrowheads*) at 24 h. Bar, 10 μm.
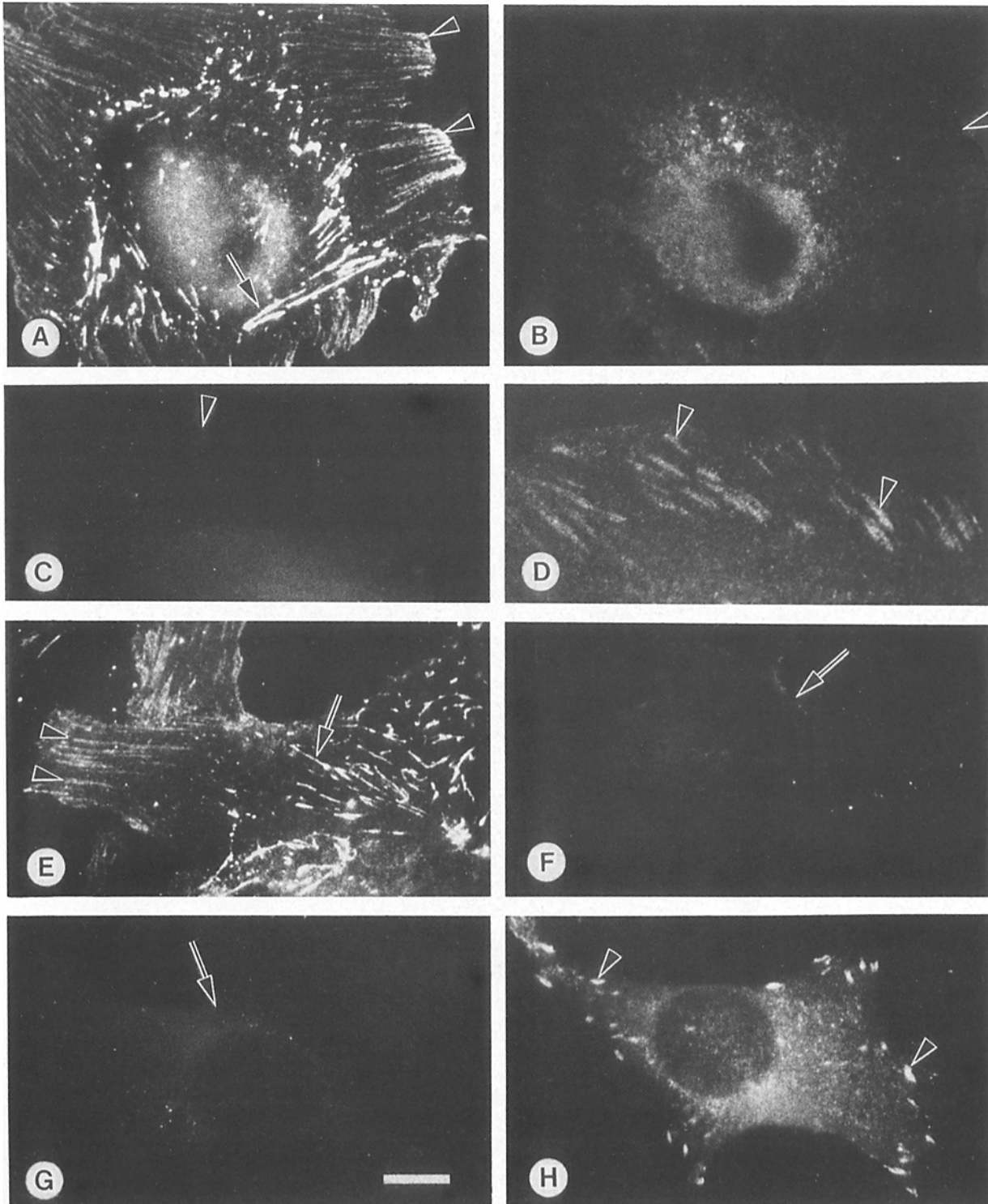
Figure 4. Control micrographs demonstrating the specificity of FNR and VNR staining. Mixtures of rabbit anti–FNR alpha chain IgG and monoclonal anti–VNR alpha subunit IgG were pretreated with purified FNR or VNR to inhibit the relevant immunostaining patterns as described in Materials and Methods. *A* and *B* depict double-IFM labeling of a Gin I fibroblast with a mixture of antibodies to FNR and VNR pretreated with purified VNR. (*A*) FNR labeling visualized with fluorescein-specific optics was concentrated at focal contacts (*arrowheads*) and at extracellular matrix contact sites (*arrow*), while the usual VNR staining at focal contacts viewed with rhodamine optics was blocked by the VNR antigen (*B, arrowhead*). *C* and *D* show a converse experiment in which similar cells were double stained with a solution of antibodies to the alpha subunits of VNR and FNR which was preincubated with purified FNR. (*C*) With fluorescein filters, no FNR staining was found at peripheral focal contacts (*arrowhead*) due to inhibition by FNR. (*D*) Under rhodamine illumination, the unblocked anti–VNR antibodies labeled focal contacts (*arrowheads*). *E* and *F* illustrate a monochromophore FNR-labeling experiment using preincubations with either relevant (FNR) or irrelevant (VNR) antigen. (*E*) Pretreatment of the anti–FNR antibodies with purified VNR did not alter FNR labeling at focal contacts (*arrowheads*) and extracellular matrix contacts (*arrow*) in 6-h fibroblast cultures, but FNR (*F*) completely blocked staining of these contacts (*arrow*). *G* and *H* depict a two-chromophore blocking experiment with 6-h M-21 human melanoma cells. (*G*) Preincubation of the antibody mixture with FNR abolished the FNR staining pattern of this cell (*arrow*), whereas the punctate VNR labeling (*H, arrowheads*) observed with rhodamine optics was unaffected. Bar, 10 μm.

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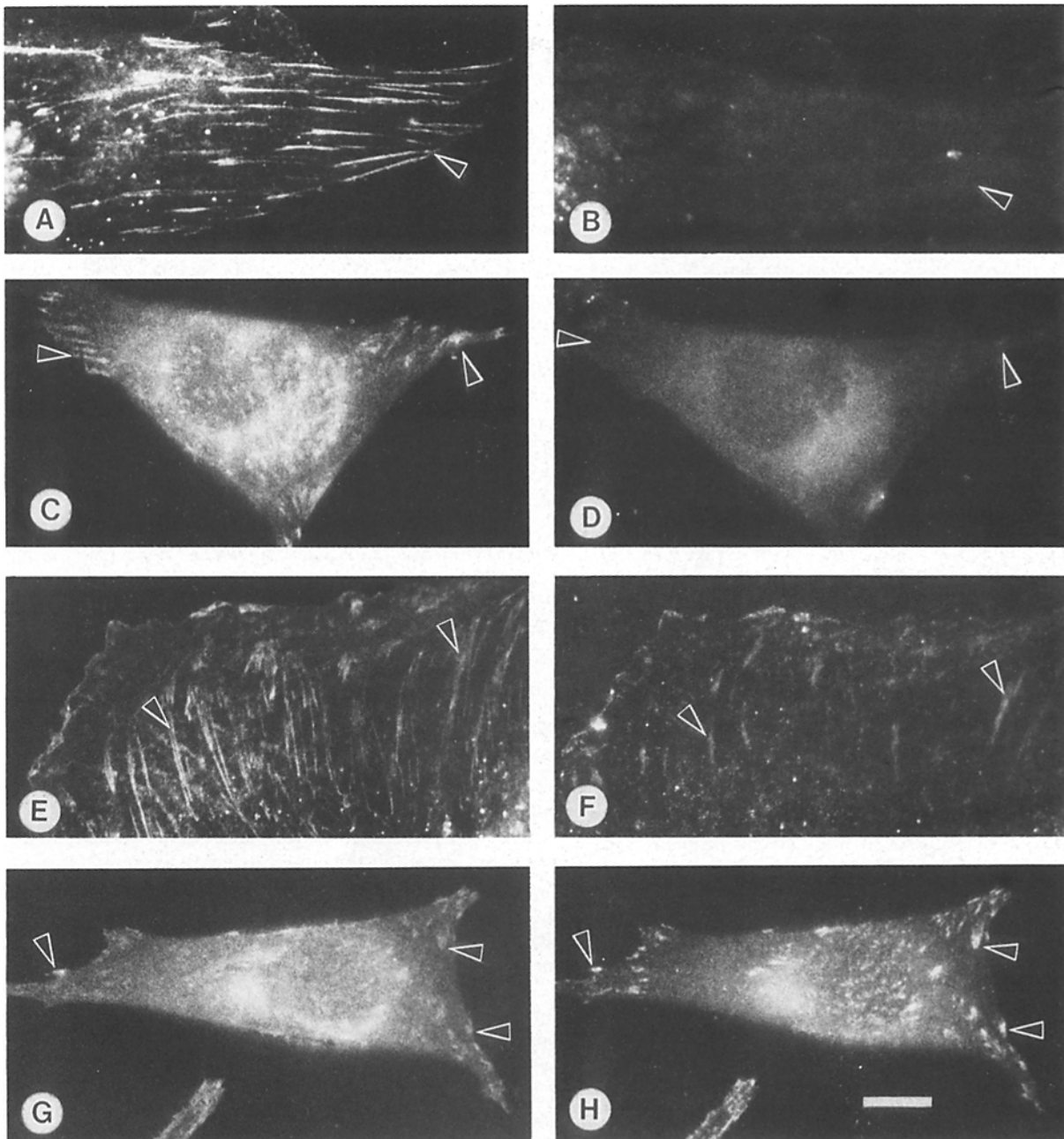
Figure 5. Effect of substrate composition upon FNR and VNR codistribution in human fibroblasts and melanoma cells. Coverglasses were coated with either purified fibronectin (*A–D*) or vitronectin (*E–H*), and serum-free cultures were established as described in Materials and Methods. FNR and VNR were studied with double-label IFM as performed in Fig. 3. Gin I fibroblasts cultured on fibronectin for 2 h (*A* and *B*) showed FNR localized at focal contacts (*A, arrowhead*) that lacked VNR staining (*B, arrowhead*). Similarly, 2-h cultures of melanoma cells on a fibronectin substrate (*C* and *D*) displayed focal concentrations of FNR (*C, arrowheads*) without detectable VNR (*D, arrowheads*). With vitronectin-coated substrates, 2-h cultures of fibroblasts (*E* and *F*) or melanoma cells (*G* and *H*) displayed coconcentration of FNR (*E* and *G*, *arrowheads*) and VNR (*F* and *H, arrowheads*) at focal contacts. Bar, 10 μm.

strates exhibited coconcentrations of FNR and VNR staining at focal contacts (Fig. 5, *E–H, matching arrowheads*; IRM micrographs not shown). Similar patterns of FNR and VNR labeling were observed after 6 h on these substrates.

Because fibronectin and vitronectin contain Arg-Gly-Asp sequences that govern the receptor recognition of these ligands (36, 39, 40, 50), we studied the effects of substrate-bound Tyr-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Cys upon the distribution of FNR and VNR in Gin I fibroblasts. To inhibit production of endogenous fibronectin which could alter the distribution of FNR, cells were pretreated with cycloheximide and seeded in the presence of sufficient polyclonal antifibronectin IgG to inhibit fibronectin-dependent cell adhesion by >90%. Only a few control cells in the 1-h cultures synthesized fibronectin fibers and these fibers were eliminated using the double block (Table I). At 4 h, all of the

Table I. Percentage of Fibroblasts with Focal Contacts (FCs) and Fibronectin Fibers (FFs) after Culture on Arg-Gly-Asp–containing Substrates*

Culture conditions	FCs, 1 h	FFs, 1 h	FCs, 4 h	FFs, 4 h
Control medium	44	7	85	100
Cycloheximide‡	40	1	98	25
Antifibronectin IgG§	24	3	98	29
Cycloheximide and antifibronectin IgG	62	0	92	0

* Human Gin I fibroblasts in serum-free medium were cultured for 1 or 4 h on coverglasses derivatized with the peptide Tyr-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Cys, and studied with IRM and IFM as described in Materials and Methods. The data represent the mean percentage of 50–159 cells counted per coverslip; two coverglasses were analyzed for each group, and the standard errors were <5.6%.
‡ 2 h before dissociation, the cells were treated with cycloheximide dissolved in the culture medium (25 μg/ml). All solutions used for subsequent treatment of the cells, including dissociation, contained this level of cycloheximide.
§ The medium contained 50 μg/ml polyvalent antifibronectin IgG, which inhibited fibronectin-mediated cell attachment by >90%.

control cells produced fibronectin fibers; the number of cells with fibers was reduced significantly by cycloheximide or antibody alone, and inhibited completely by the double block. In spite of this double-fibronectin block, 62–92% of the cells formed focal contacts at either time (Table I; Fig. 6, *A* and *B*). Even at 1 h these cells displayed substantial FNR and VNR localized precisely at focal contacts (Fig. 6, *C–F*).

Distribution of Fibronectin and Vitronectin Receptors on the Dorsal Cell Surface with Immunoscanning Electron Microscopy

Since the cells for IFM were treated with detergent to maximize access of antibodies to substrate contacts, information on the distribution of FNR and VNR was probably lost from the dorsal cell surface due to extraction. Immunoscanning electron microscopy was used to eliminate this problem, and to obtain higher resolution images of receptor patterns than possible with IFM. Fibroblasts or M-21 cells were fixed after 1, 6, or 24 h and labeled with the FNR and VNR probes. FNR (Fig. 7, *A* and *C*) and VNR (Fig. 7, *E* and *G*) were randomly distributed on the dorsal cell surface, while the plasma membrane above the nucleus was unlabeled (not shown). Microprocesses were conspicuously labeled for both receptors (Fig. 7, *A*, *C*, *D*, and *F*), and heavily stained ridges often extended centripetally from their bases (Fig. 7 *D*). Microvilli were labeled for FNR (Fig. 7 *C*) but not for VNR (Fig. 7 *G*). Similar patterns of FNR and VNR staining were seen at all time points in 10% FBS and on cells plated upon fibronectin-coated substrates in the absence of serum. The labeling was totally eliminated by replacing the FNR or VNR probes with nonimmune IgG (Fig. 7, *B* and *H*) or by preincubating the receptor antibodies with purified relevant receptor (170 μg/ml); pretreatment with the irrelevant receptor did not alter the staining (not shown).

Discussion

Our comparative analysis of FNR and VNR at the single cell level has led to several new observations regarding the expression and distribution of these adhesion receptors. First, both receptors can occupy the same focal contact when cells are seeded onto substrates coated with vitronectin, Arg-Gly-Asp peptide, or with serum. Second, FNR may accumulate in focal contacts independently of VNR on fibronectin-coated substrates. Third, VNR always remains concentrated

at focal contacts, whereas FNR appears to leave focal contacts and accumulates in extracellular matrix contacts upon prolonged culture in 10% serum.

The localization of FNR and VNR at focal contacts agrees with previous studies of mammalian cells (11, 12, 20, 22), but our work goes on to show that both receptors may coaggregate within the same focal contact. In addition, FNR and VNR were randomly distributed on the dorsal cell membrane when viewed with higher resolution immunoscanning electron microscopy. (Diffuse FNR staining of the cell surface membrane was not detected with IFM in nonpermeabilized stationary fibroblasts [16]. This difference may be attributed to the greater sensitivity and higher resolution of immunoscanning electron microscopy.) We therefore hypothesize that these receptors are not aggregated when they initially reach the cell surface, but become concentrated at focal contacts and extracellular matrix adhesion sites in apparent response to substrate-bound ligands encountered by the cell. Since focal contacts are sites of strong cell-to-substrate interaction (16, 30, 31), the accumulation of both receptors at focal contacts suggests that FNR and VNR play important roles in the generation and maintenance of stable surface adhesions. These adhesion receptors are also likely to link the cytoskeleton to the extracellular matrix at focal contacts and extracellular matrix contacts (6, 28, 42–44). In accordance with such a role, the chicken integrin complex, which is related to FNR (3, 28, 53), exhibits a cytoplasmic binding site for talin (26), an actin-associated protein which is similarly localized at focal contacts (7). Because VNR is codistributed with FNR at focal contacts, VNR might also interact with the actin cytoskeleton by a mechanism similar to that between FNR and talin. However, the mode of putative VNR–cytoskeletal binding would probably differ from that of FNR since VNR was not concentrated at extracellular matrix contacts and microvilli, while FNR was detected at these loci.

The distribution of FNR changed when the cells developed fibronectin-containing extracellular matrix fibers. At 6 h, the Gin I fibroblasts displayed streaks of FNR staining resembling extracellular matrix fibers beneath the cell center. After 24 h, this fibrous FNR pattern became extensively developed beneath the Gin I fibroblasts; a similar but less prominent FNR pattern was observed in melanoma cells at 24 h. These fibrous concentrations of FNR codistributed precisely with the gray-to-white extracellular matrix contacts
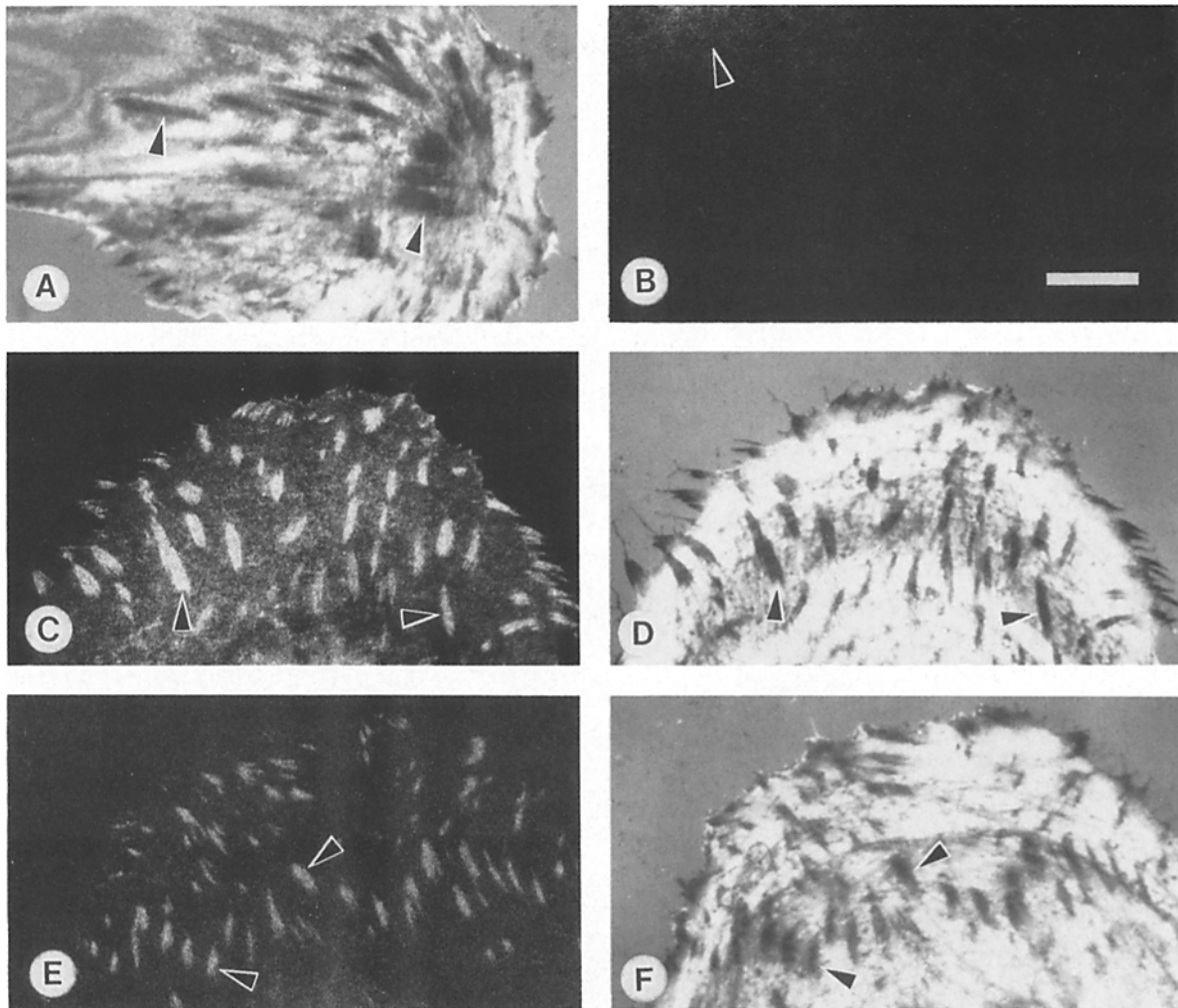
Figure 6. Accumulation of FNR and VNR at focal contacts of fibroblasts attached to Arg-Gly-Asp peptide–derivatized substrates for 1 h. Gin I cells in medium containing antifibronectin IgG and cycloheximide were seeded onto coverslips derivatized with tridecapeptide, fixed, immunostained for either fibronectin (*B*), FNR (*C*), or VNR (*E*), and studied with IFM (*B*, *C*, and *E*) and IRM (*A*, *D*, and *F*), as detailed in Materials and Methods. Striking focal contacts (*A*, *arrowheads*) that formed under these conditions did not show fibronectin labeling using 100 μg/ml polyvalent antifibronectin IgG (*B*); only muted perinuclear fluorescence (*B*, *arrowhead*) was observed. Brilliant concentrations of FNR (*C*) and VNR (*E*) entirely filled each focal contact (*corresponding arrowheads* in *D* and *F*). Bar, 10 μm.

identified with IRM (9, 10). When such extracellular matrix contacts were well developed beneath either cell type, the concentration of FNR staining within focal contacts became markedly diminished or was totally lacking. These observations imply that FNR distribution at the cell surface is strongly affected by extracellular matrix fiber production; FNR is apparently diverted from focal contacts to sites of attachment on extracellular matrix cables under these conditions. The FNR at these sites may function in the migration of cells along tracts of fibronectin-containing extracellular matrix fibers in vivo (4, 16). In contrast to FNR, VNR remained concentrated at focal contacts and was not detected at extracellular matrix adhesion sites during our experiments. Thus, VNR appears to be involved primarily in maintaining cell-to-substrate attachment at focal contacts. However, since vitronectin has been localized in some connective tissues in vivo (14, 24), VNR might also function as an extracellular matrix receptor during certain conditions.

Surprisingly, although VNR was not concentrated at focal contacts when the cells were plated on a fibronectin substrate, FNR did accumulate at focal contacts formed on a vitronectin surface. Although fibronectin is known to be localized at some types of focal contacts (43, 44), we do not think that the patching of FNR on vitronectin substrates is caused by subcellular fibronectin fibers because no fibronectin staining was observed at the surfaces of M-21 cells, and most of the Gin I fibroblasts were negative for fibronectin fibers under these conditions. Further, no fibronectin was detected by ELISA in the vitronectin used for coating the substrate, and our specificity controls exclude the possibility that accumulation of FNR at focal contacts on a vitronectin substrate might be due to putative cross-reactivity of our FNR antibodies with VNR. A possible explanation for this phenomenon is that under some circumstances, FNR might bind vitronectin through the Arg-Gly-Asp sequence common to fibronectin and vitronectin (35, 50). This hypothesis ap-
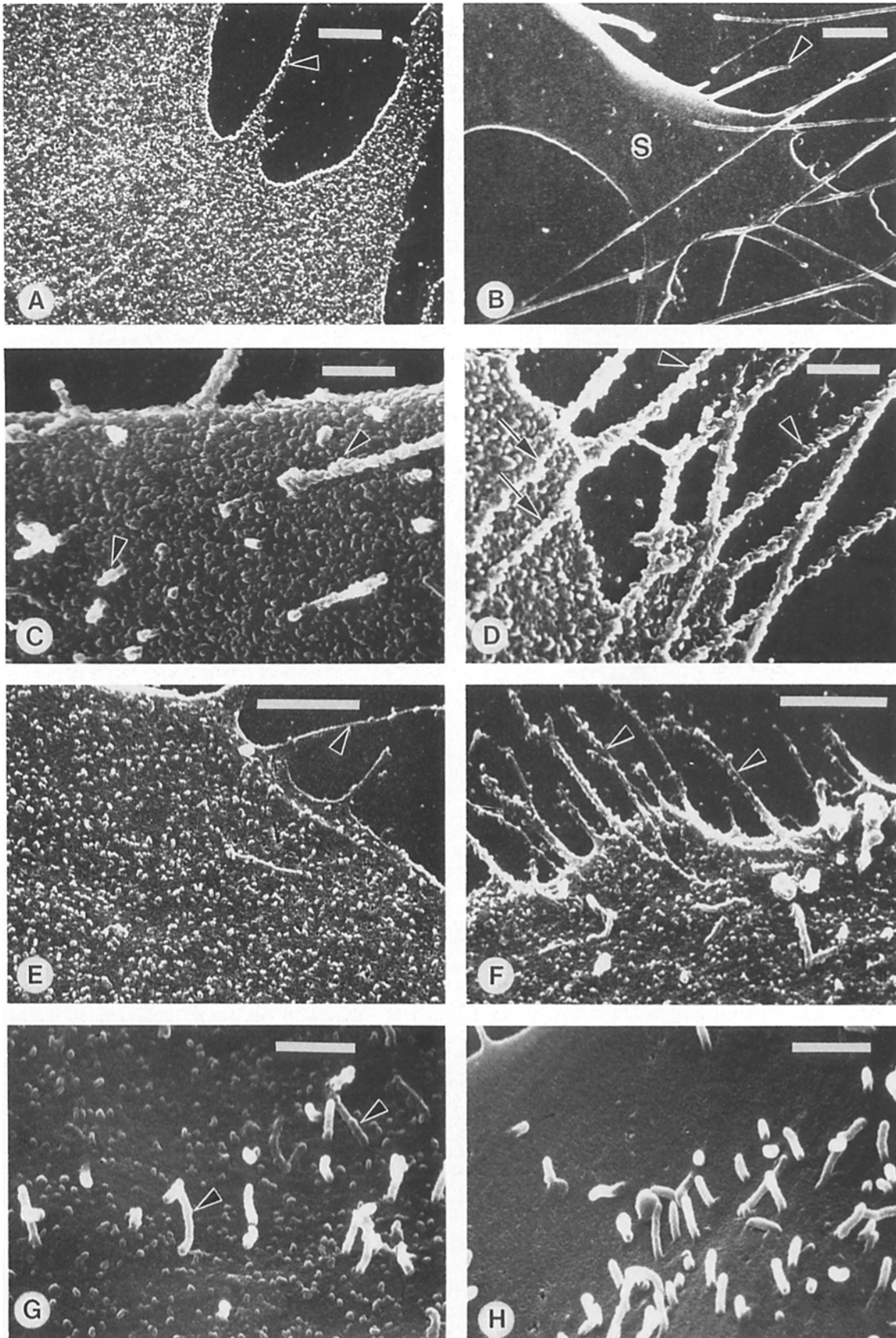
Figure 7. Immunoscanning electron micrographs of FNR and VNR on the dorsal surfaces of human fibroblasts (*A–D*) and melanoma cells (*E–H*). Cells were cultured for 6 h (*A–C, E,* and *F*) or 24 h (*D, G,* and *H*) with 10% FBS, fixed, and labeled with rabbit anti–FNR IgG (*A, C,* and *D*) or rabbit anti–VNR IgG (*E–G*), followed by 5 nm colloidal gold goat anti–rabbit IgG as described in Materials and Methods.

pears to be supported by our finding that an Arg-Gly-Asp peptide–coated substrate induced patching of both FNR and VNR at focal contacts. However, this proposal does not agree with data obtained from receptor–liposome binding assays or from affinity chromatography, in which FNR does not bind to immobilized Arg-Gly-Asp-Ser or vitronectin, but VNR does (39–41). Perhaps in the whole cell system, VNR forms aggregates after attachment to substrate-bound Arg-Gly-Asp or vitronectin and these aggregates can include FNR. Alternatively, FNR may become concentrated at focal adhesions through a programmed cell response that is independent of the substrate-bound ligand. If this is true, the processes regulating the distribution of FNR vs. VNR would be different, since VNR did not accumulate at focal adhesions on a fibronectin substrate. The redistribution of FNR but not VNR from focal contacts to extracellular matrix contacts also supports this hypothesis.

Our double-labeling observations show that a single cell can express simultaneously at least two functional extracellular matrix receptors. Since many cells adhere to various collagens and laminin in addition to fibronectin and vitronectin, it is likely that FNR and VNR are not the only extracellular matrix receptors that a single cell can express. How the multiple adhesive influences mediated by these receptors are interpreted by the cell as meaningful positional information is a major question for future studies.

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(*A*) FNR immunogold labeling is uniformly distributed on the upper surface of a Gin I fibroblast cultured for 6 h; a microprocess (*arrowhead*) is also FNR positive. (*B*) Similar preparation to A; incubated with nonimmune rabbit IgG followed by immunogold. The dorsal cell surface (*s*) and microprocesses (*arrowhead*) are unlabeled. (*C* and *D*) Higher magnification views of Gin I fibroblasts exhibit FNR receptors on microvilli (*C, lower arrowhead*) and on the basal (*D, arrows*) and distal (*D, arrowheads*) portions of microprocesses. (*E* and *F*) VNR is randomly distributed upon the upper surface and on microprocesses (*arrowheads*) of M-21 melanoma cells. (*G*) Microvilli (*arrowheads*) do not display VNR staining despite labeling on the cell membrane. (*H*) Rabbit IgG control for VNR on an M-21 cell shows a very low background. Bars: (*A, B, E,* and *F*) 2 μm; (*C, D, G,* and *H*) 1 μm.

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