The Fibronectin Receptor Is Organized by Extracellular Matrix Fibronectin: Implications for Oncogenic Transformation and for Cell Recognition of Fibronectin Matrices

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Abstract. Cells interact with extracellular fibronectin (FN) via adhesive fibronectin receptors (FNRs) that are members of the very late antigens (VLAs) subgroup of the integrin family. In stationary fibroblasts, the FNR is highly organized and distributed identically to extracellular FN fibrils. However, in highly migratory neural crest cells and embryonic somatic fibroblasts, this organization is lost and the FNR appears diffuse. Similarly, oncogenic transformation typically leads to disorganization of the FN receptor and loss of matrix FN. Two models can account for these observations. First, the FN matrix may organize the FN receptor at extracellular matrix contacts on the cell surface. Motile cells not depositing FN matrices thus lack organized receptors. Alternatively, as the FNR is required for optimal FN matrix assembly, (McDonald, J. A., B. J. Quade, T. J. Broekelmann, R. LaChance, K. Forseman, K. Hasegawa, and S. Akiyama. 1987. *J. Biol. Chem.* 272:2957–2967; Roman, J., R. M. LaChance, T. J. Broekelmann, C. J. R. Kennedy, E. A. Wayner, W. G. Carter, J. A. McDonald. 1989. *J. Cell Biol.* 108:2529–2543) and has putative cytoskeletal links, it could be organized from within the cell helping to position newly forming FN fibrils. To study this question, we developed peptide antibodies specifically recognizing the α_5 subunit of the FNR. Using these antibodies, we examined the organization of FN and of the FNR in normal, matrix assembly inhibited, and SV40-transformed human fibroblasts. On FN-coated substrates, the FNR is found in focal contacts rather than diffusely on the basal cell surface, suggesting FNR interaction with intracellular components. However, when FN fibrils are deposited, the FNR is co-distributed with these fibrils. Preventing FN matrix assembly prevents organization of the FNR. Moreover, when fibroblasts with well established FN matrices and co-distributed FNR are incubated briefly with monoclonal antibodies that block FNR binding to FN, the FNR is no longer co-distributed with the FN matrix. Thus, the FN receptor is organized in fibrils on the cell surface in response to extracellular FN. Because exogenous FN restores a FN matrix and receptor organization to SV40-transformed cells, the diffuse FN receptor phenotype appears to be related to loss of the FN matrix rather than to impaired FNR function. These results explain diffusely distributed FNRs in migratory neural crest and embryonic fibroblasts lacking well organized FN matrices and emphasize the existence of separate but related systems controlling FN deposition and recognition by receptor-armed cells.

CELL interaction with fibronectin (FN)[1] containing matrices appears critical for embryonic development and cytodifferentiation, wound healing, and tumor metastasis (Bronner-Fraser, 1986; Chen et al., 1986*b*; Duband et al., 1986; 1988; McClay and Ettensohn, 1987; Boucaut et al., 1984; Humphries et al., 1986*a*,*b*). A heterodimeric transmembrane receptor of subunit composition $\alpha_5\beta_1$ in the (very late antigens) VLAs subfamily of integrins, also termed the fibronectin receptor (FNR; we use the term "FNR" to refer to the heterodimer of $\alpha_5\beta_1$ subunit composition as defined by the cDNA sequence published by Argraves et al. [1987].) binds to an RGDS containing sequence in FN's carboxy terminal cell adhesive domain and is implicated in these interactions with FN (Pytela et al., 1987; Ruoslahti and

1. *Abbreviations used in this paper*: FN, fibronectin; FNR, FN receptor; IF, immunofluorescence; KLH, keyhole limpet hemocyanin; OSG, octylthioglucopyranoside; VN, vitronectin; VNR, VN receptor; VLA, very late antigen.

Pierschbacher, 1986; 1987; Hynes, 1987; Argraves et al., 1987). FN and the FNR are co-distributed in a fibrillar array on cultured fibroblasts (Singer et al., 1988; our observations in this work). Furthermore, the cell surface distribution of this receptor is related to cell behavior. For example, loss of the FN matrix and disorganization of members of the VLA family and probably the FNR accompanies the oncogenic transformation of chick fibroblasts (Chen et al., 1986*a*). Similarly, migrating neural crest cells and freshly cultured somatic fibroblasts lack an organized FN matrix and the VLA antigens are diffusely distributed on their surface (Duband et al., 1988).

Fibroblast monolayers also bind FN's amino terminal domain, and although this binding does not support cell adhesion it is critical for FN matrix assembly (McDonald et al., 1982, 1987; McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988; for review, see McDonald, 1988). This suggests that FN fibrils are assembled via a mechanism involving FN's amino terminus and that the FNR simply binds to these fibrils mirroring their organization. However, the FNR also contributes to matrix assembly (McDonald et al., 1987; our observations). Thus, because the FNR may interact with the cytoskeleton (Buck and Horwitz, 1987), it could be organized from within the cell and convey positional information to newly forming FN fibrils.

The FNR shares a common β_1 subunit with at least six related VLAs expressed by many cells but possesses a unique α_5 subunit (Hynes, 1987; Hemler et al., 1987). Accordingly, we raised synthetic peptide antibodies to selected polypeptides from the deduced sequence of the FNR α_5 subunit (Argraves et al., 1987). An antibody to the cytoplasmic domain specifically recognizes the FNR and has been used to study its distribution in normal and SV40-transformed fibroblasts and the mechanisms of FNR localization in fibroblasts.

Materials and Methods

Materials

All organic chemicals were of analytic grade and were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA) unless otherwise specified.

FNR Purification

Human placental FNR was purified as described (Pytela et al., 1987). Ground placenta was stirred in Dulbecco's PBS (1:1, wt/vol) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM PMSF (buffer A) plus 50 mM octylthioglucopyranoside (OSG) for 1–2 h at 4°C. After centrifuging (25,000 *g*, 30 min, 20°C) the supernatant was chromatographed on (*a*) a Sepharose-4B filter column; (*b*) an 18-ml Sepharose-4B column CNBr conjugated to 10 mg of the synthetic peptide KYAVTGRGDS to remove vitronectin receptors (VNR); (*c*) a 22-ml Sepharose-4B column CNBR conjugated to 100 mg of the 110-kD thermolysin-released cell binding domain of FN (Zardi et al., 1985) to bind the FNR. The FNR was eluted from the 110-kD fragment column with buffer A lacking divalent cations and containing 10 mM EDTA and 25 mM OSG and chromatographed on a 5-ml wheat germ agglutinin–Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ) in 10 mM Tris-HCL, pH 7.4, containing 25 mM OSG, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, and eluted with the same buffer containing 0.4 M *N*-acetyl glucosamine. Fractions were analyzed by 5–10 percent gradient SDS-PAGE and silver staining (Phast System; Pharmacia Fine Chemicals). For immunoblotting, purified FNR was electrophoresed on a 7% polyacrylamide gel, transferred to nitrocellulose and visualized as described (Towbin et al., 1979; McDonald et al., 1987).

Synthetic Peptides and Antisera

Four hydrophilic peptide sequences (residues 65-73, 811-823, 886-897, and 1039-1049) were selected (see Doolittle, 1986) from hydropathy analysis (Kyte and Doolittle, 1982; Doolittle, 1986) of the deduced cDNA sequence of the FNR α subunit (Argraves et al., 1987). Each peptide was synthesized with a cysteine residue at its amino terminus for cross-linking. After purification by C-18 reverse-phase HPLC using a 0 to 60% gradient of acetonitrile in 0.1% trifluoroacetic acid, disulfide cross-linked peptide dimers were reduced with 50 mM DTT followed by Sephadex G-10 chromatography. Peptide concentration was estimated with dithio-bis-nitrobenzoate (Janatava et al., 1968) and each peptide conjugated to keyhole limpet hemocyanin (KLH) using *N*-succinimidyl-bromoacetate (NSB) at a 1:1 (wt/wt) ratio of peptide/KLH (Bernatowicz and Matsueda, 1986). Peptide-KLH conjugates were emulsified with complete Freund's adjuvant and four rabbits were immunized subcutaneously with 400 μg each of peptide-KLH conjugate and reimmunized every 2 wk with 200 μg of peptide-KLH conjugate emulsified in incomplete Freund's adjuvant. After 2–4 weeks animals were bled, the serum titered by ELISA on purified FNR, the IgG purified (McKinney and Parkinson, 1987), and antipeptide IgG affinity purified by chromatography on a peptide-hexane-diamine-Sepharose-CLB4 column cross-linked with *N*-succinimidyl-bromoacetate.

FN Fragments and Antibodies

A mouse mAb to the α subunit of the VNR was generously provided by Dr. D. Cheresh of the Scripps Clinic Foundation (Cheresh and Harper, 1987). In IMR-90 cells, mABs to the α and β subunit of the VNR gave identical staining results with localization to focal contacts. The anticollagen receptor monoclonal antibody PlH5 to the α_2 subunit (Wayner and Carter, 1987; Takada et al., 1988) and the anti-FNR α_5 monoclonal antibody PlF8 have been described (Wayner et al., 1988).

FN and FN fragments were isolated by described methods (McDonald et al., 1987; Zardi et al., 1985) and were judged greater than 95% pure by SDS-PAGE and protein staining (Phast System; Pharmacia Fine Chemicals). VNR was purified from serum as described (Hayashi et al., 1987).

Cell Culture

The fetal human lung fibroblasts lines IMR-90, WI-38 and the SV40 transformed line WI-38 VA-13 were obtained from the ATCC and were cultured in DME (Washington University Tissue Culture Support Center) (IMR-90) or RPMI 1640 (WI-38 and WI-38 VA-13) supplemented with 10% heat-inactivated FBS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine and antibiotic–antimycotic solution (Gibco Laboratories, Grand Island, NY) in a 5% CO_2/95% air atmosphere. Antibodies or FN fragments were dialyzed against DME (three changes, 24 h) before use in cell culture. Hybridoma-conditioned medium containing the antireceptor antibodies PlF8 and PlH5 was dialyzed similarly, and fresh, heat-inactivated FBS added to 20% final concentration.

Immunofluorescence

Cells were trypsinized and plated either in 8 chamber LabTek microslides or on number 1 thickness coverslips and cultured overnight or as specified. For double label immunofluorescence (IF), staining cells were fixed with freshly prepared 1% paraformaldehyde, rinsed with 10 mM Tris-HCL containing 150 mM NaCL, pH 7.4, and 1 mg/ml of heat-denatured serum albumin (TBS-BSA), permeabilized with 0.1% Triton X-100 in TBS, and incubated with a mixture of 1 μg/ml of affinity purified Ab 33 antireceptor antibody and 10 μg/ml of a mAb (52DHl) to the EIIIA exon of FN (Vartio et al., 1987) in TBS-BSA for 30 min at room temperature. After rinsing with TBS containing 0.5% Triton X-100 and 1% normal goat serum, specimens were incubated with fluorescent-labeled secondary antibodies shown in control studies not to cross react with the irrelevant primary antibody used in the same experiment, mounted in Gelvatol (Rodriguez and Deinhardt, 1960) containing *p*-phenylenediamine and examined with a Biophot (Nikon Inc., Garden City, NY) equipped with epifluorescence optics. For interference reflection microscopy, cross polarizers were mounted and cells cultured on coverslips were illuminated using monochromatic green light supplied by the HBO 200 Hg lamp and a 543 nm filter. Film (Tmax 400; Eastman Kodak Co., Rochester, NY) was exposed at 800 ASA and developed at its rated speed (Tmax developer; Eastman Kodak Co.).

Fibronectin Receptor Immunoprecipitation

Cell surface iodination was performed with lactoperoxidase and glucose oxidase (Leiben et al., 1982). The FNR was immunoprecipitated from Triton X-100 lysates of IMR-90 cells with 100 μg/ml of Ab 33 using IgSORB (heat-killed, formalin-fixed Cowan strain *Staphylococcus aureus*; the Enzyme Center, Boston, MA) rather than protein A-Sepharose (Roberts et al., 1988). In preclearing immunoprecipitation experiments, 100 μl of a 2% Triton X-100 lysate, representing 20% of the contents of a surface-iodinated 10-cm culture dish of IMR-90 or WI-38 cells, was incubated at 4°C for 1 h with an equal volume of hybridoma supernatant containing P1H5 or P1F8 mAb or with 10 μg of Ab 33. Then, 7.5 μg of rabbit anti-mouse IgG was added (this step was omitted for Ab 33). After 30 min, the mixture was incubated with 20 μl of IgSORB for 45 min, centrifuged at 10,000 *g* for 10 min, and the supernatant reimmunoprecipitated by the addition of hybridoma-conditioned supernatant. This procedure was repeated a total of three times.

Other Reagents and Methods

MAb to vinculin was from Miles Scientific (Naperville, IL), Texas red-conjugated phalloidin was from Molecular Probes Inc. (Junction City, OR), and rabbit polyclonal antitalin antibody (Burridge and Feramisco, 1980) was a generous gift of Dr. K. Burridge, Department of Cell Biology, University of North Carolina. Glass coverslips were coated with proteins by incubation with the specified concentration of ligand overnight, followed by several rinses in sterile PBS or by covalent coupling of 25 μg/ml ligand (Aplin and Hughes, 1981).

Results

Synthetic Peptide Antibody Characterization

Synthetic peptide antibodies to an amino terminal sequence of the α_5 subunit (residues 65-73) immunoblot but do not recognize native FNR. The peptide corresponding to residues 886-897 was not immunogenic whereas the peptide sequence from 811-823 yields antibodies recognizing the purified FNR by ELISA and by immunoblotting (data not shown). Only the cytoplasmic domain peptide corresponding to residues 1039-1049 elicited rabbit antibodies (Ab 33) recognizing native FNR. By immunoblot analysis, Ab 33 recognizes unreduced purified FNR α subunit and, after reduction, the 25-kD light chain carboxyl terminal polypeptide (Argraves et al., 1987) (Fig. 1). Ab 33 immunoprecipitates a single α subunit and a β subunit from extracts of IMR-90 cells metabolically labeled overnight, but not the pre-β precursor of the mature β subunit, consistent with α subunit interaction only with the mature β subunit (Fig. 2 *A*, lane *C*). In contrast, the polyclonal antibody Ab 14.3 to the human FNR that recognizes the common β subunit as well as the α_5 subunit (Roberts et al., 1988) immunoprecipitates polypeptides of 165 kD representing the α_1 subunit of the VLA complex (data not shown), 150 kD representing at least the α_2, α_3, α_5 and α_6 subunits, the 120 kD mature β_1 subunit, and the 110-kD pre-β_1 subunit (Fig. 2 *A*, lane *B* and data not shown).

The relationship of the antigen recognized by antibody Ab 33 to other members of the integrin family of receptors was investigated by sequential immunoprecipitation with mAbs P1H5 or P1F8 that inhibit cell adhesion to collagen and FN and that recognize VLAs 2 and 5, respectively (Wayner and Carter, 1987, 1988; Takada et al., 1988) or with Ab 33 (Fig. 2 *B*). mAb P1F8 to the α_5 subunit removed all antigen recognized by Ab 33 and vice versa whereas mAb P1H5 did not remove the Ab 33 antigen (Fig. 2). Thus, P1F8 and Ab 33 recognize the $\alpha_5\beta_1$ complex. In addition, the α_2 and α_5 subunits synthesized by IMR-90 are recognized by mAb P1H5

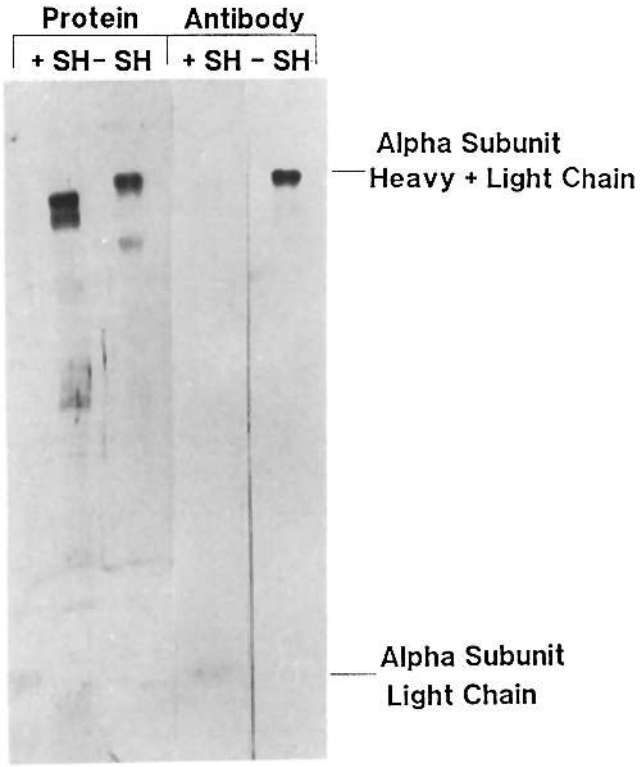


Figure 1. Immunoblot analysis of Ab 33. Purified human placental FNR was transferred to nitrocellulose and detected by protein stain (*left*) or with Ab 33 (*right*). The positions of the α subunit (*Heavy + Light Chain*) in the unreduced sample and of the light chain in the reduced sample are indicated. Ab 33 recognizes the heavy plus light chain in the unreduced sample and only the light chain in the reduced sample.

and P1F8 respectively, although as discussed below these antibodies do not stain IMR-90 cells. Similar preclearing experiments performed with Triton X-100 lysates of surface-labeled HT-1080 cells demonstrate that preclearing with an α_3 subunit specific mAb also had no effect on precipitation of the Ab 33 antigen (data not shown). We conclude that, in IMR-90 and HT 1080 cells, Ab 33 recognizes the α_5 subunit of the VLA family of integrins but not the α_1, α_2, α_3, or α_4 subunits.

Localization of the FNR in Cultured Fibroblasts

By IF, the FNR is distributed in permeabilized IMR-90 fibroblasts in a striking fibrillar pattern mirroring that of FN (Fig. 3, *A* and *B*). Nonpermeabilized cells do not stain (Fig. 3, *C* and *D*), confirming as does microinjection (data not shown) the intracytoplasmic localization of the epitope. At concentrations above 1 μg/ml, increased diffuse cell surface staining and some nonspecific nuclear staining is seen. Excess peptide antigen blocks all except the nonspecific nuclear staining by Ab 33 (Fig. 3, *E* and *F*). To establish the relationship of the FNR complex to other adhesive or cytoskeletal macromolecules, we localized actin, the VNR, vinculin, and talin by double label IF (as only a rabbit antiserum was available to talin, its distribution was compared only with that of FN). Shortly after fibroblasts are plated on serum or vi-

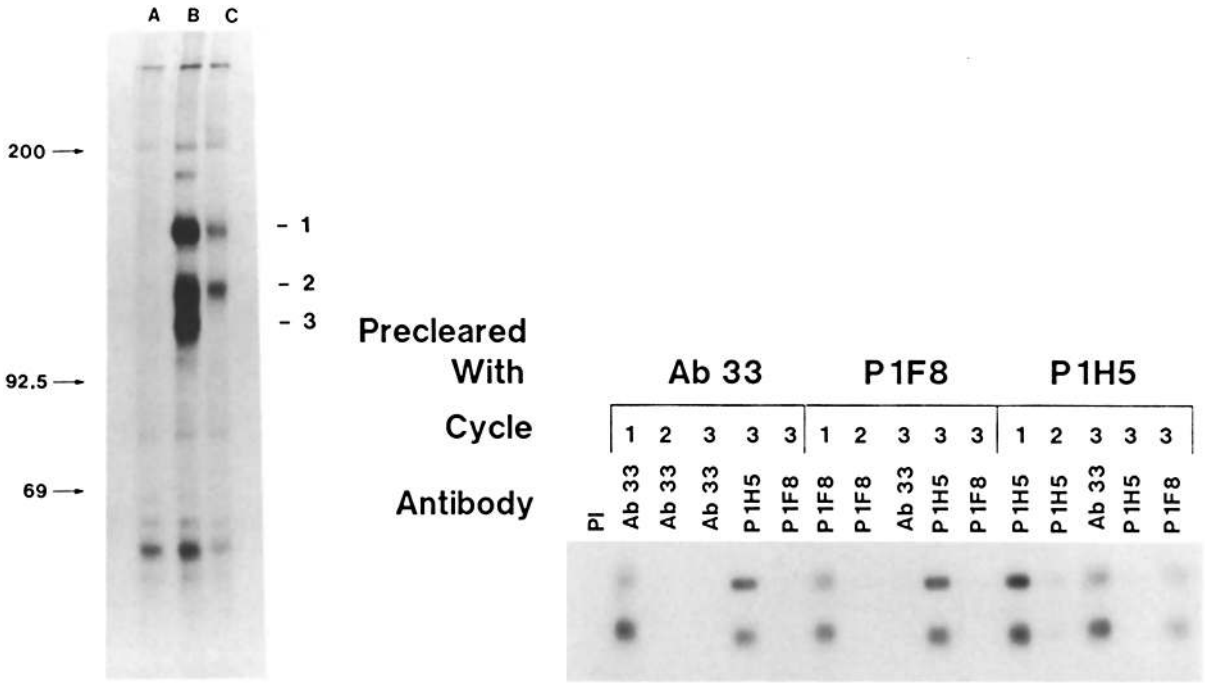


Figure 2. Immunoprecipitation of [^{35}S]methionine-labeled IMR-90 human lung fibroblasts with antibodies to the FNR or other VLAs. (*A*) IMR-90 was labeled overnight and Triton-X100 extracts immunoprecipitated (Materials and Methods). Lane *A*, preimmune IgG. Lane *B*, Ab 14.3 polyclonal antiplacental FNR (Roberts et al., 1988). Lane *C*, Ab 33. The polyclonal anti-FNR immunoprecipitates four polypeptides of ~170 kD (unlabeled) (that is, the VLA α_1 subunit [data not shown], 140 kD α [labeled 1] that includes the α 2, 3, 5, and 6 subunits [Fig. 2 *B* and data not shown], 120 kD β [labeled 2], and 110 kD pre-β [labeled 3]) whereas Ab 33 immunoprecipitates only the α_5 and mature β subunit. Addition of excess peptide antigen blocks α and β subunit immunoprecipitation by Ab 33 (not shown). (*B*) Immunoprecipitation of Triton extracts of ^{125}I surface-labeled IMR-90 fibroblasts with mAbs to the α_2 (P1H5) or α_5 (P1F8) subunits or with Ab 33. A Triton lysate was immunoprecipitated twice with Ab 33 (*left*), mAb *PIF8* to the $\alpha_5\beta_1$ complex (*center*), or P1H5 to the $\alpha_2\beta_1$ complex (*right*) (Wayner and Carter, 1987; Wayner et al., 1988). Ab 33 removes the α and β subunits cross-reacting with the α_5 subunit specific antibody P1F8, but not the α and associated β subunits cross-reacting with the α_2 specific antibody P1H5. Similarly, P1F8 removes all α and associated β subunits cross-reacting with Ab 33, but has no effect on immunoprecipitation with P1H5. Thus, P1F8 and Ab 33 recognize the same $\alpha_5\beta_1$ complex or FNR. In addition, IMR-90 expresses significant quantities of the $\alpha_2\beta_1$ complex, as well as of the $\alpha_3\beta_1$ complex (data not shown).

tronectin-coated VN glass, the FNR is localized in short stitches on the cell surface associated with FN (Fig. 4, *A* and *B*). After cell spreading is complete and an extensive FN matrix is formed, the FNR α subunit remains colocalized with extracellular FN. Actin fibers (Fig. 4, *C* and *D*) co-align with but only rarely appear to terminate at FNR fibrils present in extracellular matrix contacts. In contrast, the VNR localizes primarily at the cell periphery in focal contacts (Fig. 4, *E* and *F*) but not in extracellular matrix or close contacts. Vinculin (Fig. 4, *G* and *H*) and talin (data not shown) are present at both focal and extracellular contact sites. Thus, in IMR-90 cultured overnight in serum containing medium the VNR, vinculin, and talin but not the FNR are in focal contacts, in agreement with FN-independent attachment and spreading of IMR-90 on serum-coated surfaces (McDonald et al., 1987).

FNR and VNR Respond to Ligand-Coated Substrates by Localizing in Focal Contacts

On VN-coated substrates, the VNR is found in focal contacts, whereas the FNR remains in extracellular matrix contacts (Fig. 5, *A* and *B*). In contrast, on FN-coated glass, the FNR was organized in focal contacts 1 h after plating (Fig. 5, *D–F*). A striking, circular, wavelike pattern of FNR distribution was seen in some cells on FN (Fig. 5 *F*). By analogy to focal contact formation during fibroblast locomotion, this is presumably because of cyclic extension of nearly circumferential lamellipodia followed by focal contact formation (Abercrombie, 1980). In 10% FCS on FN-coated glass, both the FNR and VNR were found in focal contacts (Fig. 5 *C*), whereas in serum-free medium only the FNR was present (not shown).

Effect of Reagents Blocking the Amino Terminal Matrix Assembly Domain of FN, the Cell Adhesive Domain, or Both on Expression and Organization of FN and the FNR

When IMR-90 fibroblasts are cultured in 10% serum for 48 h, typically about 60–80 percent of newly synthesized FN is deposited on the cell surface and most is disulfide cross-linked into multimers. To quantify FN matrix assembly (see McDonald et al., 1987, 1988; Quade and McDonald, 1988) and cell surface FNR expression, IMR-90 were cultured for 48 h with FN fragments or anti-FN antibodies, the cell surface polypeptides iodinated, and the cell layer lysed either in SDS containing DTT (for FN immunoprecipitation, see

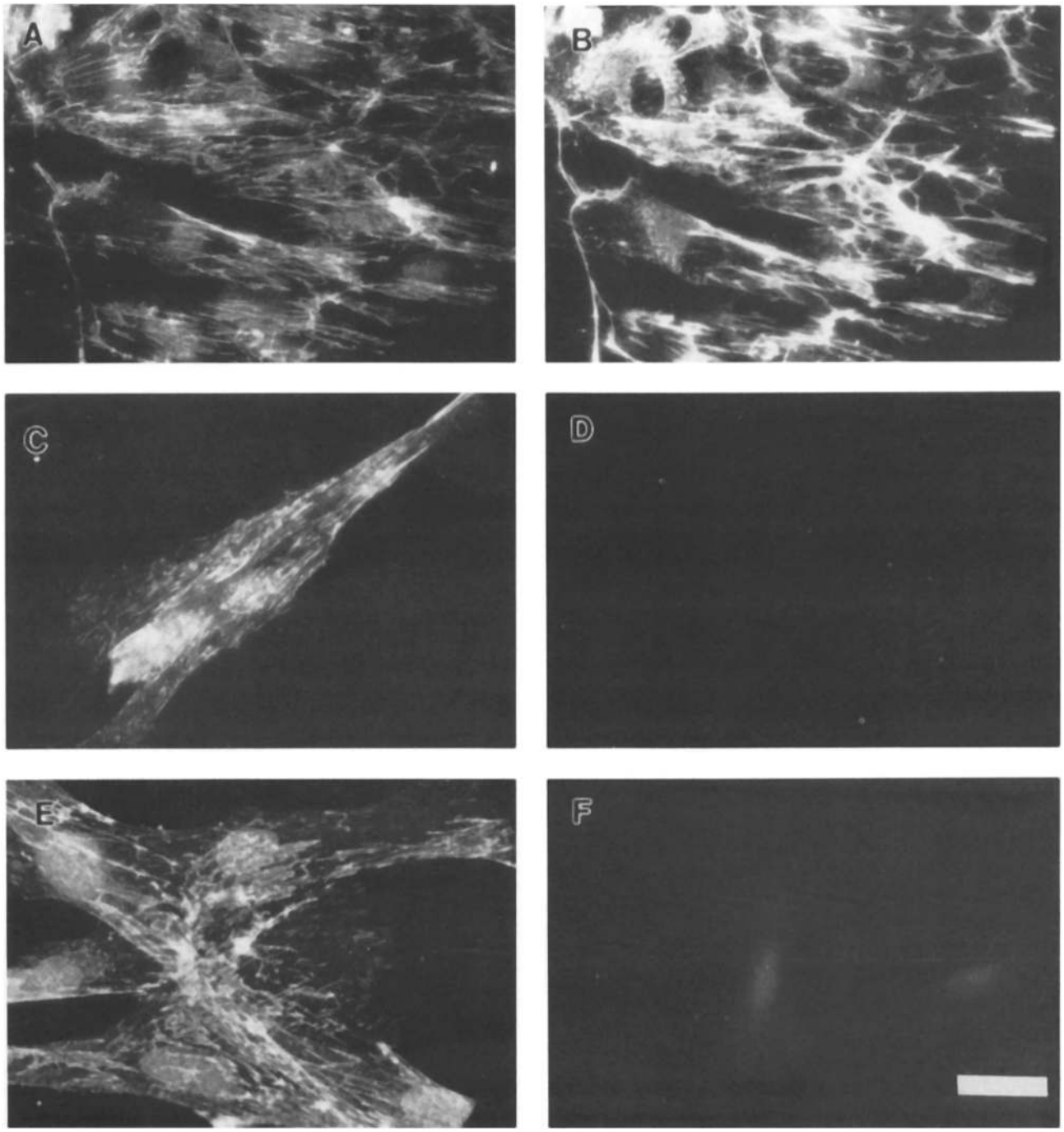
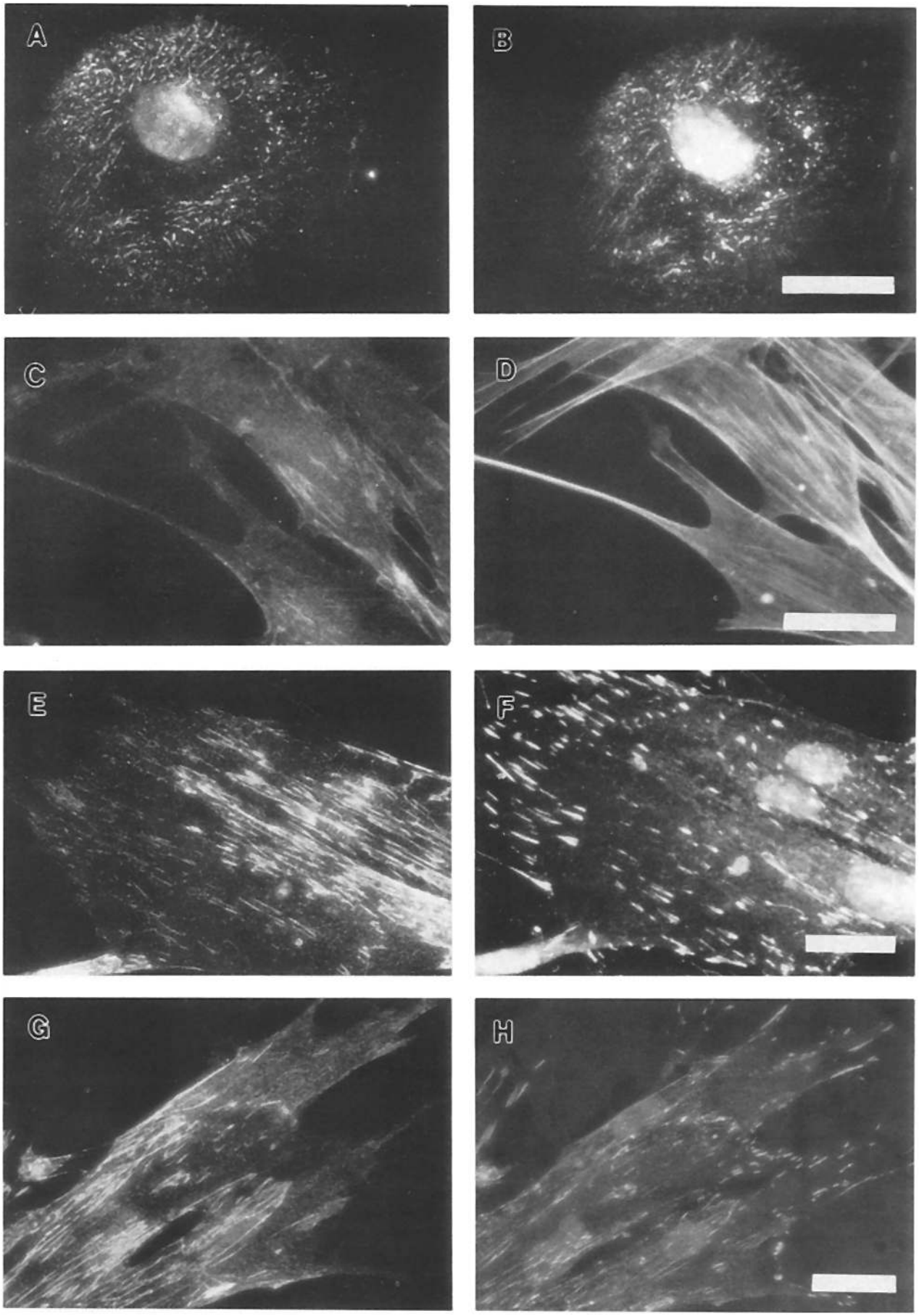


Figure 3. Distribution of the FNR and cellular FN in IMR-90 fibroblasts. FNR (*A*) and FN (*B*) in fixed and permeabilized cells. FNR in Triton permeabilized (*C*) and nonpermeabilized (*D*) fibroblasts. FNR staining in the absence (*E*) and presence (*F*) of 1 μg/ml of peptide antigen. Only residual nuclear staining remains. Bar, 20 μm.

McDonald et al., 1987) or Triton X-100 (for FNR immunoprecipitation). The lysates were counted, and samples containing the same cpm were immunoprecipitated and labeled polypeptides displayed by SDS-PAGE and autoradiography. Thus, the results reflect relative surface abundance of FN and FNR. However, total surface labeling of IMR-90 cultured with the antibodies or fragments varied <16 percent (data not shown). The autoradiograms and the results of quantitative laser scanning densitometry are shown in Fig. 6 and IF staining in Fig. 7.

The 70-kD amino terminal matrix assembly domain of FN inhibits cell surface FN accumulation by 90% (Fig. 6) and results in short streaks of FN and FNR on the cell surface but completely absence of intercellular FN fibrils (Fig. 7, *A* and *B*). In contrast, mAb N-294 to FN's cell adhesive domain inhibits FN deposition by ~40% (Fig. 6), and the cells exhibit normal appearing, albeit reduced, numbers of intercellular FN fibrils (see Fig. 8). Combining the 70 kD fragment with mAb N-294 or with a 120 kD cell adhesive fragment of FN strikingly inhibits FN deposition (Fig. 6) and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assembly (Fig. 7). The cells are completely devoid of extracellular fibrils. The altered organization of the FNR by IF staining is clearly not because of decreased FNR expression, but reflects altered distribution of the FNR on the cell surface (compare Fig. 6, *Control* and *MAb N-294 + 70 kD* lanes).

The inhibition of FN deposition is specific to these fragments or antibodies, as neither fragments encompassing the entire remaining sequence of FN apart from the amino terminus and high affinity cell adhesive fragment (viz, the 60-kD collagen binding domain or the catheptic carboxyl terminal dimer of 140 kD (Quade and McDonald, 1988) nor other mAbs to FN inhibit FN deposition (McDonald et al., 1987).

Antibodies Inhibiting FN–FNR Binding Disorganize the FNR

The need to block both the amino terminal and cell adhesive domains of FN to prevent FNR organization suggests that the FNR must bind to extracellular FN to organize in fibrils on the cell surface. To test this, we utilized two mAbs that inhibit FN-mediated cell adhesion by blocking FN–FNR interaction, N-294 to FN (McDonald et al., 1987), and PIF8 to the α_5 subunit of the FNR (Wayner et al., 1988). As a control, we used PIH5, an mAb to the VLA α_2 subunit (Takada et al., 1988) that inhibits cell adhesion to collagen but not to FN (Wayner and Carter, 1987).

Two types of experiments were performed. First, IMR-90 were cultured overnight and allowed to establish an extensive pericellular FN matrix with colocalized FNR (Fig. 8, *A* and *B*). Then, the medium was changed to one conditioned with the PIF8 hybridoma line or containing N-294 at 100 μg/ml, and at intervals fixed, permeabilized, and stained for detection of FN and FNR. By 15 min, there was a clearly visible reduction in FNR co-distribution with FN that, by 30 min, appeared virtually complete in PIF8 anti-FNR medium (Fig. 8, *C* and *D*). Thus, inhibiting FN-FNR interactions results in a redistribution of the FNR away from FN fibrils.

Second, IMR-90 were cultured either with purified IgG (N-294) or with hybridoma-conditioned medium (PIH5 or PIF8) overnight, and were fixed and stained using one of the following protocols: (*a*) rhodamine isothiocyanate goat anti-mouse IgG to visualize the monoclonal antireceptor antibodies; (*b*) Ab 33 anti-FNR followed by FITC-goat anti-rabbit IgG to detect the FNR; (*c*) the anti-FN EDIIIA mAb followed by a mixture of FITC-goat anti-rabbit IgG and rhodamine isothiocyanate goat anti-mouse IgG to detect both FNR and FN. Even though their respective antigens were abundant and readily immunoprecipitated from surface-labeled IMR-90 (see Fig. 2 *B*), neither PIF8 nor PIH5 antireceptor antibodies alone stained IMR-90 cells. This may reflect the fact that these antibodies do not recognize ligand-occupied receptors (Wayner et al., 1988). In any event, another mouse mAb could be used to detect EDIIIA containing FN.

Culturing IMR-90 with N-294 (not shown) or PIF8 (Fig. 8, *E* and *F*) reduces, but does not eliminate, FN fibril formation and largely prevents FNR co-distribution with the FN matrix. The control anti-α_2 antibody PIH5 had no effect on FN or FNR by IF (data not shown). Thus, inhibiting FN-receptor binding with antibodies directly either against the receptor binding site on FN (N-294) or to the ligand binding site of the FNR (PIF8) has two effects. If FN–FNR interaction is blocked after the FN matrix is assembled, co-localization of the FNR with FN is completely reversed within 30 min without detectable effects on the pericellular matrix. If FN–FNR interaction is inhibited beginning when the fibroblasts are plated, FN matrix assembly is impaired and the FNR is much less organized.

Effect of Cytochalasin B on FN and FNR Organization

The above results demonstrate that the FN matrix exhibits a strong influence on FNR distribution. Although the actin microfilament system is required for FN matrix formation (Lyubimov and Vasiliev, 1982), it is not clear if this system is required to maintain the association of FN and FNR after an FN matrix is established. Addition of cytochalasin B results in an accelerated loss of FN from the cell surface and cell retraction termed "arborization." However, substrate associated and pericellular FN matrix is largely retained for over 2 h (Ali and Hynes, 1977; Mautner and Hynes, 1977). Cytochalasin B treated IMR-90 completely lose organized actin. However, FN and FNR organization was not detectably altered in confluent IMR-90 with abundant matrix FN exposed to cytochalasin B for 30 min or 4 h (Fig. 9). When subconfluent cells were exposed to cytochalasin B, cells retaining organized FN fibrils also retained organized receptor networks whereas cells without detectable fibrillar FN had more diffusely distributed receptors (data not shown). Although real time imaging of individual cells is required to determine if the cells lacking an organized matrix and receptor complex did so before cytochalasin B treatment, it is clear that many cells retain more or less normal matrix and receptor organization for a considerable period despite the complete loss of organized actin.

SV40 Transformation Inhibits FN Matrix Accumulation and FNR Organization but Exogenous FN Restores a Matrix and FNR Organization

The FN matrix of WI-38 fibroblasts is identical to that of IMR-90 by IF staining, and the FNR was co-distributed with this matrix (data not shown). By contrast, the SV40-transformed line WI-38 VA13 has scant detectable surface FN fibrils and mostly diffuse FNR (Fig. 10, *A* and *B*). Culturing WI-38 VA13 with 50 μg/ml of plasma FN restores a FN matrix to the cell surface and results in complete FNR reorganization, although their more epithelial morphology is not altered (Fig. 10, *C* and *D*).

Figure 4. Localization of FNR with other adhesion receptors and cytoskeletal components. FNR (*A*) and FN (*B*) in an IMR-90 cell 2 h after attachment to a serum-coated glass substrate. The FNR and FN fibrils are co-distributed. FNR (*C*) and actin (*D*) in IMR-90 fibroblasts. The receptor complex is co-aligned with actin cables, and, in some cases, actin cables appear to terminate at FNR fibrils. FNR (*E*) and VNR (*F*) on IMR-90 cells after overnight culture on serum-coated glass substrate. The FNR is concentrated in fibrils in the central portion of the cells, whereas the VNR is primarily localized in focal contacts at the cell periphery. FNR (*G*) and vinculin (*H*) in IMR-90 cultured in serum overnight. Vinculin is found both at focal contacts and in extracellular matrix contacts containing the FNR whereas the FNR is absent from the focal contacts. Talin is distributed similarly to vinculin (data not shown). Bars, 20 μm for each pair of photomicrographs.

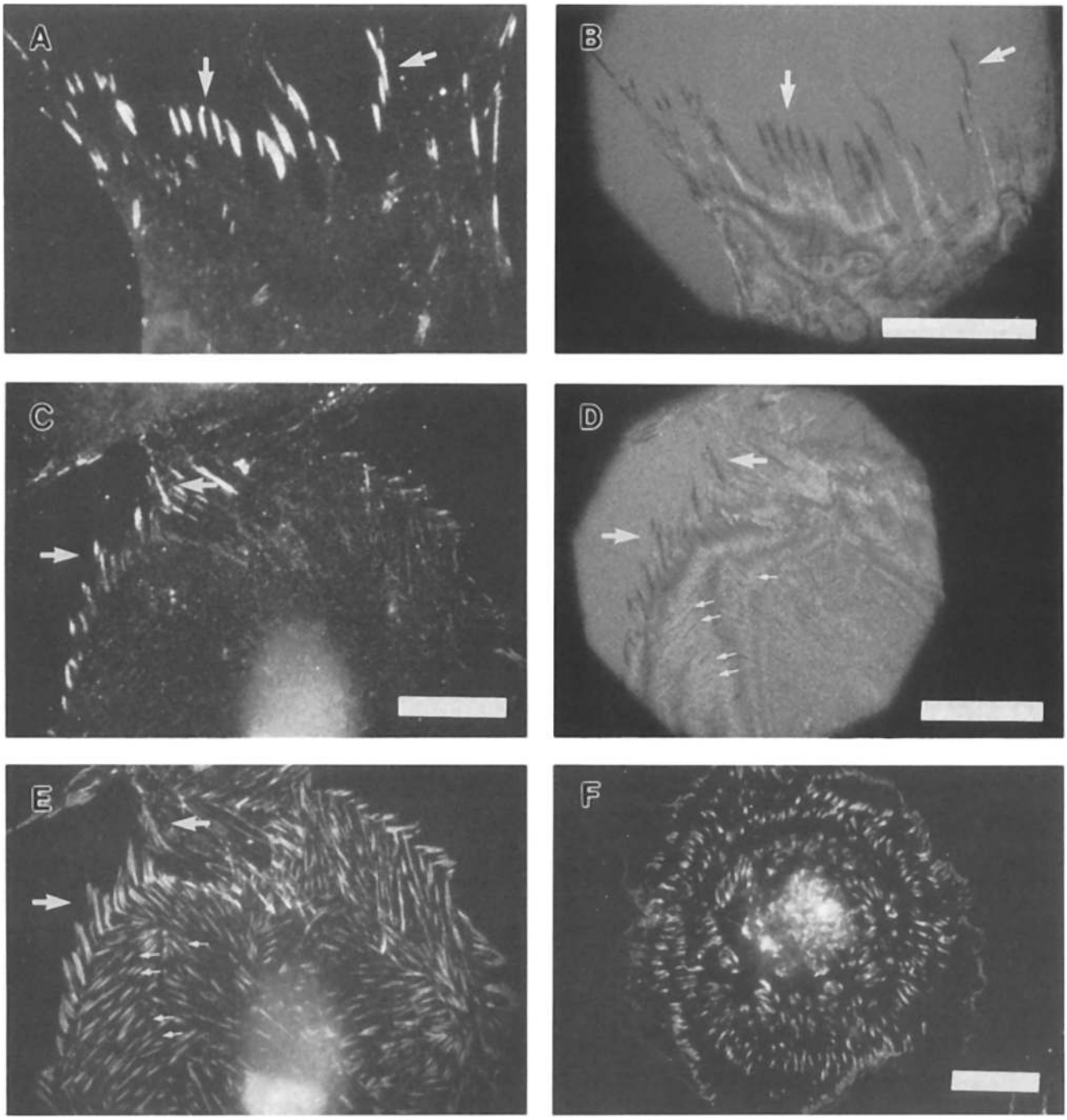

Figure 5. Localization of FNR and VNR in IMR-90 spread upon FN- or VN-coated substrates. (*A* and *B*) VNR and interference reflection image of the edge of an IMR-90 spread on a VN-coated surface for 1 h in serum containing medium. Note that the VNR (*A*) is localized almost exclusively at focal contacts (*large arrows*) as shown by the corresponding interference reflection image in *B*. The FNR was not detectable in focal contacts, but remains diffusely distributed (not shown). (*C–E*) VNR (*C*), interference reflection image (*D*), and FNR (*E*) in an IMR-90 plated on a FN-coated glass coverslip in the presence of 10% FCS. The VN and FNRs are found in focal contacts (*large arrows*), whereas the FNR is also found in extensive extracellular matrix contact sites as shown by the light grey and white lines in the interference image (*small arrows* in *D* and *E*). *F* displays a striking circular pattern of the FNR in focal and extracellular matrix contacts seen in some IMR-90 spreading on a FN substrate. Bar, 20 μm.

Discussion

Synthetic Peptide Antibody Characteristics

Synthetic peptide antibodies to a sequence comprising the last 11 residues of the cytoplasmic domain of the human FNR α_5 subunit recognize an epitope present on the light chain of ligand purified FNR preparations and cross react with native human, mouse (Holers et al., 1989) and goldfish (data not shown) FNR. Peptide antibodies to a sequence from the cytoplasmic domain of the β_1 subunit also recognize diverse species (Marcantonio and Hynes, 1988) demonstrating that

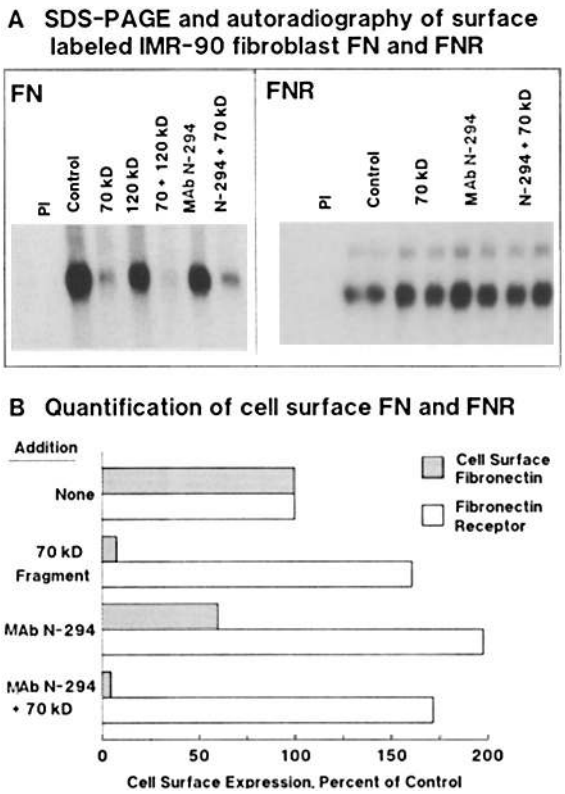

Figure 6. Cell surface FN and FNR expression in matrix inhibited IMR-90. *A* depicts autoradiography of surface iodinated IMR-90 immunoprecipitated with anti-FN (*left*) and FNR (*right*) antibodies in matrix competent and inhibited IMR-90 cells. For FN (*left*), the lanes are *PI*, preimmune control (preimmune controls were run for each condition and were identical, a representative lane is shown here); *Control*, no additions; *70 kD*, 0.75 mg/ml of 70-kD amino terminal FN fragment; *120 kD*, 1.37 mg/ml of the 120 kD cell adhesive FN fragment; *70 + 120 kD*, a mixture of the 70 kD (0.8 mg/ml) and 120 kD (1.37 mg/ml) FN fragments; *MAb N-294*, monoclonal N-294 to FN's cell binding site, 100 μg/ml; *N-294 + 70 kD*, a mixture of monoclonal N-294 (100 μg/ml) and 70 kD (0.8 mg/ml) FN fragment. For FNR (*right*), duplicate immunoprecipitates from separate cultures are shown. Concentrations of inhibitors are as above. *B* displays the relative quantities of surface FN and FNR determined by laser scanning densitometry. Cell surface FNR expression is not decreased by any of the fragments or antibodies, and may even be increased in some cases.

both cytoplasmic domains of the FNR are highly immunogenic, and yet conserved and presumably critical for function.

Preclearing experiments with mAbs demonstrate that Ab 33 appears to recognize only the α_5 subunit, agreeing with the fact that no significant identity has been detected within the cytoplasmic domains of the VLA α subunits thus far (M. E. Hemler, personal communication). Immunoprecipitation with the specific polyclonal antibody 14.3 to the FNR clearly demonstrates the need for α subunit specific immunoreagents. Antibody 14.3 immunoprecipitates VLAs 1, 2, 3, and 5 because of α–β subunit interactions. In fact, the $\alpha_5\beta_1$ complex or FNR is not even a major component of the VLAs expressed by IMR-90 fibroblasts (in Fig. 2, compare lane *B* representing all VLAs and lane *C*, containing total FNR).

Implications for Integrin Structure

Antibodies to the α_5 subunit (our observations in this work) and to the β_1 subunit putative cytoplasmic domains (Marcantonio and Hynes, 1988) only stain permeabilized cells. As these domains follow a single putative transmembrane sequence (Argraves et al., 1987), each subunit of the FNR must consist of a large amino terminal extracellular domain, a single transmembrane sequence, and a short carboxyl terminal cytoplasmic domain as previously proposed (Argraves et al., 1987; Hynes, 1987).

Localization of the FNR and VNR in Cultured Fibroblasts

An organized FN matrix is required for fibrillar organization of the FNR at extracellular matrix contact sites. FN binding is required for FNR organization at these sites as antibodies inhibiting binding displace the FNR. On serum-coated surfaces, the FNR is found in extracellular matrix sites associated with FN, vinculin, and talin as previously described (Singer et al., 1988). However, on FN- or VN-coated substrates in the absence of serum, the cognate receptor localizes in adhesive focal contacts, whereas the other remains diffusely distributed and is not detectable at focal contacts (our observations in this work; Singer et al., 1988; Dejana et al., 1988). As the substrate is more or less uniformly coated with ligand under these circumstances the adhesive receptor might be expected to be diffusely distributed on the basal cell surface. However, both VNR and FNR accumulate at focal contacts, suggesting additional interactions within the cell. In endothelial cells, clustering of the β_1 subunit and of the VNR receptor precedes focal contact formation (Dejana et al., 1988). Using the fluorescence recovery after photobleaching technique, it has further been demonstrated that the mobility of VLA complexes is decreased by association at focal or matrix contacts. Receptors associated with ECM streaks or focal contacts are largely immobile (18% mobile fraction) whereas diffuse receptors in the plasma membrane are much more mobile (66% mobile fraction) (Duband et al., 1988). This is also true for the FNR. When mAb PIF8 was added to IMR-90 cells with established FN matrices and highly organized FNR, there was a complete loss of organized FNR structures by 30 min.

Disorganization of the FNR in SV40 Transformed Fibroblasts Is not because of Deficient Ligand Binding

The FNR is largely, but not completely, disorganized in the SV40 transformed WI38 VA13 line. However, restoring the FN matrix with exogenous FN also restores FNR organization. This effect could be related to associated transforming growth factor β rather than the FN per se (Fava and McClure, 1987), but it seems unlikely that the diminished FNR organization is related to deficient ligand binding. Although it has been suggested that phosphorylation of the FNR β_1 subunit (Hirst et al., 1986) in cells transformed by viruses encoding tyrosine kinases is responsible for the abnormal distribution of the FNR, impaired FNR function need not be invoked as the absence of an organized FN matrix is sufficient to account for diffusely organized FNR. Thus, it is not suprising that migratory neural crest cells lacking a FN matrix also have diffusely distributed FNRs without significantly increased VLA phosphorylation (Du-

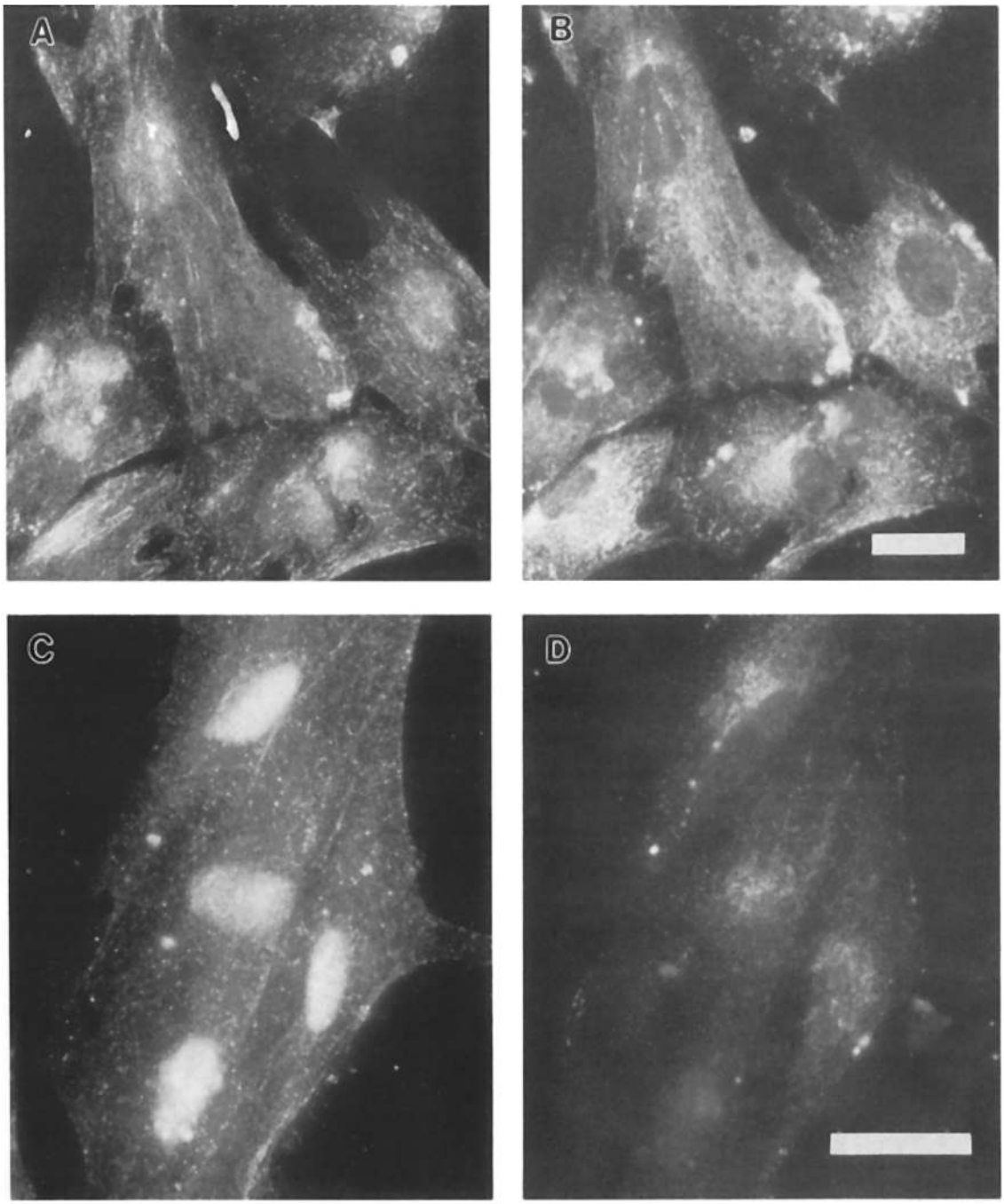

Figure 7. Effect of FN fragments and mAb on FNR distribution and FN matrix assembly. FNR (*A*) and FN (*B*) in IMR-90 cultured overnight with 0.75 mg/ml of the 70 kD amino-terminal fragment of FN. The controls for this experiment are shown in Fig. 3, *A* and *B*. FN fibrils spanning between cells are absent but shorter stitches remain on the cell surface (*B*) and the FNR is organized similarly (*A*). There are few uninterrupted linear receptor arrays, but many interrupted streaks are retained. The bright staining in *B* is intracellular FN as nonpermeabilized cells showed only scant stitches on their surface. In addition, cells cultured with the 70-kD fragment variably exhibit apparent increases in staining of intracellular FN as seen here. FNR (*C*) and FN (*D*) in IMR-90 cultured overnight with a mixture of 0.75 mg/ml of 70-kD fragment and 100 μg/ml of mAb N-294 directed to FN's receptor binding site. Note the absent FN matrix and the corresponding lack of FNR organization. Bars, 20 μm for each pair.

band et al., 1988). Similarly, other cells lacking a FN-containing pericellular matrix should lack organized FN receptors and this is true for CHO-K1 and L-cells (our unpublished results).

The diminished FN matrix in most transformed cells may be because of deficient FN synthesis, cell surface binding, assembly, or FN proteolysis (Wagner et al., 1981; Yamada, 1978; Fegan et al., 1981; Chen and Chen, 1987) but probably not to defective FN (Wagner et al., 1981). In SV40 transformed WI-38 fibroblasts, deficient amino terminal binding appears to be important (McDonald et al., unpublished data).

FNR Cytoskeletal Interactions

Avian integrins of uncertain subunit composition that bind

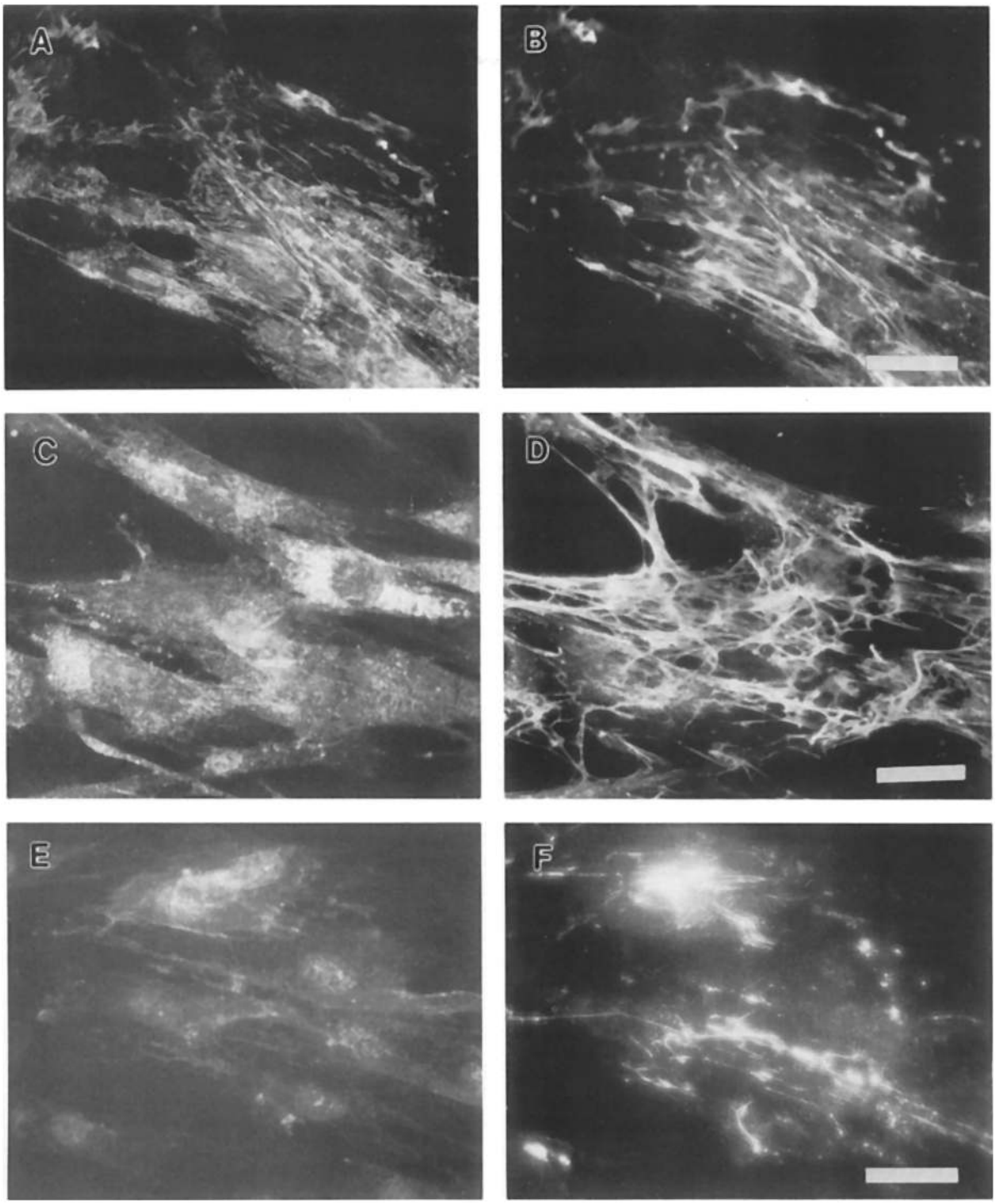

Figure 8. Inhibiting FN–FNR binding with mAbs to FN reverses FNR organization. (*A* and *B*) IMR-90 cultured overnight and the FNR (*A*) and FN (*B*) detected by IF. *C* and *D* show a parallel well that was rinsed and incubated with hybridoma-conditioned medium containing the anti-FNR P1F8 for 30 min. Although the FN matrix remains intact (*D*), there is almost no associated FNR (*C*). *E* and *F* show the results of culturing IMR-90 overnight with the anti-FNR P1F8. There is a significant decrease in FN matrix deposition, and FNR co-distribution. Bar, 20 μm.

FN also bind weakly to talin, and the talin-receptor complexes bind vinculin (Horwitz et al., 1986). However, the molecules involved in mammalian FNR-cytoskeletal interactions remain unknown. Both talin and vinculin are present at the extracellular matrix contacts containing FNR and FN, but it is not known if they interact directly with the FNR. It does seem, however, that actin microfilaments do not play a significant short term role in maintaining (as opposed to initiating) FNR organization as collapsing the actin cytoskeleton had no obvious effects on FN or FNR distribution. Syn-

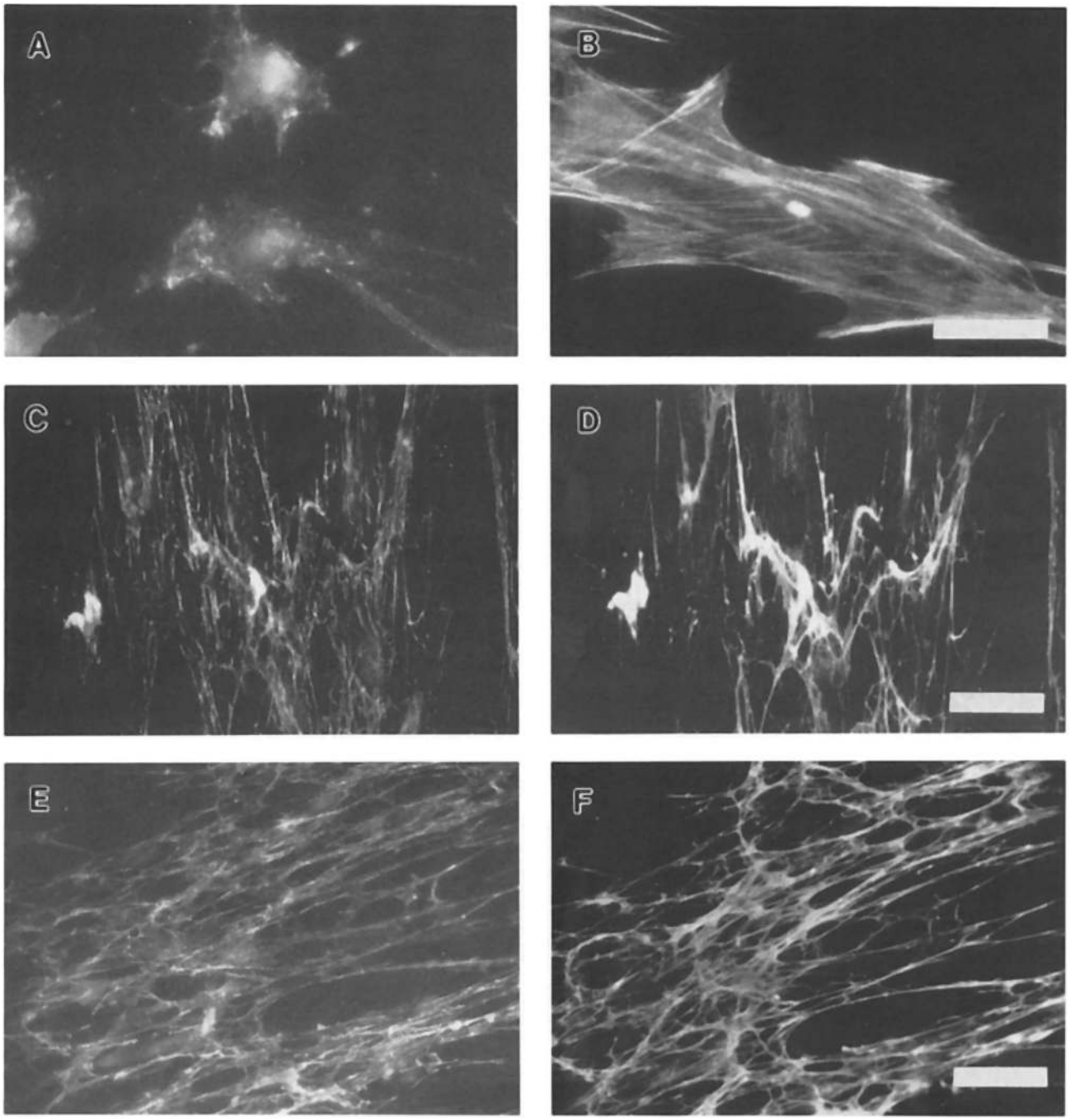

Figure 9. Effect of cytochalasin B on FNR and FN distribution in IMR-90. (*A*) Actin staining in IMR-90 exposed to 10 μg/ml of cytochalasin B for 30 min or not (*B*). FNR (*C*) and and FN (*D*) in control cells. (*E* and *F*) Cytochalasin B–treated (30 min, 10 μg/ml) cells. The FNR (*E*) and FN matrix (*F*) remain orgnized despite extensive cell arborization (not shown). Similar results were obtained with confluent monolayers exposed to cytochalasin B for 4 h (not shown).

thetic peptide antibodies to cytoplasmic domains should be useful tools to dissect FNR interactions with putative cytoskeletal links.

A Model for FN Matrix Assembly Incorporating Bidirectional Information Transfer between the FN Matrix and the FNR

Previous studies demonstrated that FN fragments binding to the FNR did not inhibit binding of exogenous radiolabeled FN to fibroblast monolayers (McKeown-Longo and Mosher, 1985) even though FN binding to suspended fibroblasts is mediated via the FNR binding site (Akiyama and Yamada, 1985). This paradox may be explained by the observation that suspended fibroblasts do not bind to FN's amino terminal matrix assembly domain (Quade and McDonald, 1988), possibly because an intact actin microfilament system is required (Barry and Mosher, 1988). Although we cannot conclude that the FNR is absolutely required for FN matrix

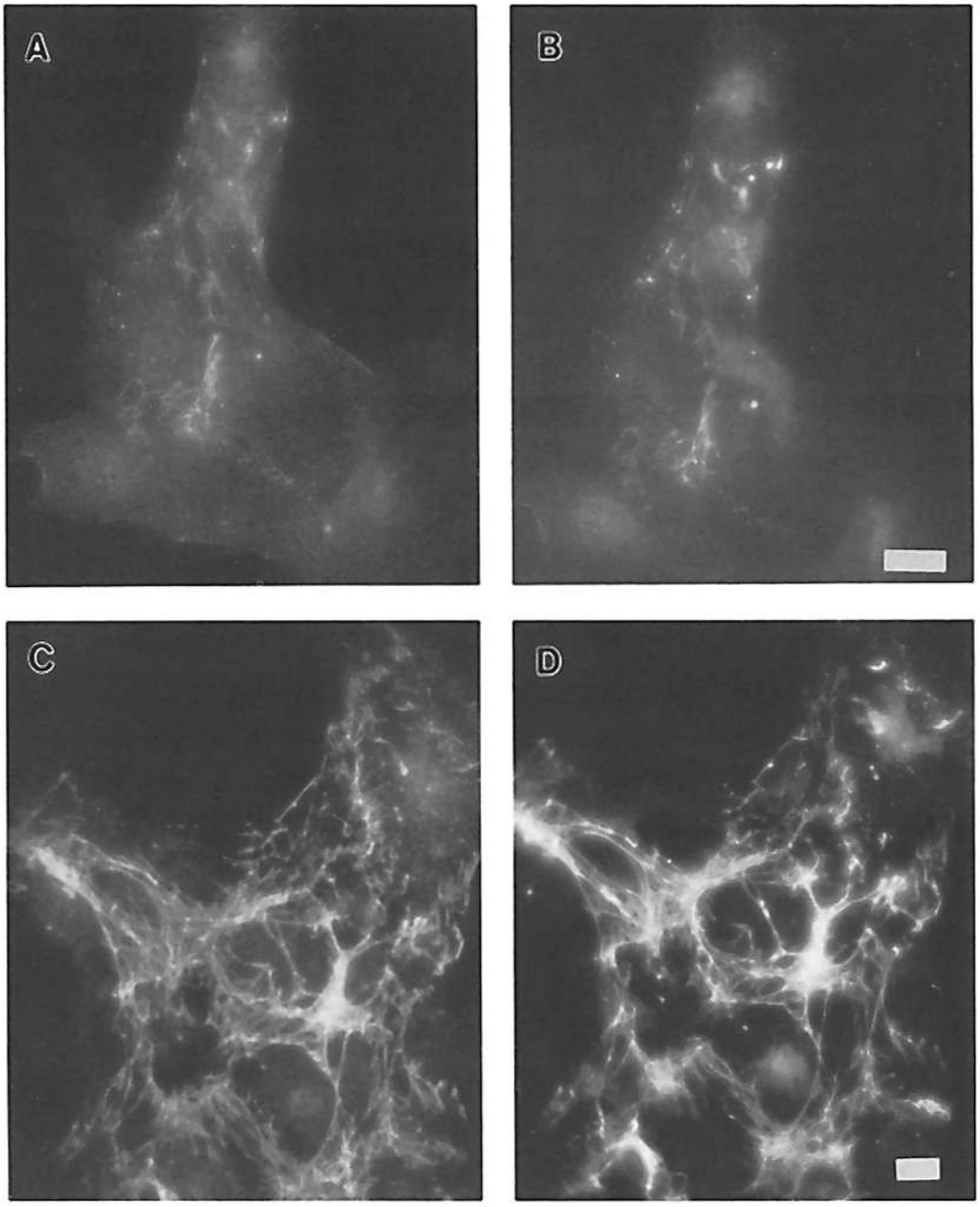

Figure 10. FN and FNR in SV40-transformed human fibroblasts. (*A* and *B*) FNR and FN in WI38 VA13 SV40-transformed human lung fibroblasts. The FNR is largely disorganized, and there is a scant FN matrix. (*C* and *D*) FNR and FN in WI38 VA13 fibroblasts cultured with 50 μg/ml of human plasma FN overnight. Note that the FNR is co-distributed with the now abundant FN matrix. Bars, 20 μm for each pair.

assembly, cell surface FN accumulation is reproducibly inhibited by high concentrations of cell adhesive fragments (either the 120 kD catheptic fragment (our observations in this work) or the similar chymotryptic peptide (data not shown)) or mAb N-294 (Fig. 6). This effect is specific (McDonald et al., 1987; Quade and McDonald, 1988). Moreover, reagents inhibiting both amino terminal and RGD binding sites produce additive effects on FN accumulation, and both are required to prevent all traces of FN (and FNR) organization on the cell surface. However, until we are confident that complete inhibition of FNR function is achieved, quantitative estimates of its importance in FN matrix assembly are not possible (McDonald, 1988).

Inhibiting amino terminal binding by the 70 kD fragment in IMR-90 leaves ~10% of surface FN as short streaks on the cell surface associated with the FNR (Fig. 7, *A* and *B*). Assuming this reflects the phenotype associated with FN binding mediated via an RGD and adjacent affinity site (Obara et al., 1988) dependent mechanism, then FN organization occurs without the involvement of FN's amino terminal domain. Clearly the FNR is not critical for fibril elongation, as amino terminal inhibitors alone are very efficient at

blocking formation of intercellular FN fibrils (McDonald et al., 1987; our observations in this work) whereas FNR inhibitors do not seem to be. Although by no means proof, our results are compatible with a model in which fibril "nucleation" occurs via the FNR followed by elongation and cross-linking requiring participation of the amino terminal matrix assembly domain (McDonald et al., 1987; McDonald 1988). Alternatively, it is possible that the occupied FNR interacts with a distinct "matrix assembly receptor" proposed by McKeown-Longo and Mosher (1985), enhancing cell binding to FN's amino terminal matrix assembly domain (McDonald, 1988). Regardless of formal mechanism(s), there is clearly a mutual interdependence of the FN adhesive receptor complex, amino terminal binding activity and FN matrix assembly (McDonald, 1988).

There Are Dual Systems of FN Deposition in the Extracellular Matrix and Recognition

Organization of the FNR by the extracellular matrix is sensible viewed in the context of dual systems of FN matrix deposition and recognition (McDonald, 1988). The profound change in FNR organization associated with FN matrix recognition suggests that a major role of the FNR is to transmit information about the state of the extracellular matrix to the cell. Rather than alterations in FNR function somehow dictating cell behavior (e.g., by phosphorylation of the FNR inducing decreased FN binding and hence increased cell motility [Duband et al., 1988]) the FNR may serve as a signal transducer between the external FN matrix and the cell regulating adhesive interactions and motility (Duband et al., 1986), cytodifferentiation (Menko and Boettiger, 1987; Patel and Lodish, 1988), growth promotion (Bitterman et al., 1983) and the regulation of gene expression (Holderbaum and Ehrhart, 1986). Accordingly, it is important to understand the mechanisms of FN deposition by mesenchymal cells and signal transduction by the FNR. Finally, it scarcely must be emphasized that the extracellular matrix is a rich composite with marked regional heterogeneity. Although we have focused on FN and the FNR, multiple cellular receptors are utilized for adhesion even to matrices formed in vitro (Wayner and Carter, 1987), and doubtless greater complexity exists in vivo.

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