Cells interact with extracellular fibronectin (FN) via adhesive fibronectin receptors (FNRs) that are members of the very late antigens (VLAs) subfamily 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 matrixFN. 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 α5 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 the diffuse distribution of 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.
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 (Pytel et al., 1987). Ground placenta was stirred in Dulbecco's PBS (1:1, wt/vol) containing 1 mM CaCl₂, 1 mM MgCl₂, and 1 mM PMSF (buffer A) plus 50 mM octylglycoside (OGS) 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 human placental FNR; (c) a 22-ml Sepharose-4B column CNBr conjugated to 103 mg of the 110-kD fragment of FN (Zardi et al., 1985) to bind the FNR. Both the FNR and FN fragments were isolated by described methods (McDonald et al., 1987; Zardi et al., 1987; Zardi et al., 1988) and were judged greater than 95% pure by SDS-PAGE and protein staining. A mouse mAb to the α subunit of the VNR was generously provided by Dr. D. Cheresi 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 anticonnective tissue monoclonal antibody PIH3 to the α subunit (Wayner and Carter, 1987; Takada et al., 1988) and the anti-FNR α monoclonal antibody PIF8 have been described (Wayner et al., 1988).

FN Fragments and Antibodies

A mouse mAb to the α subunit of the VNR was generously provided by Dr. D. Cheresi 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 anticonnective tissue monoclonal antibody PIH3 to the α subunit (Wayner and Carter, 1987; Takada et al., 1988) and the anti-FNR α monoclonal antibody PIF8 have been described (Wayner 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; Quad 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 subunit with at least six related VLA subtypes expressed by many cells but possesses a unique α subunit (Hynes, 1987; Hemler et al., 1987). Accordingly, we raised synthetic peptide antibodies to selected polypeptides from the deduced sequence of the FNR α 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.

Synthetic Peptides and Antisera

Four hydrophilic peptide sequences (residues 65-73, 811-823, 886-897, and 1039-1049) were selected (see Doolittle, 1986) from hydrophathy analysis (Kyte and Doolittle, 1982). These sequences include a characteristic repeat of the 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-nitrobenzozoate (Laemmli and Doherty, 1968) and each peptide conjugated to keyhole limpet hemocyanin (KLH) using N-succinimidyl-bromooacetate (NSB) at a 1:1 ratio of peptide/KLH. 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 reinoculated 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 titrated by ELISA on purified FNR, the IgG purified (McKinney and Parkinson, 1987), and antipeptide IgG affinity purified by chromatography on a peptide-hexa-dimesphosphor-CLB4 column cross-linked with N-succinimidyl-bromooacetate.

FN Fragments and Antibodies

A mouse mAb to the α subunit of the VNR was generously provided by Dr. D. Cheresi of the Scripps Clinic Foundation (Cheresh and Harper, 1987). 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, 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 Ab33 antireceptor antibody and 10 μg/ml of a mAb (52DH1) 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 microscope (Nikon Inc., Garden City, NY) equipped with epiillumination 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 Hz 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.).

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 (52DH1) 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 microscope (Nikon Inc., Garden City, NY) equipped with epiillumination 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 Hz 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.).
Cell surface iodination was performed with lactoperoxidase and glucose oxidase (Leibenzon 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 PIH5 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 α5 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 (Arggraves 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 α5 subunit (Roberts et al., 1988) immunoprecipitates polypeptides of 165 kD representing the α5 subunit of the VLA complex (data not shown), 150 kD representing at least the α2, α5, α3 and α6 subunits, the 120 kD mature β, subunit, and the 110-kD pre-β, 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 PIH5 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 α5 subunit removed all antigen recognized by Ab 33 and vice versa whereas mAb PIH5 did not remove the Ab 33 antigen (Fig. 2). Thus, P1F8 and Ab 33 recognize the α5β3 complex. In addition, the α3 and α5 subunits synthesized by IMR-90 are recognized by mAb PIH5 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 α5 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 α5 subunit of the VLA family of integrins but not the α1, α2, α3, or α6 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 [35S]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 α1 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 ~25I surface-labeled IMR-90 fibroblasts with mAbs to the α 2 (PIH5) or α 5 (PIF8) subunits or with Ab 33. A Triton lysate was immunoprecipitated twice with Ab 33 (left), mAb PIF8 to the α5β complex (center), or PIH5 to the α3β1 complex (right) (Wayner and Carter, 1987; Wayner et al., 1988). Ab 33 removes the α 5 and associated β subunits cross-reacting with the α 5 specific antibody PIF8, but not the α and associated β subunits cross-reacting with the α 5 specific antibody PIH5. Similarly, PIF8 removes all α and associated β subunits cross-reacting with Ab 33, but has no effect on immunoprecipitation with PIH5. Thus, PIF8 and Ab 33 recognize the same α5β1 complex or FNR. In addition, IMR-90 expresses significant quantities of the α3β1 complex, as well as of the α5β1 complex (data not shown).

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 FN 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...
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.

Roman et al. Fibronectin Receptor Organization

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
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 α5 subunit of the FNR (Wayner et al., 1988). As a control, we used P1H5, an mAb to the VLA α2 subunit (Takada et al., 1988) that inhibits cell adhesion to collagen but not to FN (W en 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 FN (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 (PIHS 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 FN and FNR. Even though their respective antigens were abundant and readily immunoprecipitated from surface-labeled IMR-90 (see Fig. 2 B), neither PIF8 nor PIHS 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-α5 antibody P1H5 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 FN 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 FN 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 FN Rs 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 $\alpha_5$ 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 $\beta_1$ subunit also recognize diverse species (Marcantonio and Hynes, 1988) demonstrating that
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 α5 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 α5β1 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 α5 subunit (our observations in this work) and to the β1 subunit putative cytoplasmic domains (Mar-cantonio 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 β1 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 PIP8 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 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 surprising 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.

The Journal of Cell Biology, Volume 108, 1989 2538

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
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-
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 assembly, synthetic peptide antibodies to cytoplasmic domains should be useful tools to dissect FNR interactions with putative cytoskeletal links.

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 organized despite extensive cell arborization (not shown). Similar results were obtained with confluent monolayers exposed to cytochalasin B for 4 h (not shown).
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
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, 1989). 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.

Dr. G. D. Wilner of the Department of Pathology, Washington University School of Medicine, synthesized the peptides used. Dr. E. J. Brown, Department of Internal Medicine, Washington University School of Medicine, suggested the use of the N-succinimidyl bromoacetate conjugation method, and Dr. R. P. Mecham, Department of Cell Biology and Physiology and Respiratory and Critical Care Division, Washington University School of Medicine, performed amino acid analyses. T. M. Birkenmeier, J. B. Fegan, J. B., M. E. Sobel, K. M. Yamada, B. de Crombrugghe, and I. Pastan, received for publication 21 November 1988 and in revised form 8 February 1989.

References

Abercrombie, M. 1980. The crawling movement of metazoan cells. Proc. R. Soc. Lond. B 207:127-137.

Akiyama, S. K., and K. M. Yamada. 1985. The interaction of plasma fibronectin with fibroblastic cells in suspension. J. Biol. Chem. 260:4492-4500.

Ali, I. U., and R. O. Hynes. 1977. Effects of cytochalasin B and colchicine on arrangement of a major surface protein of fibroblasts. Biochim. Biophys. Acta. 471:16-24.

Aplin, J. D., and R. C. Hughes. 1981. Protein-derivatized glass cover slips for the study of cell-to-substratum adhesion. Anal. Biochem. 113:144-148.

Argraves, W. S., S. Susuki, H. Arai, K. Thompson, M. D. Pierschbacher, and E. Rauskalahti. 1987. Amino acid sequence of the human fibronectin receptor. J. Cell Biol. 105:1183-1190.

Barry, E. L. R., and D. F. Mosher. 1988. Factor XIII cross-linking of fibronectin at cellular matrix assembly sites. J. Biol. Chem. 263:10464-10469.

Bernardow, M., S. M., and G. R. Matsuda. 1986. Preparation of peptide-protein immunogens using N-succinimidyl bromoacetate as a bifunctional cross-linker reagent. Anal. Biochem. 153:95-102.

Bitterman, P. B., S. I. Remund, S. Adelberg, and R. G. Crystal, 1983. Role of fibronectin as a growth factor for fibroblasts. J. Cell Biol. 97:1925-1932.

Boucaut, J. C., T. Darribere, T. J. Poole, H. Aoyama, K. M. Yamada, and J. P. Thiery. 1984. Biologically active synthetic peptides as probes of erythropoietic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest cell migration in avian embryos. J. Cell Biol. 99:1822-1830.

Brommer-Fraser, M. 1986. An antibody to a receptor for fibronectin and laminin promotes cranial neural crest development in vivo. Dev. Biol. 117:528-536.

Buck, C. A., and A. F. Horwitz. 1987. Cell surface receptors for extracellular matrix molecules. Annu. Rev. Cell Biol. 3:179-205.

Burrage, K., and J. R. Feramisco. 1980. Microinjection and localization of a 130k protein in living fibroblasts: a relationship to actin and fibronectin. Cell. 19:587-595.

Chen, J.-M., and W.-T. Chen. 1987. Fibronectin-degrading proteases from the membranes of transformed cells. Cell. 48:193-203.

Chen, W.-T., J. Wang, T. Hasegawa, S. S. Yamada, and K. M. Yacandra. 1986. Regulation of fibronectin receptor distribution by transformation, exogenous fibronectin, and synthetic peptides. J. Cell Biol. 103:1649-1661.

Chen, W.-T., J. M. Chen, and S. C. Mueller. 1986b. Coupled expression and colocalization of 140k cell adhesive molecules, fibronectin, and laminin during morphogenesis and cytodifferentiation of chick lung cells. J. Cell Biol. 103:1073-1090.

Chereshe, D. A., and J. R. Harper. 1987. Arg-Gly-Asp recognition by a cell adhesive receptor requires its 130 kDa alpha subunit. J. Biol. Chem. 262:1434-1437.

Dejana, O., S. Colella, G. Conforti, M. Abbadini, M. Gaboli, and P. C. Marchisio. 1988. Fibronectin and vitronectin regulate the organization of their respective Arg-Gly-Asp receptors in cultured human endothelial cells. J. Cell Biol. 107:1215-1223.

Doolittle, R. F. 1986. Of Urfs and Orfs. A Primer on How to Analyze Derived Amino Acid Sequences. University Science Books, Mill Valley, CA. 103 pp.

Duband, J.-L., S. Rocher, W.-T. Chen, K. M. Yamada, and J. P. Thiery. 1986. Cell adhesion and migration in the early vertebrate embryo: location and possible role of the putative fibronectin receptor. J. Cell Biol. 102:160-178.

Duband, J.-L., S. Dufour, K. M. Yamada, and J. P. Thiery. 1988. The migratory behavior of avian embryonic cells does not require phosphorylation of the fibronectin receptor complex. FEBS (Fed. Eur. Biochem. Soc.) Lett. 230:181-185.

Fava, R. A., and D. B. McClure. 1987. Fibronectin associated transforming growth factor. J. Cell Physiol. 131:184-189.

Feghali, B. J., M. E. Sobel, K. M. Yamada, B. de Crombrugghe, and I. Pastan. 1981. Effects of transformation on fibronectin gene expression using cloned fibronectin cDNA. J. Biol. Chem. 256:520-525.

Hayashi, M. T., Yatohgo, H. Kashiwagi, and M. Izumi. 1987. Heparin-binding properties and a new method for purification of a cell-adhesive glycoprotein vitronectin. Zool. Sci. (Tokyo). 4:1025.

Hemler, M. E., C. Huang, and L. Schwarz. 1987. The VLA protein family. Characterization of five distinct cell surface heterodimers each with a common 130,000 molecular weight beta subunit. J. Biol. Chem. 262:3300-3309.

Hirst, R., A. Horwitz, C. Buck, and L. Rohrschneider. 1986. Phosphorylation of the fibronectin receptor in cells transformed by oncogenes that encode tyrosine kinases. Proc. Natl. Acad. Sci. USA. 83:6470-6474.

Holderbaum, D., and L. A. Ehrhart. 1986. Substratum influence on collagen and fibronectin biosynthesis by arterial smooth muscle cells in vitro. J. Cell. Physiol. 126:216-224.

Hofers, V. M., T. G. Ruff, D. L. Parks, J. A. McDonald, L. L. Ballard, and E. J. Brown. 1989. Murine fibronectin receptor expression (VLA-5) is increased in activated peritoneal macrophages. J. Exp. Med. in press.

Horwitz, A. K., Duggan, C. Buck, M. C. Beckerle, and K. Burrage. 1986. Interaction of the plasma membrane fibronectin receptor with talin: a transmembrane linkage. Nature (Lond.) 320:531-533.

Humphries, M. J., S. K. Akiyama, A. Komoriya, K. Olden, and K. M. Yamada. 1986a. Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion. J. Cell Biol. 103:2673-2674.

Humphries, M. J., K. Olden, and K. M. Yamada. 1986b. A synthetic peptide...
from fibronectin inhibits experimental metastasis of murine melanoma cells. Science (Wash. DC). 233:467–470.

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. Cell. 48: 549–554.

Janatova, J., J. Fuller, and M. J. Hunter. 1968. The heterogeneity of bovine albumin with respect to sulphydryl and dimer content. J. Biol. Chem. 243: 3612–3622.

Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105–132.

Leiben, T. W., D. R. Boue, J. G. Bradley, and J. H. Kersey. 1982. Antibody affinity may influence antigenic modulation of the common acute lymphoblastic leukemia antigen in vitro. J. Immunol. 129:2287–2292.

Lyubimov, A. V., and J. M. Vasiliev. 1982. Distribution of fibronectin-containing structures on the surface of lamelloplasm and endoplasm of fibroblast: hypothesis of receptor-mediated assembly of fibronectin structures. Cell Biol. Int. Rep. 6:105–112.

Marcantonio, E. H., and R. O. Hynes. 1988. Antibodies to the conserved cytoplasmic domain of the integrin B subunit react with proteins in vertebrates, invertebrates, and fungi. J. Cell Biol. 106:1765–1772.

Mautner, V., and R. O. Hynes. 1977. Surface distribution of fibronectin receptor ligand protein in relation to the cytoskeleton of normal and transformed cells. J. Cell Biol. 75: 743–768.

McClay, D. R., and C. A. Ettensohn. 1987. Cell adhesion in morphogenesis. Annu. Rev. Cell Biol. 3:319–345.

McDonald, J. A., B. J. Quade, and T. J. Broekelmann. 1982. Role of fibronectin in collagen deposition: fab' to the gelatin-binding domain of fibronectin inhibits both fibronectin and collagen organization in fibroblast extracellular matrix. J. Cell Biol. 92:485–492.

McDonald, J. A., B. J. Quade, T. J. Broekelmann, R. LaChance, K. Forseman, K. E. Hasegawa, and S. Akiyama. 1987. Fibronectin cell-adhesive domain and an amino terminal matrix assembly domain participate in its assembly into fibroblast pericellular matrix. J. Biol. Chem. 272:2957–2967.

McDonald, J. A. 1988. Extracellular matrix assembly. Annu. Rev. Cell Biol. 4:183–207.

McKeown-Longo, P. J., and D. F. Mosher. 1985. Interaction of the 70,000-mol-wt amino-terminal fragment of fibronectin with the matrix-assembly receptor of fibroblasts. J. Cell Biol. 100:364–374.

McKinney, M. M., and A. Parkinson. 1987. A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascites fluid. J. Immunol. Methods. 96:271–278.

Menko, A. S., and D. Boettiger. 1987. Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. Cell. 51:51–57.

Obara, M., M. S. Kang, and K. M. Yamada. 1988. Site-directed mutagenesis of the cell-binding domain of human fibronectin—separable, synergistic sites mediate adhesive function. Cell. 53:649–657.

Patel, V. P., and H. F. Lodish. 1988. A fibronectin matrix is required for differentiation of murine erythroleukemia cells into reticulocytes. J. Cell Biol. 105:3105–3118.

Patela, R., M. D. Pierschbacher, S. Argraves, S. Susuki, and E. Ruoslabhi. 1987. Arginine-glycine-aspartic acid recognition receptors. Methods Enzymol. 144:475–489.

Quade, B. J., and J. M. McDonald. 1988. Fibronectin's amino-terminal matrix assembly site is located within the 29-kDa amino-terminal domain containing five type I repeats. J. Biol. Chem. 263:19602–19609.

Robert, C. J., T. M. Birkenmeier, J. J. McQuillan, S. K. Akiyama, S. S. Yamada, W. Chen, K. M. Yamada, and J. A. McDonald. 1988. Transforming growth factor beta stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. J. Biol. Chem. 263:4586–4592.

Rodriguez, J., and F. Deinhardt. 1960. Preparation of a semipermanent mounting medium for fluorescent antibody studies. Virology. 12:316–317.

Ruoslabhi, E., and M. D. Pierschbacher. 1986. Arg-Gly-Asp: a versatile cell recognition signal. Cell. 44:517–518.

Ruoslabhi, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. Science (Wash, DC). 28:491–497.

Singer, I. I., S. Scott, D. W. Kawka, D. M. Kazazis, J. Gallit, and E. Ruoslabhi. 1988. Cell surface distribution of fibronectin and vitronectin receptors depend on substrate composition and extracellular matrix accumulation. J. Cell Biol. 106:2171–2182.

Takada, Y., E. A. Wayner, W. G. Carter, and M. E. Hemler. 1988. Extracellular matrix receptors, ECMRII and ECMRI, for collagen and fibronectin correspond to VLA-2 and VLA-3 in the VLA family of heterodimers. J. Cell Biol. 107:385–393.

Towbin, H., T. Staehelein, and J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350–4354.

Varino, T., L. Laitinen, O. Nanvenen, M. Cutolo, L.-E. Thornell, L. Zardi, and I. Virtanen. 1987. Differential expression of the ED sequence containing form of cellular fibronectin in embryonic and adult human tissues. J. Cell Sci. 88:419–430.

Wagner, D. D., R. Ivatt, A. T. Destree, and R. O. Hynes. 1981. Similarities and differences between the fibronectins of normal and transformed hamster cells. J. Biol. Chem. 256:11708–11715.

Wayner, E. A., and W. G. Carter. 1987. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α subunits. J. Cell Biol. 105:1873–1884.

Wayner, E. A., W. G. Carter, R. S. Piotrowicz, and T. J. Kuniccki. 1988. The function of multiple extracellular matrix receptors (ECMRs) in mediating cell adhesion to ECM: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-Ila. J. Cell Biol. 107:1881–1891.

Yamada, K. M. 1978. Immunological characterization of a major transforming-sensitive fibroblast cell surface glycoprotein. Localization, redistribution, and role in cell shape. J. Cell Biol. 78:520–541.

Zardi, L., B. Cannemalla, E. Balza, L. Borsii, P. Castellani, M. Roccia, and A. Siri. 1985. Elution of fibronectin proteolytic fragments from a hydroxypatite chromatography column. Eur. J. Biochem. 146:571–579.