β1 Integrin–dependent and –independent Polymerization of Fibronectin

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Abstract. The mouse cell line GD25, which lacks expression of the β1 family of integrin heterodimers due to disruption of the β1 integrin subunit gene, was used for expression of full-length cDNA coding for splice variant A of the mouse β1 integrin subunit. In a stably transformed clone (GD25-β1A), the expressed protein was found to form functional heterodimeric receptors together with the subunits α3, α5, and α6. Both GD25 and GD25-β1A attached to fibronectin and formed focal contacts which contained αvβ3, but no detectable α5β1A. The presence of GRGDS peptide allowed α5β1A to locate to focal contacts of GD25-β1A cultured on fibronectin, while the β1-null GD25 cells were unable to attach under these conditions. Affinity chromatography revealed that α5β1A and αvβ3 could bind to a large cell-binding fragment of fibronectin. α5β1A strongly promoted polymerization of fibronectin into a fibrillar network on top of the cells. Whereas little αvβ3 was colocalized with the fibronectin fibrils in GD25-β1A cells, this integrin was able to support fibronectin fibril polymerization in GD25 cells. However, the αvβ3-induced polymerization was less efficient and occurred mainly in dense cultures of the GD25 cells. Thus, while both α5β1A and αvβ3 are able to support adhesion to fibronectin, αvβ3 dominates in the formation of focal contacts, and α5β1A has a prime function in fibronectin matrix assembly. This is the first report on fibronectin matrix assembly in the absence of β1 integrins.

THE cell-adhesive protein fibronectin (FN)[1] is secreted in soluble form by many normal and tumorigenic cells (Hynes, 1990). The protein can polymerize into a disulfide-linked pericellular network in a multistep process which is under cellular control (Mosher et al., 1992). The nucleation step occurs on the cell surface and appears to involve several membrane components (Moon et al., 1994; Woods et al., 1988; Wu et al., 1993). Tumor cells do not assemble an FN network, a feature thought to be of importance for their invasive growth and formation of metastases (Giancotti and Ruoslahti, 1990; Hynes, 1990). Among the membrane components implicated in the FN polymerization process, integrins have been firmly demonstrated to have a central role (Dzamba et al., 1994; Wu et al., 1993).

Integrins are a family of transmembrane receptors mediating adhesion to both extracellular matrix (ECM) and cell surface molecules (Heino, 1993; Hynes, 1992). Each integrin is composed of an α and a β subunit. Binding of integrins to immobilized ECM ligands commonly results in cytoskeleton reorganization and formation of focal contacts (FC), where the actin cytoskeleton is anchored to the integrins via a complex of proteins including α-actinin, talin, and vinculin (Burridge et al., 1990). The formation of FC complexes, which also contain the regulatory enzymes $pp125^{FAK}$ (Guan and Shalloway, 1992; Kornberg et al., 1992) and protein kinase C (Jaken et al., 1989; Woods and Couchman, 1992), triggers signal cascades which act in concert with signals from growth factor stimulation and which can alter gene expression (Damsky and Werb, 1992; Hedin et al., 1988; Schlaepfer et al., 1994).

Several integrins have been reported to interact with FN (e.g., α3β1, α4β1, α5β1, αvβ1, αvβ3, αvβ6) (Busk et al., 1992; Hynes, 1992). Among these, α5β1 has been shown to be able to promote FN polymerization (Wu et al., 1993). It binds FN to the cell surface and possibly induces the conformational change which appears to be required for polymerization of FN. A transition from the folded soluble form to a more extended state has been shown to result in exposure of an FN binding site ("self assembly site") and spontaneous polymerization in vitro (Morla et al., 1994). However, the finding that mouse embryos deficient in α5

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; FC, focal contact; FN, fibronectin; LN-1, laminin-1; VN, vitronectin.

subunit expression still deposited an FN matrix network indicated that other integrins could also support this process (Yang et al., 1993). The αvβ1 and α4β1 integrins were both shown to mediate adhesion of CHO cells to FN, but failed to induce FN polymerization in these cells (Wu et al., 1995; Zhang et al., 1993).

In this study we have used a β1-deficient cell line (GD25) derived from the embryonic stem cell line G201 (Fässler et al., 1995) to study the role of β1 integrins in the FN polymerization process. This was done by expressing the integrin subunit β1A in the GD25 cells. β1A was the first described splice variant of the integrin β1 protein, and it has been demonstrated to have a broad distribution in vivo (Balzac et al., 1993; Languino and Ruoslahti, 1992). We report that this variant of β1 in complex with α5 strongly promotes FN matrix assembly, but that the process can occur, although less efficiently, in the absence of β1 integrins due to the compensatory activity of integrin αvβ3.

Materials and Methods

Proteins and Peptides

FN was purified from human plasma as previously described (Smilenov et al., 1992). Bovine vitronectin (VN) and rat collagen type I was purchased from Telios Pharmaceuticals (San Diego, CA) and Invitrogen (San Diego, CA), respectively. Laminin-1 (LN-1) was kindly provided by Dr. Rupert Timpl (Max-Planck-Institute for Biochemistry, Martinsried, Germany). GRGDS peptide was obtained from Calbiochem-Novabiochem GmbH (Bad Soden, Germany).

Antibodies

The rabbit anti–human FN serum, rabbit anti–β1 serum, and chicken anti–human FN antibodies were prepared in our laboratory and have been described previously (Bottger et al., 1989; Johansson and Höök, 1984; Woods et al., 1986). The hamster anti–mouse β3, rat anti–mouse α4, and rat anti–mouse α5 mAbs were purchased from Pharmingen (San Diego, CA). The mouse anti–human vinculin mAb was purchased from Sigma Immunochemicals (St. Louis, MO). The rabbit anti–human β5 serum, rabbit anti–human β4 serum, and rabbit anti–human α2 serum were purchased from Chemicon Intl., Inc. (Temecula, CA). The rabbit anti–rat α1 serum, the rabbit anti-cytoplasmic αv serum, the rabbit anti-cytoplasmic α3 serum, the rat anti–human α6 mAb (GoH3), the rabbit anti-cytoplasmic β5 serum, and the affinity-purified rabbit anti-cytoplasmic α9 IgG were kindly provided by Drs. K. Löster (Freie Universitat, Berlin, Germany), G. Tarone (University of Torino, Italy), M. Johansson (University of Uppsala, Sweden), A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam, Netherlands), L. Reichardt (University of California, San Francisco) and D. Sheppard (University of California, San Francisco), respectively. The fluorescence-labeled secondary antibodies were all affinity purified: Cy3 goat anti–rat IgG, DTAF goat anti–rabbit IgG, Cy3 goat anti–rabbit IgG, Cy3 goat anti–mouse IgG, DTAF goat anti–mouse IgG (all of multiple labeling quality) from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA); FITC sheep anti–chicken IgG from Binding Site Inc. (San Diego, CA); and FITC goat anti–hamster IgG from Cappel Laboratories (Malvern, PA).

cDNA

Full-length mouse cDNA coding for the integrin subunit β1A was generated by adding the missing start codon to the clone mFNRβ (Holers et al., 1989). This was done by ligation with the 5′-end of the clone pMINTβ (obtained from Dr. R. O. Hynes, MIT, Cambridge, MA) through a common unique HindIII site. The resulting β1A-cDNA, which contained the 3′ untranslated sequence of the β1A-mRNA including the polyadenylation signal, was cloned behind the PGK promotor in pGEM(KJ1)SalI (obtained from Dr. M. A. Rudnicki, MIT, Cambridge, MA) after removal of the neomycin cassette by a PstI/SalI digestion. The resulting β1A cassette and a puro-cassette encoding puromycin resistance under control of the PGK promotor were ligated next to each other into pBSKSII between the SacI and SalI sites of the vector. The two cassettes were separated by a unique XbaI site to allow linearization of the construct before transformation of cells. The puro-cassette was obtained from the vector pPGKPuro (obtained from Dr. P. W. Laird, MIT, Cambridge, MA).

Cells

The GD25 cells were derived from the embryonic stem cell clone G201, which are deficient in the integrin subunit β1 due to the introduction of a null mutation in the β1 integrin gene via homologous recombination (Fässler et al., 1995). By differentiating G201 cells in the presence of 0.5% DMSO into a mixed population of cells, followed by transformation with SV-40 large T antigen and ring cloning, immortalized β1-deficient cell lines were obtained. The GD25 cell line was chosen for these studies because of its rapid growth. The stably transformed cell line GD25-β1A was obtained by electroporating wild-type integrin β1A cDNA into GD25 cells.

The GD25 cells were cultured in DME + 10% FCS + L-glutamine (2 mM) + penicillin-streptomycin + fungizone (i.e., nonselection medium). The established β1 integrin-expressing cell lines were continuously cultured in nonselection medium + puromycin (10 μg/ml) (selection medium). The cells were harvested by trypsin-EDTA treatment and resuspended in serum-containing medium to inactivate the trypsin.

Transfection

The integrin β1A vector was linearized with Xba I, EtOH-precipitated, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at a concentration of 1 μg/μl. GD25 cells were harvested and resuspended in PBS at a concentration of 1×10^7 cells/ml. The cell suspension (300 μl) was mixed with 20 μg of linearized vector and electroporated at 2.0 kV and 0.9 μF. After electroporation the cells were kept on ice for 10 min, resuspended in 50 ml nonselection medium, and put into 24-well plates (two plates/electroporation). The cells were cultivated for 3 d before selection with 5 μg/ml puromycin was started. Surviving clones were picked after 1–2 wk of selection and were cultured continuously in selection medium.

FACS® Analysis

Transfected clones were harvested and suspended in PBS containing 10% goat serum for 30 min at 4°C. The cells (1×10^6) were then incubated on ice for 20 min with primary antibody (rabbit anti-β1) diluted in FACS®-PBS (PBS containing 2% goat serum and 0.001% NaN_3). The cells were washed twice with ice-cold FACS®-PBS and incubated on ice for 20 min with secondary antibody (fluorescein-labeled goat anti–rabbit IgG) diluted in FACS®-PBS and washed twice with ice-cold FACS®-PBS. The cells (5,000/sample) were analyzed in a FACScan® (Becton Dickinson and Co., Mountain View, CA) equipped with 5-W argon laser at 488 nm.

Immunoprecipitation of Integrins

Integrins were immunoprecipitated from cells labeled with ^{125}I. The labeling of the membrane proteins was done by incubating the cells of a confluent 25-cm^2 flask with 1 ml PBS containing 5 mM β-D-glucose + 50 μg lactoperoxidase + 2 μg glucose peroxidase + 0.5 mCi ^{125}I for 20 min, followed by washing of the cells five times with PBS and solubilization with 1% Triton X-100 in 10 mM Tris buffer, pH 7.5, containing pepstatin A (1 μg/ml), pefabloc SC (0.4 mM), and N-ethylmaleimide (2 mM). The lysate was centrifuged at 13,000 rpm for 10 min, and the supernatant was used for the immunoprecipitations.

The lysates were precleared with preimmune serum and a mixture of protein A–Sepharose (Pharmacia, Uppsala, Sweden) and protein G–Sepharose for 2 h at 4°C. The precleared lysate was used for immunoprecipitation by incubating with antibodies overnight. The antibodies were precipitated with protein G–Sepharose, and the pellets were washed three times with HS-TBS (500 mM NaCl, 10 mM Tris-HCl, pH 7.4) + 0.1% Triton X-100 + protease inhibitors (PI; pepstatin A [1 μg/ml], pefabloc SC [0.1 mM] and N-ethylmaleimide [0.2 mM]), three times with TBS + 0.1% Triton X-100 + PI, and once with TBS + PI. The samples were run on a 6–10% SDS-PAGE gel in the absence of reducing agent. The immunoprecipitated material was visualized by autoradiography on x-ray film (Fuji Photo Film Co., Ltd.) or in a Phosphoimager (Fuji Photo Film Co., Ltd.).

Purification of FN-binding integrins was performed from the lysate of surface iodinated cells as previously described (Forsberg et al., 1994) by

sequential affinity chromatography of the lysate on a WGA-Sepharose column and a column of Sepharose conjugated with a 120-kD FN fragment. Material retained on the FN fragment–Sepharose after washing with 10 mM Tris buffer, pH 7.4, containing 50 mM NaCl, 2 mM Mn^{2+}, 0.2% Triton X-100, and PI, was eluted with EDTA and ^{125}I labeled further by the chloramine-T method using Iodobeads (Pierce Chemical Co., Rockford, IL). After reiodination of the material, immunoprecipitations for integrins were performed.

Cell Attachment Assay

The assay used was a modification of an earlier described method (Aumailley et al., 1989). The wells of 96-well microtiter plates (No. 67008; Nunc, Roskilde, Denmark) were coated with ECM proteins in DME for 1 h at 37°C. Coating with 15% FCS was used as a positive control. As a negative control, the wells were incubated with DME only. After coating, the wells were washed twice with PBS and blocked with 1% heat-treated BSA in PBS for 2 h at 37°C. After washing with PBS, 1×10^5 cells suspended in DME were added to each well, and the cells were allowed to attach for 1 h at 37°C. Unattached cells were removed by washing twice with PBS, and remaining cells were fixed in 96% EtOH for 10 min and stained with 0.1% crystal violet in H_2O for 30 min. The excess stain was washed away with water, and after lysis of the fixed cells with water containing 0.2% Triton X-100, the absorbance was read in a microtiter plate reader (Multiskan PLUS; Labsystems, Stockholm, Sweden) at 595 nm. All samples were analyzed in triplicates.

RNA Preparation and Northern Analysis

Total RNA was purified from GD-25 and GD25-β1A using a modified method by Auffray and Rougeon (1980). Briefly, cells were resuspended in 3 M LiCl + 6 M Urea + 0.2% SDS and homogenized in ultrathurax. Subsequently, RNA was precipitated on ice overnight. The pellet was solubilized in TE containing 0.5% SDS, extracted once in phenol and once in chloroform/isoamyl alcohol (24:1), followed by precipitation of the RNA by addition of NaAc and EtOH to 0.1 M and 70% final concentrations, respectively. After dissolving the precipitate in TE, the RNA concentration was determined by UV spectroscopy at 260 nm.

For Northern blot analysis, 10 μg of total RNA was electrophoretically separated on a 1% agarose gel containing 1.8% formaldehyde and 1 × MOPS buffer (20 mM MOPS, 5 mM NaAc, 1 mM EDTA, pH 7.0), partly hydrolyzed in 50 mM NaOH, neutralized in 100 mM Tris-HCl, pH 7.5, and blotted to Hybond-N^{plus} membrane (Amersham Intl., Little Chalfont, UK) by capillary transfer. The RNA was UV cross-linked to the membrane in a Stratalinker (Stratagene, La Jolla, CA) and prehybridized according to the recommendations of the manufacturer. After hybridization with ^{32}P-labeled probes at 65°C in prehybridization buffer, the membranes were washed twice in 2 × SSC + 0.1% SDS at room temperature, once in 1 × SSC + 0.1% SDS at 65°C for 15 min, and once in 0.5 × SSC + 0.1% SDS at 65°C for 10 min. The membranes were then exposed to x-ray film for 24–72 h at −80°C.

^{32}P-labeled probes for FN were generated by random hexamer-primed DNA synthesis (Boehringer Mannheim Biochemicals, Indianapolis, IN) with a 1.7-kb BsaI/SmaI fragment of human FN-cDNA as template.

FN Extraction from ^{35}S-labeled Media

Cells were grown to 90% confluency and were then incubated for 24 h in MCDB medium (-Met, -Cys; Flow Laboratories, Irvine, Scotland) containing 5% FCS, L-glutamine, penicillin-streptomycin, fungizone, and 2.5 MBq ^{35}S-Met and ^{35}S-Cys.

The media were collected, and after addition of protease inhibitors, the samples were centrifuged to remove cell debris. After preclearing by passage through a preimmune IgG–Sepharose column, 1.5 ml of the samples were incubated overnight with gelatin–Sepharose at 4°C and washed three times with HS-TBS + PI and three times with TBS + PI. The gelatin-bound samples were run on a 6–10% SDS-PAGE gel in the presence of reducing agents. The material on the gel was visualized by autoradiography on an x-ray film. The bands seen on the gel correlated with the size of reduced FN.

Immunofluorescent Staining of Cells

Cells (10,000) were seeded in serum-containing medium and grown on coverslips (diam 19 mm) in 12-well plates for 5–7 d to ~75% confluency. Alternatively, 50,000 cells were cultured for 30 min to 3 h in DME on glasses coated with ECM proteins. Coating with FN was done by incubating the glasses overnight at 4°C in PBS with 10 μg/ml of FN. The LN-1 coatings were done by adding 100 μl of PBS containing 100 μg/ml of LN-1 to the glasses and incubating them overnight at 4°C. The coated glasses were blocked with 1% BSA in PBS. The cells were fixed with 2% paraformaldehyde in PBS for 10 min, washed 3 × 10 min with PBS, and stored in PBS containing 0.02% NaN_3.

When needed, the cells were permeabilized with 0.5% Triton X-100 for 20 min (see figure legends). The coverslips were blocked with 10% goat serum in PBS overnight at 4°C. Subsequently, 40 μl of the antibody diluted in 10% goat serum was added to the coverslips and incubated for 1 h. After each antibody incubation, the coverslips were washed 3 × 10 min with PBS. The coverslips were then mounted onto microscope slides with Vectashield (Vector Cloning Systems, San Diego, CA) and studied in a fluorescence microscope for fluorescein and/or Cy3 staining. All double stainings were tested to ensure that no undesired cross-reactivity existed between the primary and secondary antibodies.

Results

Establishment of the Cell Lines

The cDNA construct of murine integrin β1A was transfected by electroporation into GD25 cells. Selection was made for stably transfected cells, and clones expressing high levels of integrin β1 were identified by FACS® analysis and chosen for further studies. Several clones expressing similar levels of β1 were obtained. While the results described in this study were obtained with one clone (GD25-β1A), two other high expressing clones were tested and behaved in the same way as the one described in detail.

Integrin Analysis and Attachment to Matrix Substrates

GD25 and GD25-β1A were analyzed for their integrin expression by immunoprecipitation of surface-iodinated cells using antibodies specific for various integrin subunits (Fig. 1). The results demonstrate that the transfected β1 cDNA encoded a protein with expected properties regarding size, immunoreactivity, and ability to form heterodimers with endogenous α subunits. Three β1-containing integrins were identified on the GD25-β1A cells, namely α3β1, α5β1, and α6β1, while α1, α2, α4, α9, and αv in complex with β1 were not detected. In addition, these cells expressed αvβ3, avβ5 and small amounts of α6β4 as seen by the immunoprecipitations. The untransfected GD25 cells

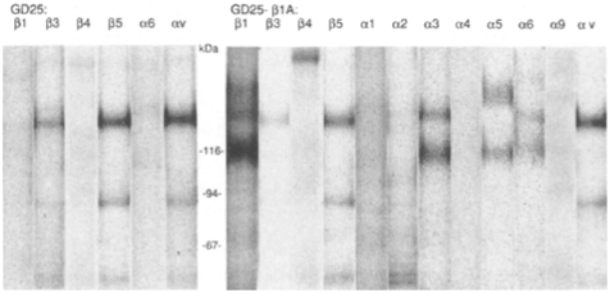

Figure 1. SDS-PAGE of integrins immunoprecipitated from surface ^{125}I-iodinated GD25 and GD25-β1A cells. The antibodies used were specific for β1, β3, β4, β5, α1, α2, α3, α4, α5, α6, α9, and αv integrin subunits, respectively. Material from all immunoprecipitates was run under nonreducing conditions. Immunoprecipitations were carried out as described in *Materials and Methods.*

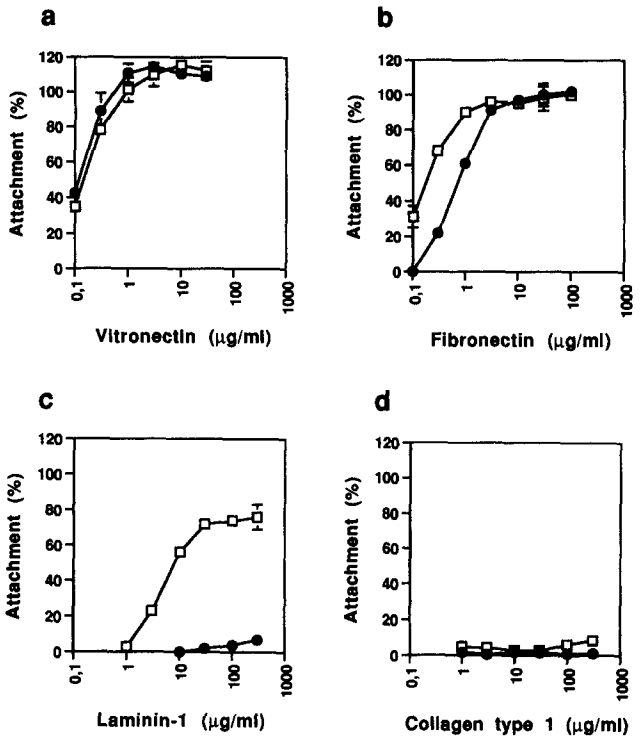

Figure 2. Attachment of GD25 (*filled circles*) and GD25-β1A (*open squares*) to ECM proteins. The proteins tested were VN (*a*), FN (*b*), LN-1 (*c*), and collagen type I (*d*). Attachment is expressed as percentage of the number of cells bound to serum-coated wells. Cell attachment assay was done as described in *Materials and Methods.*

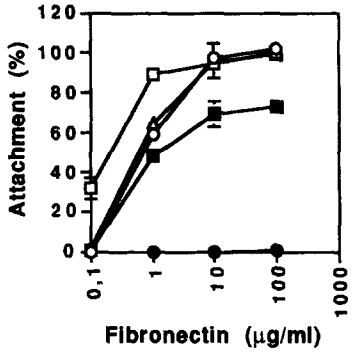

Figure 3. Attachment to FN of GD25 in the absence (*open circles*) or presence of GRGDS peptide (0.5 mg/ml) (*filled circles*), GD25-β1A in the absence (*open squares*) or presence of GRGDS peptide (0.5 mg/ml) (*filled squares*), and GD25-β1A in the presence of rabbit anti-β1 IgG (0.5 mg/ml) (*open triangles*). Attachment is expressed as percentage of the number of cells bound to serum-coated wells.

expressed only the latter three integrins among those investigated in this study. No obvious differences in morphology were seen in light microscopy between GD25 and the β1A-expressing clones (not shown).

Next, the attachment of GD25 and GD25-β1A to the ECM substrates VN, FN, collagen type I, and LN-1 was compared (Fig. 2). As expected, both cell types attached well to VN, probably by use of αv integrins. This interaction was strongly inhibited by the presence of GRGDS peptide in the medium (not shown). Surprisingly, the attachment to FN was almost as good for GD25 cells as for GD25-β1A. Only at low coating concentrations of FN, GD25-β1A was found to attach better than GD25. Addition of antibodies specific for integrin β1 to GD25-β1A reduced the attachment to FN down to the level of GD25 (Fig. 3). Thus, both cell lines apparently express an FN receptor which does not belong to the β1 subfamily. However, the attachment of the β1-deficient cells to FN was much more susceptible to inhibition by GRGDS peptide than the attachment of GD25-β1A (Fig. 3). More than 1.5 mg/ml of the peptide was required to inhibit the attachment of GD25-β1A by 50% while the attachment of GD25 cells was completely inhibited at 0.1 mg/ml (not shown). Collagen type I did not support adhesion of any of the cells, despite the presence of α3β1 on GD25-β1A. The adhesion to LN-1 was markedly dependent on the expression of β1; GD25-β1A cells attached well to LN-1, while GD25 cells showed only a weak binding to LN-1 at high coating concentrations of the protein. This interaction of GD25 cells was possibly due to binding of integrin α6β4 to LN-1. The ability of antibodies specific for β1 to inhibit the attachment of GD25-β1A to LN-1 to the level of the GD25 cells confirmed that the attachment of GD25-β1A was mediated by a β1-integrin (not shown).

Immunostaining of GD25 cells spread on FN in serum-free medium showed that the αv and the β3 subunits were located in FC (Fig. 4). Similar results were obtained with the GD25-β1A cells, and notably, β1A could not be detected in FC (Fig. 5) at any time between 30 min and 3 h of spreading. In contrast, when the cells were seeded on FN in the presence of GRGDS peptide in the medium (0.5 mg/ml), the GD25-β1A had integrin α5β1A and only small amounts of αv integrins in FC (Fig. 6). Under these conditions, the GD25 cells did not attach at all to FN. When spread on LN-1, GD25-β1A had α6β1A and no αv integrins in FC (not shown). Inhibition of protein synthesis by preincubation with cycloheximide (25 μg/ml) did not alter any of these results.

β1A Integrins Can Strongly Enhance, But Are Not Required for FN Polymerization

To test the role of integrin subunit β1A in FN polymerization, cells were grown on noncoated coverslips in serum-containing medium and immunostained for FN and various integrin subunits.

GD25-β1A cells were found to produce a dense FN network on top of and between the cells (Fig. 7 *B*). Staining for FN in GD25 showed that these cells could promote polymerization of FN, but to a much lower extent than β1A expressing cells (Fig. 7 *A*). An FN network was formed by the GD25 cells mainly when they were growing densely or in multilayers and often consisted of fibers that were shorter and thicker than those in GD25-β1A cultures. In subconfluent or monolayer GD25 cultures, polymerized FN was mostly seen as occasional single fibrils. In addition, FN was observed associated with the basal side of both cell lines. This FN was seen as punctuate deposits which in some cases resembled short fibrils (Figs. 7, *C* and *D*).

To investigate whether the differences in FN matrix assembly between the two cell lines were due to differences

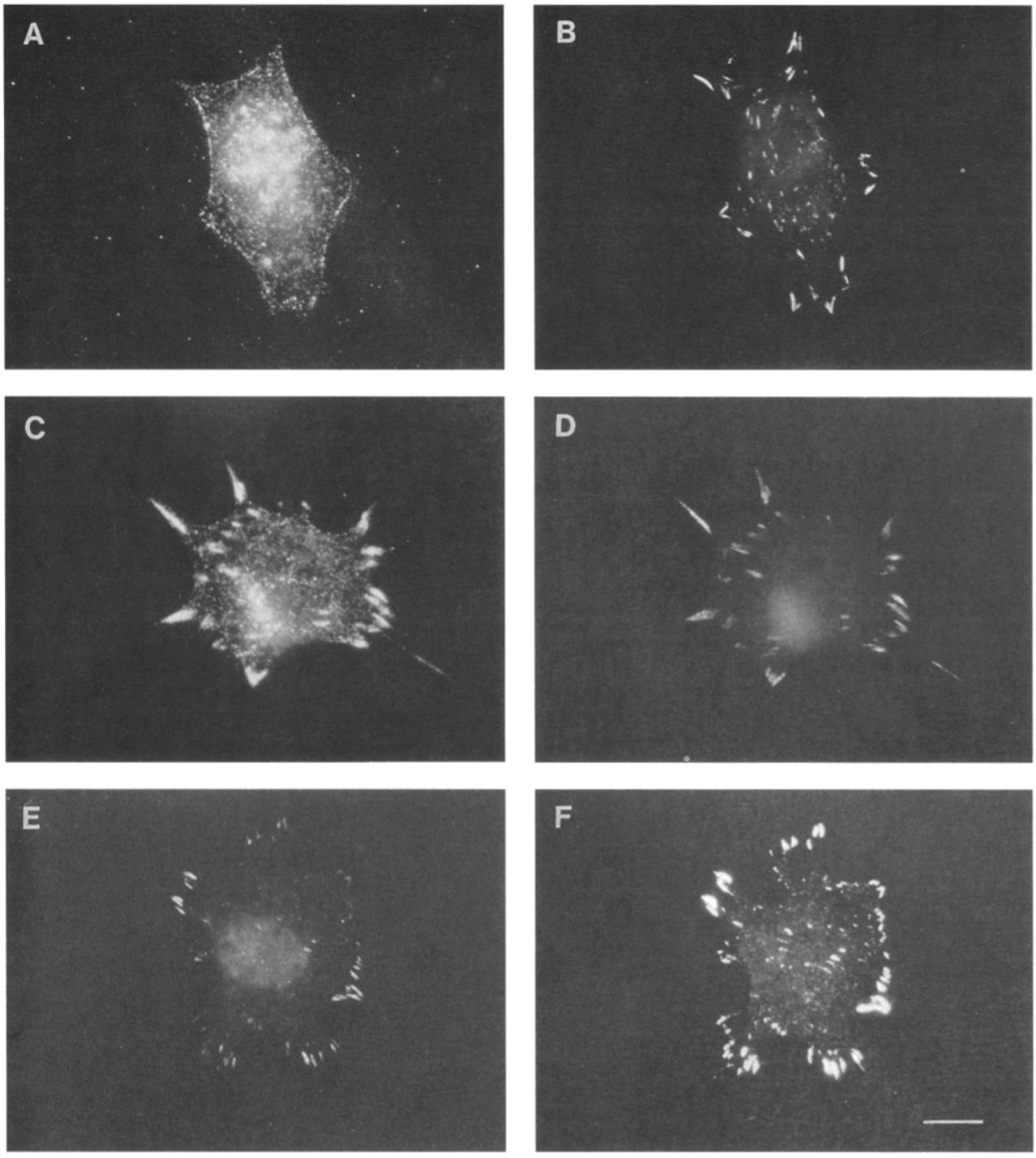

Figure 4. Immunofluorescent detection of FC in GD25 cells spread for 2 h on FN. Double stainings for β1 (*A*) and vinculin (*B*), for αv (*C*) and vinculin (*D*), and for β3 (*E*) and vinculin (*F*), respectively, are shown. The immunofluorescent stainings were carried out on permeabilized cells as described in *Materials and Methods.* Bar, 10 μm.

in the amounts of FN produced, Northern blotting of RNA derived from both cell lines was performed. Similar levels of FN mRNA were detected in both cell lines (not shown). Furthermore, metabolically labeled FN secreted into the media was measured after affinity purification on gelatin-Sepharose. The amount of radiolabeled FN in the medium was somewhat higher from the GD25 cells than from the GD25-β1A cells (not shown), probably due to the more extensive incorporation of FN into matrix by the GD25-β1A cells. These results show that FN secretion is not dependent on the presence of β1 integrins and that the observed differences between the two cell lines in FN matrix assembly were not due to differences in FN production.

The integrins involved in FN polymerization in the two cell lines were investigated by immunofluorescent stainings. In the GD25-β1A cells, double-staining for β1 (not shown) and α5 (Fig. 8, *D* and *C*) and FN showed a clear

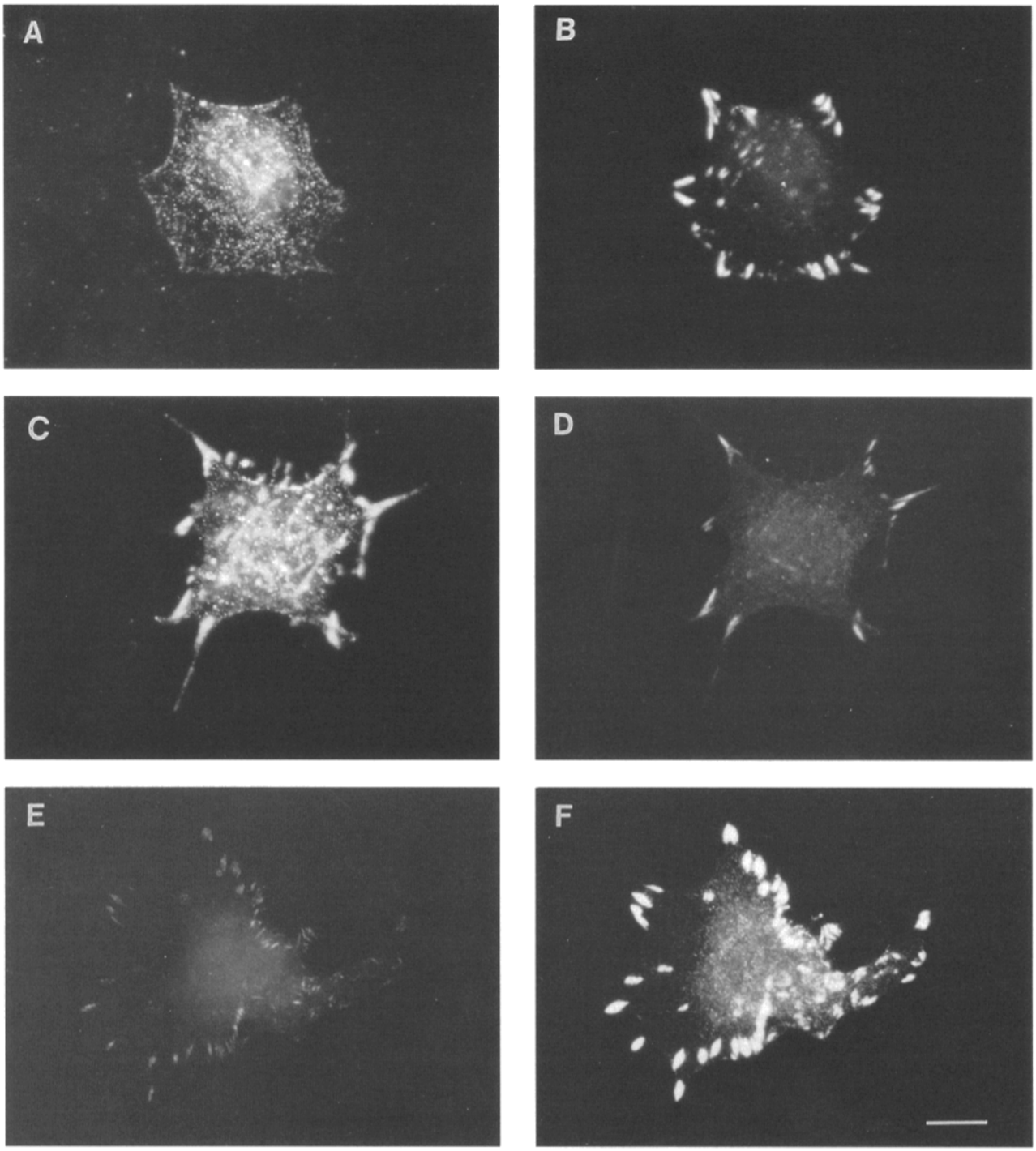

Figure 5. Immunofluorescent detection of FC in GD25-β1A cells spread for 2 h on FN. Double stainings for β1 (*A*) and vinculin (*B*), for αv (*C*) and vinculin (*D*), and for β3 (*E*) and vinculin (*F*), respectively, are shown. Cells were permeabilized before the immunostainings. Bar, 10 μm.

colocalization of the integrin subunits and FN with α5β1A concentrated to the ends of the fibrils. As expected, the GD25 cells showed no staining for β1 or α5 (Fig. 8). The GD25 showed a colocalization of αv and FN, analogous to the α5β1A-FN localization in the GD25-β1A cells (Fig. 9, *A* and *B*), indicating that an αv-containing integrin could be involved in the β1-independent FN polymerization. In the GD25-β1A cultures, αv was only weakly detected along the fibers (αv was mainly localized to FC) (Fig. 9, *C* and *D*). To test which β subunit was associated with the FN fibrils, GD25 cells were stained for β3 and β5. Whereas β3 was found to colocalize with the FN fibrils in the same way as αv (Fig. 9, *E* and *F*), the staining for β5 was diffusely distributed over the cell surface (not shown). The same result was obtained independently with two different β5 antibodies. In GD25-β1A cells, β3 staining could only barely

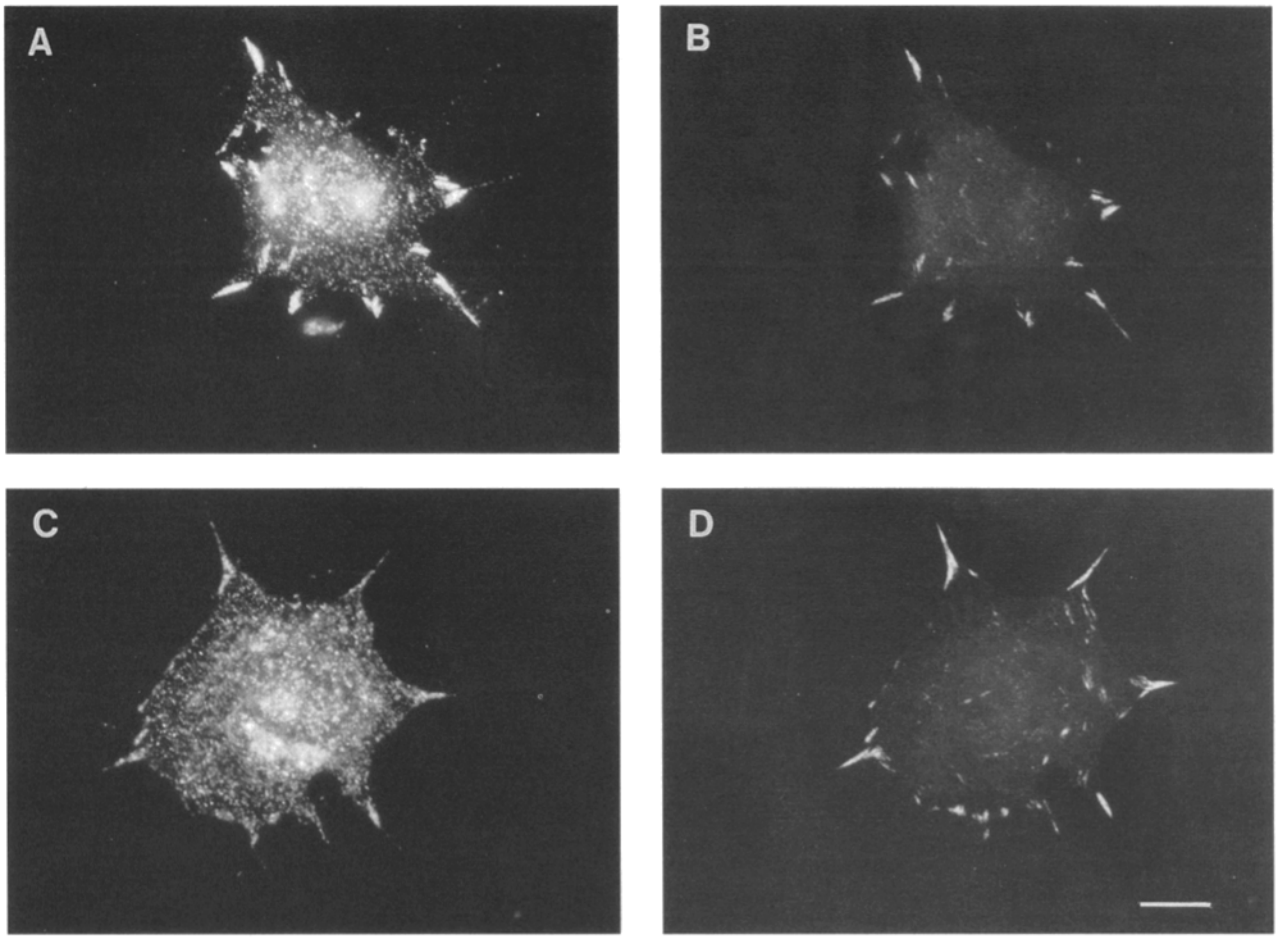

Figure 6. Immunofluorescent detection of FC in GD25-β1A cells spread for 2 h on FN in the presence of GRGDS peptide in the medium (0.5 mg/ml). Double stainings for β1 (*A*) and vinculin (*B*) and for αv (*C*) and vinculin (*D*), respectively, are shown. Cells were made permeable before the immunostainings. Bar, 10 μm.

be detected along some of the fibrils (Fig. 9, *G* and *H*) by use of this relatively weak antibody. Stainings for α3 integrin showed no specific localization of the integrin in either GD25 or GD25-β1A (not shown). Taken together, the results clearly indicate that FN polymerization in GD25-β1A cells is primarily dependent on integrin α5β1A, whereas the less efficient FN polymerization in the GD25 relies on αvβ3.

Affinity chromatography was applied to confirm the identity of the β1-independent FN receptor on the cells. After surface iodination and solubilization of the cells, the cell lysates were run on a column conjugated with a 120-kD FN fragment containing the RGD site. The eluted material was reiodinated, and immunoprecipitation for integrins was performed. The results showed that α5β1A and small amounts of αvβ3 (Fig. 10), but not α3β1A or αvβ5 (not shown), bound to the column from the GD25-β1A lysate, while only αvβ3 could be detected from the GD25 material. A band of ~250 kD coprecipitated with α5β1, the identity of which is not known at present.

Discussion

The integrin subunit β1 is expressed in most mammalian cells except mature erythrocytes (Hynes, 1992). Three splice variants of the protein have been described so far and some cells express more than one variant at the same time (Balzac et al., 1993; Languino and Ruoslahti, 1992). The lack of β1-deficient cells suitable as hosts for transformation of β1 cDNA has hampered the detailed functional analysis of the different forms of the protein. Most studies of β1 integrins in the past have been assumed to deal with the A form of the protein, but this has to be verified in each case. The different forms of the protein most likely have specific functions, as indicated by the inability of β1B to promote cell migration, activate $pp125^{FAK}$, or to localize to focal contacts. Furthermore, β1B has been shown to exert a dominant negative effect on the function of β1A in these processes (Balzac et al., 1993; Balzac et al., 1994). Recently, targeted disruption of the β1 gene has been accomplished in F9 cells (Stephens et al., 1993) and in embryonic stem cells of mouse (Fässler et al., 1995). The availability of cells deficient in integrin β1 allowed us to establish cell lines that express one specific splice variant of the protein, β1A, by transfection of the corresponding cDNA.

The expressed β1A was shown to form functional heterodimers together with endogenous α subunits when ex-

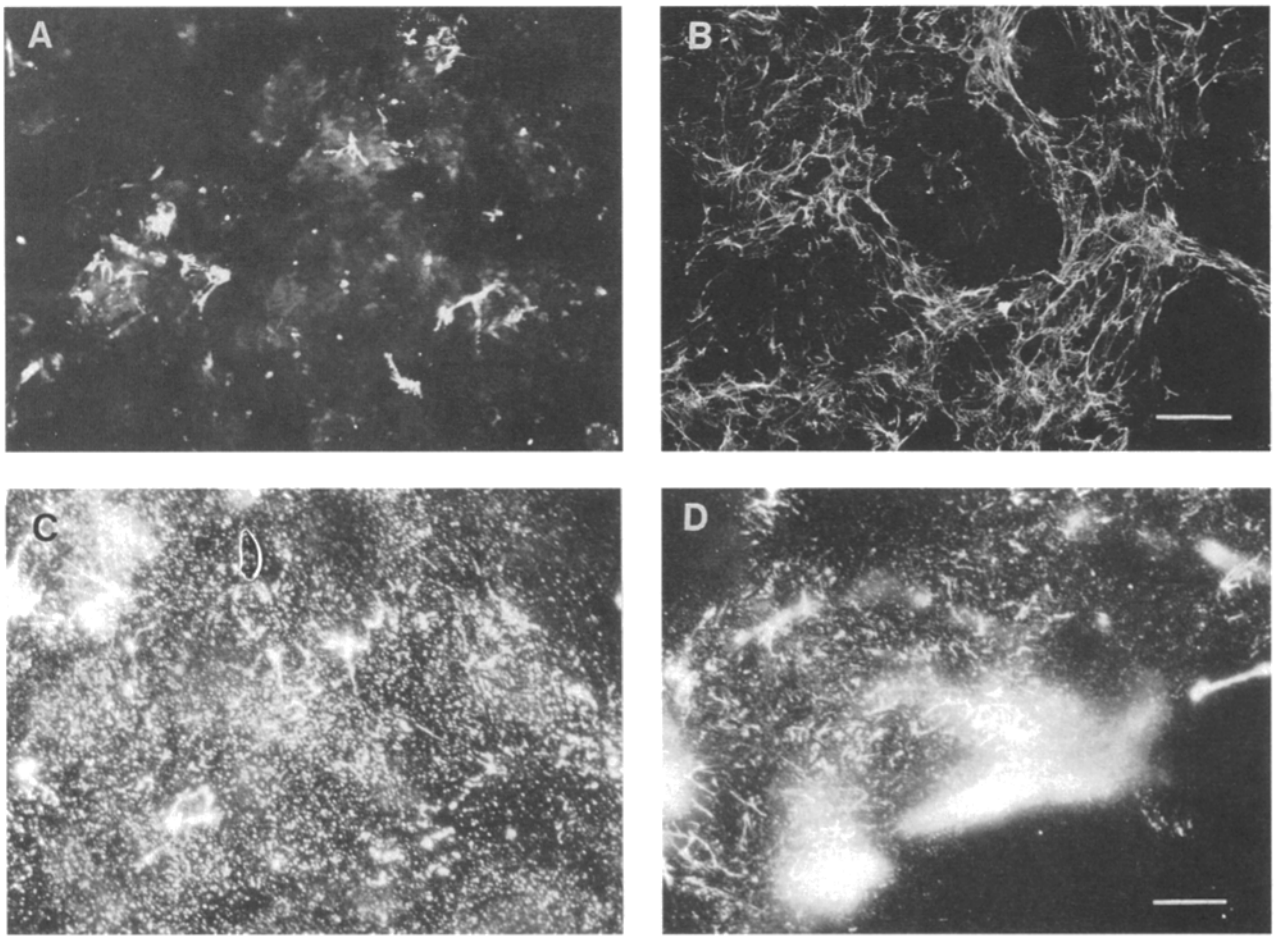

Figure 7. Immunofluorescent staining for FN in GD25 cells (*A* and *C*) and GD25-β1A cells (*B* and *D*). In *A* and *B* the focus is set at the apical side of the cell layer, and in *C* and *D* the focus is at the basal side. Cells were grown on coverslips for 5 d to ~75% confluency before fixation and staining. Bars, 50 μm (*A* and *B*) and 10 μm (*C* and *D*).

pressed in GD25 cells. Attachment and spreading of these cells on LN-1 was strongly dependent on β1 integrins. The main LN-1 receptor in GD25-β1A cells was α6β1A as shown by essentially complete inhibition of attachment to LN-1 by β1 antibodies and staining for α6β1 in FC. None of the two cell lines attached to collagen type I, consistent with the fact that they did not express the collagen-binding integrins α1β1 or α2β1. Apparently, integrin α3β1A was not an active collagen receptor in these cells or was present in insufficient amount to allow stable adhesion to collagen. Surprisingly, the attachment to FN was almost as efficient for the β1-deficient GD25 cells as for the GD25-β1A cells. However, at low coating concentrations β1A integrins clearly enhanced the attachment to FN.

Immunostaining of cells spread on FN showed that GD25 cells adhered via integrin αvβ3. This is consistent with the results obtained with melanoma cells and human myoblasts (Charo et al., 1990; Gullberg et al., 1995). GD25-β1A cells also used primarily αvβ3 for adhesion to FN, and this was the only detectable integrin present in FC in GD25-β1A plated on FN. This indicates that the integrin αvβ3 can keep the classical FN receptor α5β1A from forming FC on FN substrata. Whether this effect is due to a high binding avidity of αvβ3 for FN, overrepresentation of αvβ3 on the cell surface compared to α5β1A, or is mediated via intracellular regulation is unclear. After addition of GRGDS peptide to the medium, the GD25 cells could no longer attach to FN. In contrast, the GD25-β1A cell attachment to FN was only partially inhibited at the peptide concentration used, the remaining attachment being dependent on β1 integrins. Interestingly, FC were still present and now contained α5β1A. These results are in accordance with the reported higher affinity of GRGDS for αvβ3 than for α5β1 (Koivunen et al., 1993; Pytela et al., 1985).

When the ability of the cells to assemble FN matrix was tested, the β1A-expressing cells deposited a dense FN network with integrin α5β1A concentrated to the ends of the FN fibrils. This result confirms earlier reports that α5β1 can promote FN matrix assembly. Surprisingly, the GD25 cells also assembled FN into a matrix, albeit in lower amounts than the GD25-β1A and mainly when the cells were growing densely or in more than one layer. In these cells αvβ3 was concentrated at the ends of FN fibrils in the same way as α5β1A in GD25-β1A. A similar, but considerably weaker, staining of αvβ3 was seen also in GD25-β1A. This indicates that integrin αvβ3 could promote FN polymerization but not as efficiently as α5β1A and that,

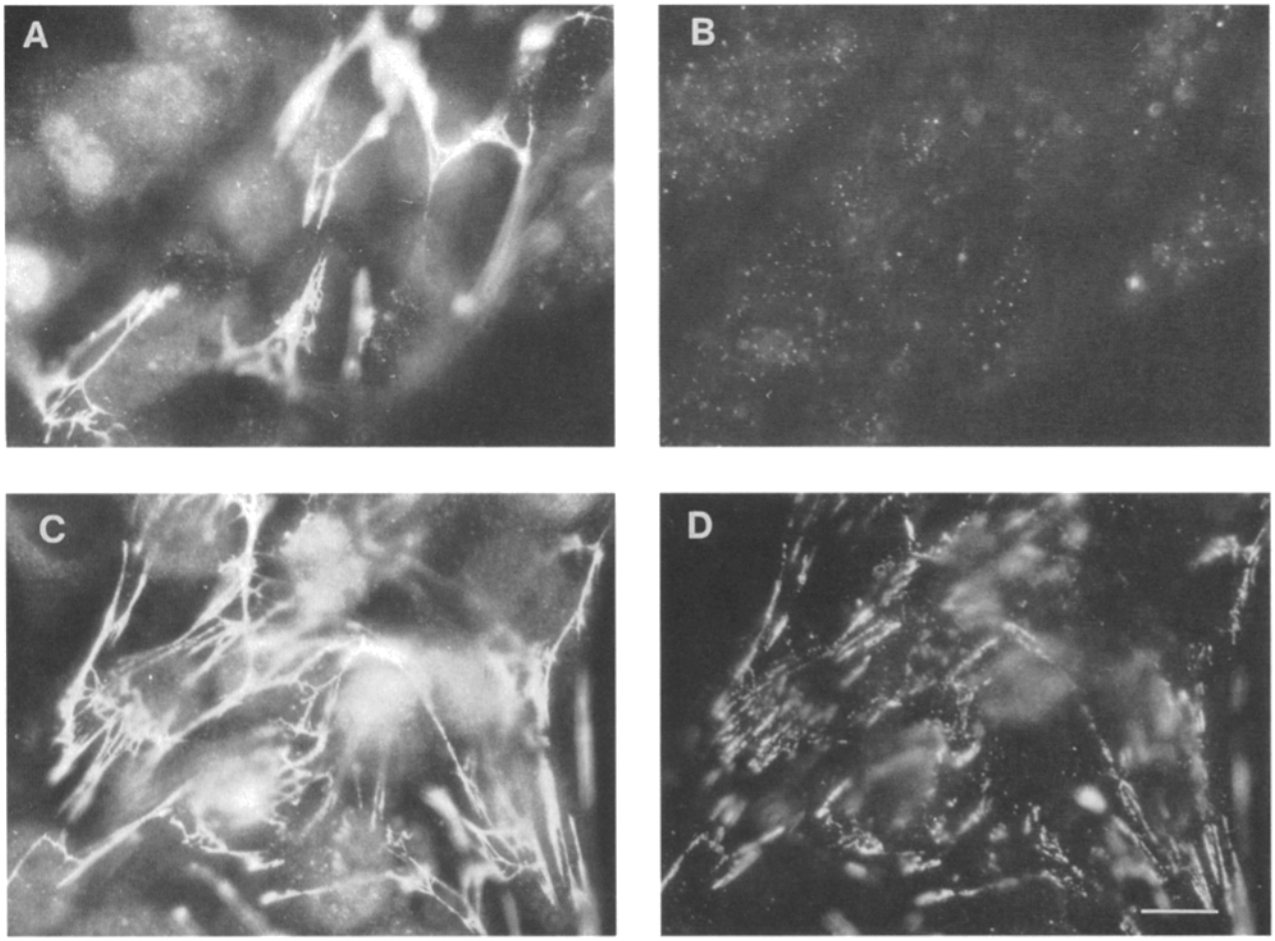

Figure 8. Immunofluorescent detection of FN matrix in contact with α5 integrin, showing double stainings for FN and α5 in GD25 cells (*A* and *B*) and GD25-β1A cells (*C* and *D*). Bar, 10 μm.

when present, α5β1A dominates over αvβ3 in the FN polymerization process. These findings could explain the presence of FN matrix in embryonal development of mice deficient in the integrin subunit α5 (Yang et al., 1993).

Integrin αvβ3 was initially reported to interact specifically with VN and several other RGD-containing proteins, but not with FN (Pytela et al., 1985). Later, αvβ3 from some cell types, e.g., melanoma cells (Charo et al., 1990), fetal skeletal muscle cells (Gullberg et al., 1995), and trophoblast cells (Schultz and Armanti, 1995), has been shown to contribute to the adhesion to FN. In other studies, αv was not found in FC of endothelial cells and fibroblasts on FN substrata (Dejana et al., 1988; Singer et al., 1988). The β subunits were not analyzed in the latter studies, but most likely β3 was expressed in those cells. The variable results regarding the FN-binding ability of αvβ3 may be due to splice variations of the proteins in different cells (two variants of β3 have been reported [van Kuppevelt et al., 1989]) or to differences in the regulation of the receptor. Regarding the cellular contacts with FN fibrils, αvβ3 has repeatedly been reported to be absent from these sites (Roman et al., 1989; Singer et al., 1988). In our study, the colocalization of αvβ3 with FN fibrils in GD25-β1A cells may have been overlooked, except for our awareness of the prominent staining in GD25 cells.

The property of αvβ3 to promote polymerization of FN was unexpected also from the fact that the related VN/FN receptor αvβ1 was found to lack this ability when expressed in CHO cells (Zhang et al., 1993). However, it cannot be excluded that this receptor may behave differently in another cellular host.

The observation that αvβ3 can exclude α5β1A from forming FC on FN while α5β1A, and not αvβ3, has the primary role in promoting FN polymerization is of particular interest. The mechanism behind this functional specialization is not clear, but some possible explanations exist. One would be that the binding of FN to the two integrins induces different conformational changes in the FN molecule. Interaction with α5β1 may induce an FN conformation that has a greater tendency to polymerize with other FN molecules, e.g., due to exposure of the self assembly site in FN. This would probably be of no importance in the formation of FC on surface-adsorbed FN. Alternatively, the avidity of the two integrins for FN may also affect their distribution. A third possibility is that the cytoplasmic proteins associated with integrin cytoplasmic domains in the two situations are different and are affected by which FN receptor is engaged. For example, clustering of integrins is required for binding of $pp125^{FAK}$ and formation of FC (Guan and Shalloway, 1992; Kornberg et al., 1992; Miya-

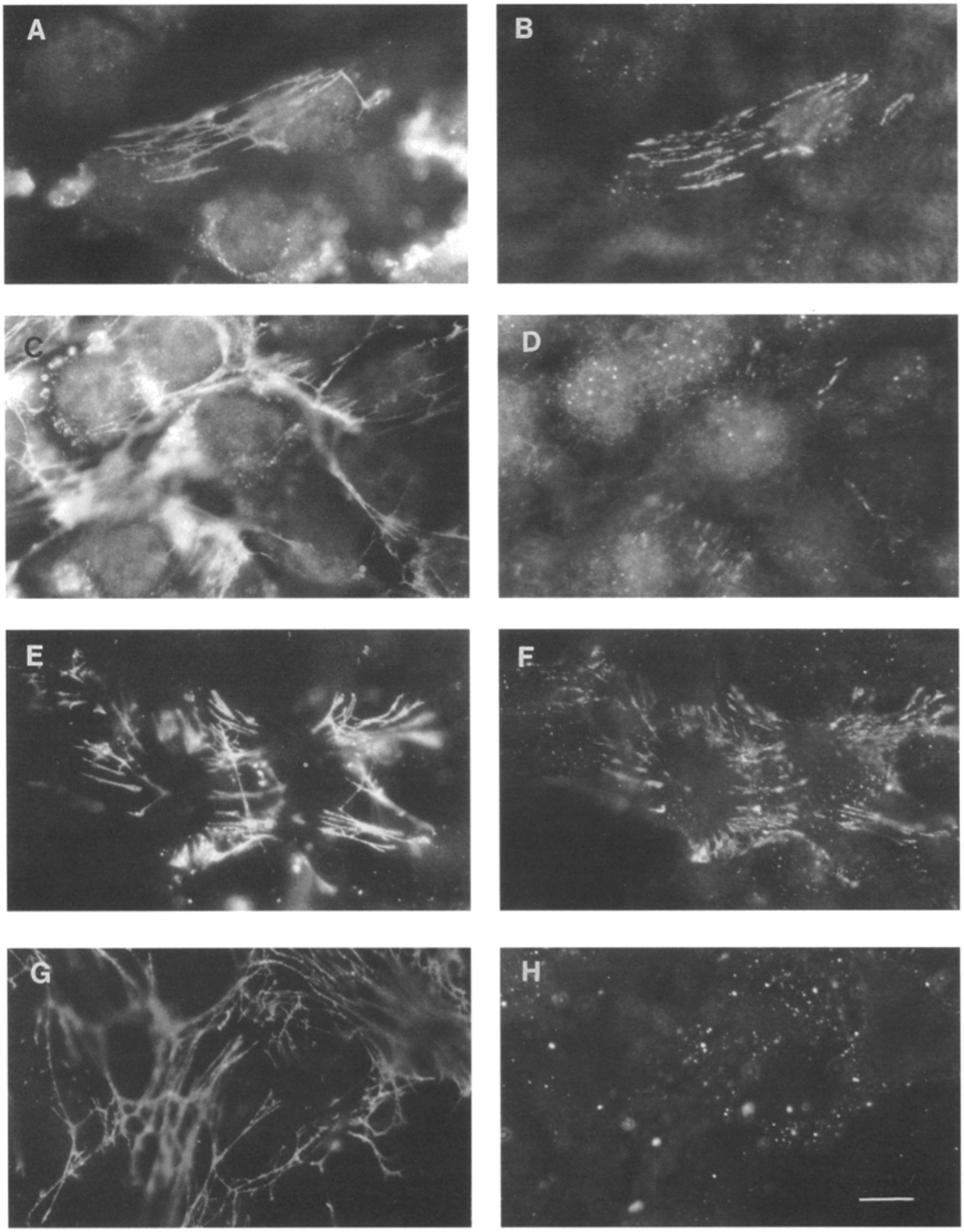

Figure 9. Detection of integrin αv and β3 subunits in contact with FN fibrils. Double stainings in GD25 (*A*, *B*, *E*, and *F*) and in GD25-β1A (*C*, *D*, *G*, and *H*) for FN (*A* and *C*) and αv (*B* and *D*), and for FN (*E* and *G*) and β3 (*F* and *H*). In *A–D*, the cells were permeabilized before the stainings. Bar, 10 μm.

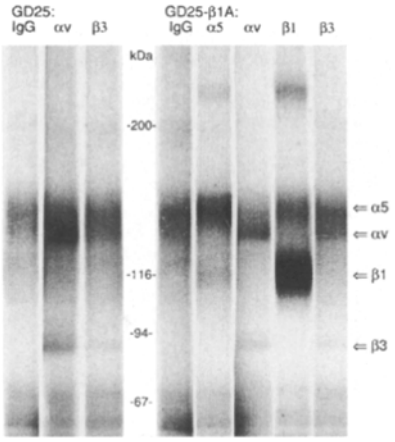

Figure 10. Immunoprecipitations of material eluted from sequential affinity chromatography of surface-iodinated cell lysate on WGA–Sepharose and Sepharose conjugated with 120-kD FN fragment. Immunoprecipitations with preimmune rabbit serum, α5, αv, β1, and β3 antibodies are shown. The positions of the immunoprecipitated subunits are marked in the figure. Note that a nonspecifically precipitated component migrates close to the position of the α subunits. The affinity chromatography was done as described in *Materials and Methods.*

moto et al., 1995), but it is not known if this is the case also for FN fibril assembly. In this context, it is interesting that pp125^{FAK} has been reported to be absent from membrane sites that contact FN fibrils (Katoh, K., Y. Kano, M. Masuda, and K. Fujiwara. 1994. *Mol. Biol. Cell.* 5 (Suppl.) 48a).

The presence on most cells of a large number of integrins, often with overlapping ligand specificities, makes it difficult to investigate the specific functions of these receptors. Generation of cells with defined expression of integrins is one way to overcome these problems. The β1-deficient cell line GD25, as well as other cells derived from the embryonic stem cell line G201, should be useful for further studies of the role of the αv integrin family in the formation of an FN matrix and other postattachment events. For example, it will be important to investigate if the ability of αvβ3 to support FN matrix assembly is general or limited to certain cell types. Similarly, the β1A-expressing cell line described in the present study will make it possible to define the interactions and functions of this splice variant of β1 in the absence of any influence of other forms of the protein.

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References

Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* 107:303–314.

Aumailley, M., K. Mann, H. von der Mark, and R. Timpl. 1989. Cell attachment properties of collagen type VI and Arg-Gly-Asp dependent binding to its α2(VI) and α3(VI) chains. *Exp. Cell Res.* 181:463–474.

Balzac, F., A. M. Belkin, V. E. Koteliansky, Y. V. Balabanov, F. Altruda, L. Silengo, and G. Tarone. 1993. Expression and functional analysis of a cytoplasmic domain variant of the β1 integrin subunit. *J. Cell Biol.* 121:171–178.

Balzac, F., S. F. Retta, A. Albini, A. Melchiorri, V. E. Koteliansky, M. Geuna, L. Silengo, and G. Tarone. 1994. Expression of β1B integrin isoform in CHO cells results in a dominant negative effect on cell adhesion and motility. *J. Cell Biol.* 127:557–565.

Bottger, B. A., U. Hedin, S. Johansson, and J. Thyberg. 1989. Integrin-type fibronectin receptors of rat arterial smooth muscle cells: isolation, partial characterization and role in cytoskeletal organisation and control of differentiated properties. *Differentiation.* 41:158–167.

Burridge, K., G. Nuckolls, C. Otey, F. Pavalko, K. Simon, and C. Turner. 1990. Actin-membrane interaction in focal adhesions. *Cell Differ. Dev.* 32:337–342.

Busk, M., R. Pytela, and D. Sheppard. 1992. Characterization of the integrin αvβ6 as a fibronectin-binding protein. *J. Biol. Chem.* 267:5790–5796.

Charo, I. F., L. Nannizzi, J. W. Smith, and D. A. Cheresh. 1990. The vitronectin receptor αvβ3 binds fibronectin and acts in concert with α5β1 in promoting cellular attachment and spreading on fibronectin. *J. Cell Biol.* 111:2795–2800.

Damsky, C. H., and Z. Werb. 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol.* 4:772–781.

Dejana, E., 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 adhesion receptors in cultured human endothelial cells. *J. Cell Biol.* 107:1215–1223.

Dzamba, B. J., H. Bultmann, S. K. Akiyama, and D. M. Peters. 1994. Substrate-specific binding of the amino terminus of fibronectin to an integrin complex in focal adhesions. *J. Biol. Chem.* 269:19646–19652.

Fässler, R., M. Pfaff, J. Murphy, A. A. Noegel, S. Johansson, R. Timpl, and R. Albrecht. 1995. Lack of β1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. *J. Cell Biol.* 128:979–988.

Forsberg, E., B. Ek, A. Engström, and S. Johansson. 1994. Purification and characterization of integrin α9β1. *Exp. Cell Res.* 213:183–190.

Giancotti, F. G., and E. Ruoslahti. 1990. Elevated levels of the α5β1 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell.* 60:849–859.

Guan, J. L., and D. Shalloway. 1992. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature (Lond.).* 358:690–692.

Gullberg, D., G. Sjöberg, T. Velling, and T. Sejersen. 1995. Analysis of fibronectin and vitronectin receptors on human skeletal muscle cells upon differentiation. *Exp. Cell Res.* 220:112–123.

Hedin, U., B. A. Bottger, E. Forsberg, S. Johansson, and J. Thyberg. 1988. Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. *J. Cell Biol.* 107:307–319.

Heino, J. 1993. Integrin-type extracellular matrix receptors in cancer and inflammation. *Ann. Med.* 25:335–342.

Holers, V. M., T. G. Ruff, D. L. Parks, J. A. McDonald, L. L. Ballard, and E. J. Brown. 1989. Molecular cloning of a murine fibronectin receptor and its expression during inflammation. Expression of VLA-5 is increased in activated peritoneal macrophages in a manner discordant from major histocompatibility complex class II. *J. Exp. Med.* 169:1589–1605.

Hynes, R. O., editor. 1990. Fibronectins. Springer-Verlag, New York. 554 pp.

Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11–25.

Jaken, S., K. Leach, and T. Klauck. 1989. Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. *J. Cell Biol.* 109:697–704.

Johansson, S., and M. Höök. 1984. Substrate adhesion of rat hepatocytes: on the mechanism of attachment to fibronectin. *J. Cell Biol.* 98:810–817.

Koivunen, E., D. A. Gay, and E. Ruoslahti. 1993. Selection of peptides binding to the α5β1 integrin from phage display library. *J. Biol. Chem.* 268:20205–20210.

Kornberg, L., H. S. Earp, J. T. Parsons, M. Schaller, and R. L. Juliano. 1992. Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J. Biol. Chem.* 267:23439–23442.

Languino, L. R., and E. Ruoslahti. 1992. An alternative form of the integrin β1 subunit with a variant cytoplasmic domain. *J. Biol. Chem.* 267:7116–7120.

Miyamoto, S., S. K. Akiyama, and K. M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science (Wash. DC).* 267:883–885.

Moon, K. Y., K. S. Shin, W. K. Song, C. H. Chung, D. B. Ha, and M. S. Kang. 1994. A candidate molecule for the matrix assembly receptor to the N-terminal 29-kDa fragment of fibronectin in chick myoblasts. *J. Biol. Chem.* 269: 7651–7657.

Morla, A., Z. Zhang, and E. Ruoslahti. 1994. Superfibronectin is a functionally distinct form of fibronectin. *Nature (Lond.)* 367:193–196.

Mosher, D. F., J. Sottile, C. Wu, and J. A. McDonald. 1992. Assembly of extracellular matrix. *Curr. Opin. Cell Biol.* 4:810–818.

Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. A 125/115 kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. *Proc. Natl. Acad.*

Sci. USA. 82:5766–5770.

Roman, J., R. M. LaChance, T. J. Broekelmann, C. J. Kennedy, E. A. Wayner, W. G. Carter, and J. A. McDonald. 1989. The fibronectin receptor is organized by extracellular matrix fibronectin: implications for oncogenic transformation and for cell recognition of fibronectin matrices. *J. Cell Biol.* 108: 2529–2543.

Schlaepfer, D. D., S. K. Hanks, T. Hunter, and P. van der Geer. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature (Lond.).* 372:786–791.

Schultz, J. F., and D. R. Armanti. 1995. β1- and β3-class integrins mediate fibronectin binding activity at the surface of developing mouse peri-implantation blastocysts. *J. Biol. Chem.* 270:11522–11531.

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

Smilenov, L., E. Forsberg, I. Zeligman, M. Sparrman, and S. Johansson. 1992. Separation of fibronectin from a plasma gelatinase using immobilized metal affinity chromatography. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 302:227–230.

Stephens, L. E., J. E. Sonne, M. L. Fitzgerald, and C. H. Damsky. 1993. Targeted deletion of β1 integrins in F9 embryonal carcinoma cells affects morphological differentiation but not tissue-specific gene expression. *J. Cell Biol.* 123:1607–1620.

van Kuppevelt, T., L. R. Languino, J. O. Gailit, S. Suzuki, and E. Ruoslahti. 1989. An alternative cytoplasmic domain of the integrin β3 subunit. *Proc. Natl. Acad. Sci. USA.* 86:5415–5418.

Woods, A., and J. R. Couchman. 1992. Protein kinase C involvement in focal adhesion formation. *J. Cell Sci.* 101:277–290.

Woods, A., J. R. Couchman, S. Johansson, and M. Höök. 1986. Adhesion and cytoskeletal organisation of fibroblasts in response to fibronectin fragments. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:665–670.

Woods, A., S. Johansson, and M. Höök. 1988. Fibronectin fibril formation involves cell interactions with two fibronectin domains. *Exp. Cell Res.* 177: 272–283.

Wu, C., J. S. Bauer, R. L. Juliano, and J. A. McDonald. 1993. The α5β1 integrin fibronectin receptor, but not the α5 cytoplasmic domain, functions in an early and essential step in fibronectin matrix assembly. *J. Biol. Chem.* 268: 21883–21888.

Wu, C., A. J. Fields, B. A. E. Kapteijn, and J. A. McDonald. 1995. The role of α4β1 integrin in cell motility and fibronectin assembly. *J. Cell Sci.* 108:821–829.

Yang, J. T., H. Rayburn, and R. O. Hynes. 1993. Embryonic mesodermal defects in α5 integrin-deficient mice. *Development (Camb.).* 119:1093–1105.

Zhang, Z., A. O. Morla, K. Vuori, J. S. Bauer, R. L. Juliano, and E. Ruoslahti. 1993. The αvβ1 integrin functions as a fibronectin receptor but does not support fibronectin matrix assembly and cell migration on fibronectin. *J. Cell Biol.* 122:235–242.