The Fibronectin Receptor on Mammalian Erythroid Precursor Cells: Characterization and Developmental Regulation

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Abstract. The plasma membrane of murine erythroleukemia (MEL) cells contains a 140-kD protein that binds specifically to fibronectin. A ^{125}I-labeled 140-kD protein from surface-labeled uninduced MEL cells was specifically bound by an affinity matrix that contained the 115-kD cell binding fragment of fibronectin, and specifically eluted by a synthetic peptide that has cell attachment-promoting activity. The loss of this protein during erythroid differentiation was correlated with loss of cellular adhesion to fibronectin. Both MEL cells and reticulocytes attached to the same site on fibronectin as do fibroblasts since adhesion of erythroid cells to fibronectin was specifically blocked by a monoclonal antibody directed against the cell-binding fragment of fibronectin and by a synthetic peptide containing the Arg-Gly-Asp-Ser sequence found in the cell-binding fragment of fibronectin. Erythroid cells attached specifically to surfaces coated either with the 115-kD cell-binding fragment of fibronectin or with the synthetic peptide-albumin complex. Thus, the erythroid 140-kD protein exhibits several properties in common with those described for the fibronectin receptor of fibroblasts. We propose that loss or modification of this protein at the cell surface is responsible for the loss of cellular adhesion to fibronectin during erythroid differentiation.

During erythropoiesis, plasma membrane proteins of the nucleated erythroblasts are replaced by membrane proteins of the mature erythrocyte. In mammals, the synthesis of erythrocyte membrane proteins spectrin, the anion-exchange protein Band III, and glycophorin is completed well before the non-nucleated reticulocytes are released from the bone marrow into the circulation (4, 9, 10, 17). Erythroid differentiation also involves elimination of glycoproteins characteristic of erythroblasts, such as the transferrin receptor (24). Recently we showed that uninduced murine erythroleukemia (MEL)[1] cells, reticulocytes from anemic rabbits, and erythrocytes from splenectomized mice attach tightly and specifically to immobilized fibronectin (25, 26). Adhesion to fibronectin is lost upon dimethyl sulfoxide-induced erythroid differentiation of MEL cells in vitro, and upon maturation of reticulocytes to erythrocytes in vivo.

Fibronectin is a disulfide-linked dimeric glycoprotein that is associated with extracellular matrices of many tissues, and a soluble form is found in the plasma. It has been implicated in numerous biological processes including cell adhesion (13), cellular differentiation (32, 42), and morphogenetic events (2, 3). Each of the similar but not identical 220-kD subunits of fibronectin contains several protease-resistant domains that represent functional binding sites for collagen (gelatin), fibrin, heparin, and cell surface receptors involved in the adhesion of cells to fibronectin (16, 45). Adhesion of fibroblasts to fibronectin involves the recognition of a tetrapeptide, Arg-Gly-Asp-Ser, which is found within the 115-kD cell-binding domain (28, 46).

Several types of plasma membrane components have been proposed to serve the function of fibronectin receptor (reviewed in reference 47). Photoaffinity cross-linking experiments reveal that chondroitin sulfate proteoglycans (27) and a 47-kD glycoprotein (1) are associated with fibronectin at the cell surface. Membrane glycolipids are also implicated in cell-fibronectin interactions. Sialic acid-rich gangliosides inhibit cell attachment (18, 48). Several investigators have shown that antibodies that interfere with cell adhesion recognize cell surface glycoproteins in the 140-kD range (reviewed in reference 6). Recently, the cell-binding domain of fibronectin was used as an affinity matrix to identify putative receptors in human MG-63 osteosarcoma cells and normal rat kidney (NRK) cells (33). Liposomes containing a 140-kD glycoprotein isolated from these cells attach specifically to fibronectin.

Fibronectin is a major component of the extracellular matrix in the bone marrow (43, 49). We postulated that some of the membrane components that mediate adhesion of erythrocyte precursors to fibronectin are lost in the bone marrow, whereas the rest are lost in the spleen as the reticulocyte

[1] *Abbreviations used in this paper:* MEL, murine erythroleukemia; NRK, normal rat kidney; PMSF, phenylmethylsulfonyl fluoride; WGA, wheat germ agglutinin.

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membrane is remodeled to erythrocyte membrane (26). But the region of the fibronectin molecule recognized by the erythrocyte precursors and the cell surface receptors that mediate such interactions remained unknown. We show here that MEL cells and rabbit reticulocytes attach to the same site on fibronectin as do fibroblasts. Affinity chromatography was used to identify a putative fibronectin receptor in uninduced MEL cells. This 140-kD protein was not detected in MEL cells after 4 d of differentiation. We propose that loss or modification of this protein at the cell surface is responsible for the loss of cellular adhesion to fibronectin during erythroid differentiation.

Materials and Methods

Materials

Cyanogen bromide-activated Sepharose 4B, α-chymotrypsin type VII, N_α-*p*-tosyl-L-lysine chloromethylketone (TLCK), phenylmethylsulfonyl fluoride (PMSF), and wheat germ agglutinin (WGA) were all purchased from Sigma Chemical Co., St. Louis, MO. Lactoperoxidase and octyl-β-D-glucopyranoside were purchased from Calbiochem-Behring Corp., San Diego, CA. Monoclonal antibodies against human plasma fibronectin were purchased from Mallinckrodt Inc., St. Louis, MO. Peptide I was synthesized by the Peptide Synthesizing Facility in the Department of Chemistry, University of California, San Diego. Peptide II was a gift from Dr. Samuel H. Barondes, University of California, San Diego. Peptides III, IV, and V were purchased from Sigma Chemical Co. The structures of these peptides are shown in Table II.

Preparation of Fibronectin Fragments and Peptide I-Albumin Complex

Fibronectin was purified from outdated human plasma by gelatin-Sepharose affinity chromatography according to Engvall and Ruoslahti (8). The 115-kD cell-binding and 45-kD gelatin-binding fragments were isolated from a chymotryptic digest of fibronectin according to Ruoslahti et al. (35). These fibronectin fragments were purified to >95% homogeneity by gel filtration on Sephadex G-200 and Sephacryl S-200 columns. Synthetic peptide I containing [^{14}C]alanine in its sequence was further purified by Sephadex G-15 gel filtration chromatography, and its sequence was confirmed by quantitative amino acid analyses. Peptide I was cross-linked to bovine albumin (peptide/albumin molar ratio, 50:1) with fresh glutaraldehyde as described (41). After the remaining free aldehyde groups were blocked with glycine, the peptide-albumin complex was isolated by gel filtration on a Sephadex G-25 column. The efficiency of cross-linking, calculated from the amount of radiolabeled peptide recovered with albumin, was ~62%.

Cell Culture and Induction of Anemia

MEL cells were grown in suspension culture in Dulbecco's modified Eagle's medium supplemented with 13% heat-inactivated fetal bovine serum. Induction of MEL cell differentiation by dimethyl sulfoxide was carried out as described (25). NRK fibroblasts were grown in Dulbecco's modified Eagle's medium. Reticulocytes were obtained from rabbits made anemic by phenylhydrazine injection (34). In brief, New Zealand White rabbits, 2.5–3.5 kg, were injected subcutaneously with freshly prepared 2% phenylhydrazine hydrochloride (Eastman Kodak Co., Rochester, NY) solution in PBS according to the following schedule: 2 ml on day 1, 2 ml on day 2, 1.5 ml on day 4, and 1 ml on day 6. The rabbit was bled on day 8.

For cell adhesion assays, MEL and NRK cells were metabolically labeled by incubation in Dulbecco's modified Eagle's medium containing 30 μCi/ml of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) for 30 h at 37°C in a tissue culture incubator. Reticulocytes were first washed three times with Hanks' balanced salt solution as described (26). Approximately 5×10^6 reticulocytes were incubated in 2 ml leucine-free Eagle's minimum essential medium containing 20 mM Hepes, 10 mM inosine, 5 mM adenosine, 10 mg/ml bovine albumin, and 30 μCi/ml [^{3}H]leucine (Amersham Corp.) for 1 h at 37°C in a tissue culture incubator.

Cell Attachment Assays

The cell adhesion assays were performed in 96-well polystyrene microtiter plates (Linbro) according to Ruoslahti et al. (36). In brief, wells were coated with fibronectin by incubating 0.1 ml of fibronectin at 5 μg/ml in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.5) for 1 h at 22–24°C. The remaining protein adsorption sites were saturated with albumin by incubation with 0.1 ml of a 10 mg/ml solution of fibronectin-free albumin for an additional 30 min. In some experiments, wells were coated with the peptide I-albumin complex instead of fibronectin, and the free sites were blocked with albumin as described above. To determine the effect of anti-fibronectin monoclonal antibodies on cell attachment, we preincubated fibronectin-coated wells with 0.1 ml of adhesion medium (Dulbecco's modified Eagle's medium without serum, but supplemented with 2 mg/ml albumin) containing monoclonal antibody for 6 h at 4°C. Approximately 10^4 [^{35}S]-methionine-labeled NRK and MEL cells and 3×10^4 [^{3}H]leucine-labeled reticulocytes suspended in 0.1 ml adhesion medium were added to each well. Cells were allowed to attach in the presence of antibody for 45 min at 37°C. The unattached cells were washed away and the trichloroacetic acid-precipitable radioactivity in the attached fraction was determined by counting in a scintillation spectrophotometer. The cell adhesion promoting activity of peptide I was measured by incubating cells in wells that had been coated with peptide I-albumin complex and glutaraldehyde-treated albumin (control) for 60 min at 37°C. After unattached cells were washed away, radioactivity in the attached fraction was determined as above.

Cell Surface Labeling

Uninduced and 4-d-induced MEL cells were washed four times with ice-cold Hank's balanced salt solution containing 20 mM Hepes buffer (wash buffer). Approximately 10^8 washed cells were suspended in 3 ml Hanks' balanced salt solution and iodinated according to Sefton et al. (38) in the presence of 3 mCi ^{125}I-sodium iodide (100 mCi/ml; Amersham Corp.) and 0.29 mg/ml of lactoperoxidase. After 30 min of incubation on ice, cells were washed three times with wash buffer containing 5 mM sodium iodide and 2 mM PMSF, and once with column buffer (10 mM sodium phosphate, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.5). Cells were lysed in 1 ml of column buffer containing 200 mM octylglucoside, 2 mM PMSF, and 1 mM N_α-*p*-tosyl-L-lysine chloromethylketone by vortexing for 30 sec and incubating on ice for 20 min. The cell extract was centrifuged at 13,000 *g* for 15 min at 4°C to remove insoluble cytoskeletal and nuclear components. In some experiments, a suspension of rabbit erythrocytes (10% hematocrit) in Hanks' balanced salt solution was also iodinated as above.

Isolation of Fibronectin Receptor

The 115-kD cell-binding fragment of fibronectin was immobilized on CNBr-activated Sepharose 4B beads according to the technical handbook of Pharmacia Fine Chemicals, Piscataway, NJ. About 4 mg of the 115-kD fragment of fibronectin was bound per ml Sepharose.

The detergent extract of labeled cells was subjected to 115-kD affinity chromatography according to Pytela et al. (33). In brief, 1 ml of cell extract was applied to 1 ml of affinity matrix that had been equilibrated with column buffer containing 50 mM octylglucoside, 1 mM PMSF, and 1 mM N_α-*p*-tosyl-L-lysine chloromethylketone (starting buffer). Material unbound to the column was recovered upon washing the column with 3 ml of starting buffer. Bound components were eluted by washing the column sequentially with 2 ml of starting buffer containing 1 mg/ml of peptide IV, 1 mg/ml of peptide I, and 6 M urea. 0.5-ml fractions were collected, and aliquots (25 μl) were mixed with an equal volume of Laemmli (20) gel sample buffer, boiled for 3 min, and subjected to SDS PAGE analysis. For autoradiography, the dried gel was exposed to Kodak XAR X-ray film with an intensifying screen for 1–2 d at −70°C.

Results

Inhibition of Cell Attachment by Anti-Fibronectin Monoclonal Antibody

Adhesion of fibroblasts to immobilized fibronectin can be blocked by antisera to fibronectin (14). In our initial studies we used a panel of monoclonal antibodies raised against human plasma fibronectin to identify domains of the fibronectin molecule involved in the adhesion of MEL cells and reticulocytes. These antibodies bind to different parts of the fibronectin molecule[2] (reference 7 and Fig. 1).

[2] McDonald, J. A. Personal communication.

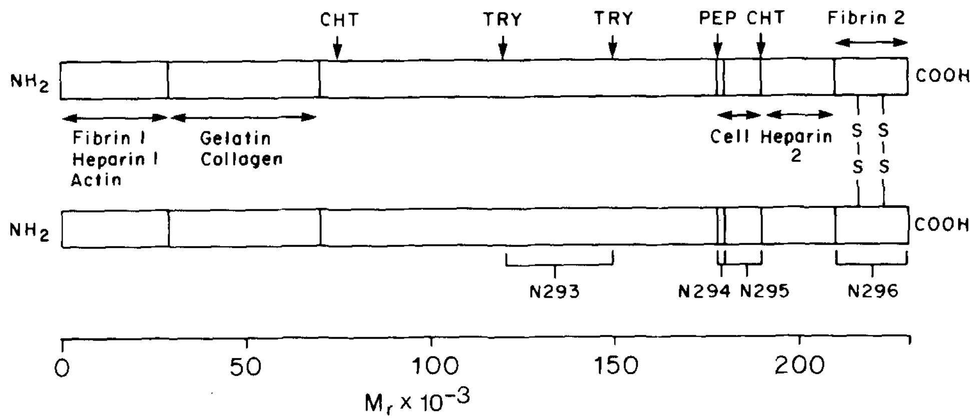

Figure 1. A model of a fibronectin dimer showing the relative positions of various functional domains and proteolytic cleavage sites. Also shown are the regions of fibronectin molecule recognized by various monoclonal antibodies (N293, N294, N295, and N296) used in this study; after Hynes and Yamada (16) and Pierschbacher et al. (30). *CHT*, chymotrypsin, *TRY*, trypsin, and *PEP*, pepsin.

Antibody N294 recognizes a pepsin cleavage site at the amino-terminus of the 11.5-kD cell adhesion fragment of fibronectin.[2] It inhibited adhesion of MEL cells and reticulocytes to fibronectin (Fig. 2, *B* and *C*). This antibody also inhibited the adhesion of NRK fibroblasts to fibronectin (Fig. 2*A*). The degree of inhibition was dependent on the cell type: 50% inhibition of MEL cell and reticulocyte adhesion occurred at 7 μg of antibody per ml, and of NRK cells at 3 μg/ml (Fig. 2). The inhibitory effect of the N294 antibody was specific, since antibodies N293 and N296 that recognize other parts of the fibronectin molecule did not perturb cell adhesion (Fig. 2). Antibody N295 binds to the 11.5-kD cell adhesive fragment of fibronectin and inhibits cell adhesion weakly.[2] In our assays, we could not detect any effects on cell attachment (Fig. 2).

Identification of MEL Cell and Reticulocyte-binding Domains of Fibronectin

Adhesion of fibroblasts to immobilized fibronection occurs primarily by interaction with the 115-kD chymotryptic fragment that contains the cell-binding domain of the fibronectin and that lacks other known binding activities of the molecule (28). This fragment and the 45-kD gelatin-binding fragment were purified from the chymotryptic digest of fibronectin. Both MEL cells and reticulocytes attached specifically to dishes coated with intact fibronectin and its 115-kD chymotryptic fragment, but not to dishes coated with bovine serum albumin or the gelatin-binding fragment of fibronectin (Table I). As expected, a similar specificity of attachment was observed with NRK cells (Table I). Thus, MEL cells, reticulocytes, and fibroblasts attach to the 115-kD cell binding domain of fibronectin.

Localization of the MEL Cell and Reticulocyte Attachment Site on Fibronectin

Adhesion of fibroblasts to fibronectin is mediated by the Arg-Gly-Asp-Ser sequence located at the carboxy-terminal portion of the 115-kD chymotryptic fragment of fibronectin (30). To determine whether this amino acid sequence is also involved in the adhesion of MEL cells and reticulocytes, we used two types of assays. First, we determined the capacity of synthetic peptides to inhibit attachment of cells to a substrate coated with intact fibronectin. Second, we determined directly the

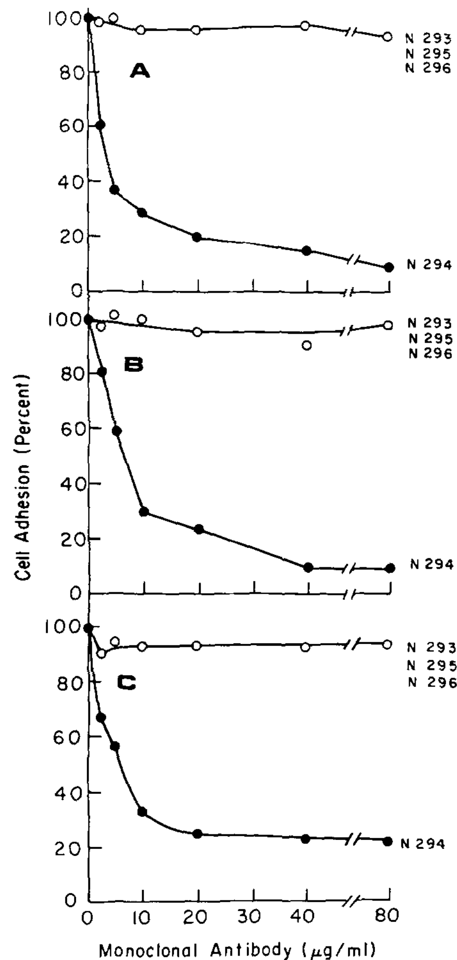

Figure 2. Effect of anti-fibronectin monoclonal antibodies on cell attachment. Adhesion of NRK (*A*), MEL (*B*), and reticulocytes (*C*) to fibronectin-coated wells in the presence of the indicated amounts of antibodies was measured as described in Materials and Methods. In albumin-coated wells, <10% of the plated cells attached either in the presence or absence of antibodies (not shown). Data shown are means of values derived from quadruplet assays done at each indicated concentration of the antibody. Cell attachment in the presence of each of the antibodies N293, N295, N296 (○), and N294 (●) was measured in parallel experiments. Maximum attachment of cells to fibronectin in the absence of antibodies was ~50% of the plated cells, and this is normalized to 100%.

ability of a synthetic peptide to promote cell adhesion by cross-linking the peptide to albumin with glutaraldehyde.

As shown in Table II, fibronectin-mediated adhesion of MEL cells and reticulocytes was inhibited by an octapeptide (peptide I) that contains the Arg-Gly-Asp-Ser sequence. Half-maximal inhibition of attachment occurred at ~70 μg/ml. A pentapeptide (peptide II) containing Arg-Gly-Asp-Ala sequence was also inhibitory. Adhesion of these cells to fibronectin was maximally (>90%) inhibited by both the peptides at 200 μg/ml (data not shown). As expected, adhesion of NRK cells to fibronectin was inhibited by both peptides (Table II). Three other nonrelated peptides (III, IV, and V; Table II) did not interfere with cell adhesion. Inhibition of cell attachment is probably not due to the cytotoxicity of the peptides, since >90% of the cells recovered after the assay were viable as determined by trypan blue exclusion.

Table I. Uninduced MEL Cells and Rabbit Reticulocytes Attach to Cell-binding Domain of Fibronectin

Additions	Attached (%)		
	NRK	MEL	Reticulocytes
BSA	5	5	3
Intact fibronectin	95	90	45
Cell binding domain	87	81	40
Gelatin binding domain	6	5	6

Falcon petri dishes (35-mm diam, Falcon Labware, Oxnard, CA) were coated with 2 ml bovine serum albumin (BSA) at 10 mg/ml, intact fibronectin at 6 μg/ml, the 115-kD cell-binding fragment of fibronectin at 10 μg/ml, and the 45-kD gelatin-binding fragment of fibronectin at 12 μg/ml as described elsewhere (26). Approximately 1×10^5 NRK and MEL cells and 1×10^6 red cells from an anemic rabbit (which contained 50% reticulocytes) in 2 ml adhesion medium were incubated at 37°C for 1 h. Unattached cells were washed away. The attached cells were detached by a 5-min incubation in 0.2% trypsin/0.02% EDTA at 37°C and counted in the Coulter Counter (Coulter Electronics, Hialeah, FL). Anemia was induced in rabbits by repeated bleeding; the blood contained ~50% reticulocytes and ~80% of the cells that attached were reticulocytes as judged by new methylene blue staining. Data presented are mean for three replicate dishes.

Table II. Effects of Synthetic Peptides on Attachment of Cells to Immobilized Fibronectin

Peptides	Amino acid sequence	Concentration for half-maximal inhibition (μg/ml)		
		NRK	MEL	Reticulocytes
I Octapeptide	Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ser.	52	60	70
II Pentapeptide	Arg-Gly-Asp-Ala-Asp.	60	80	85
III Fibrinopeptide-A	Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg.	>700	>700	>700
IV	Gly-Pro-Arg-Pro.	>700	>700	>700
V	Arg-Gly-Pro-Phe-Pro-Ile.	>700	>700	>700

Cell attachment assay was performed in polystyrene microtiter wells coated with 5 μg/ml plasma fibronectin. Approximately 10^4 [^{35}S]methionine-labeled NRK and MEL cells and 3×10^4 [^{3}H]leucine-labeled reticulocytes were incubated at 37°C in the presence of various concentrations of peptides. After 30 min, unattached cells were washed away and the radioactivity in the attached cell fraction was determined as described in experimental procedures. Indicated concentrations of peptides are means of values derived from two separate experiments. Maximum attachment in the absence of peptides was ~50% of the cells plated.

When cross-linked to albumin, peptide I also promoted adhesion of cells to plastic surface. MEL cells, reticulocytes, and NRK cells attached specifically to wells coated with peptide-albumin complex, but not to wells coated with glutaraldehyde-treated albumin (Fig. 3). Erythrocytes do not attach to fibronectin (26), nor do they attach to wells coated with peptide I-albumin complex or glutaraldehyde-treated albumin (Fig. 3*B*). Albumin coupled to a nonrelated tetrapeptide Gly-Pro-Arg-Pro did not promote cell adhesion (data not shown). Adhesion of cells to peptide I-albumin complex was dose dependent: half-maximal adhesion of NRK cells and MEL cells was observed at ~2 μg peptide, whereas half-maximal attachment of reticulocytes occurred at 10 μg (Fig. 3). These results indicate that most MEL cells and reticulocytes attach to the same Arg-Gly-Asp-Ser sequence of fibronectin implicated in the interaction with fibroblasts.

Detection and Developmental Regulation of a Cell Surface Fibronectin-binding Proteins

Since erythroid differentiation of MEL cells is accompanied by a dramatic loss of cellular adhesion to fibronectin, loss of adhesion probably results from the loss or modification of a cell surface fibronectin receptor (25). To identify and isolate a putative fibronectin receptor in these cells we used affinity chromatography, an approach described recently for the isolation of the fibronectin receptor from fibroblasts (33). Uninduced or 4-d dimethyl sulfoxide-induced cells were surface-labeled with ^{125}I by extracellular lactoperoxidase. An octylglucoside extract of these cells was then applied to a column of Sepharose to which was coupled the 115-kD cell attachment fragment of fibronectin. Specific elution of bound components was achieved by washing with peptide I.

A 140-kD protein labeled on uninduced MEL cells was bound to the column and specifically eluted by peptide I (Fig. 4*A*, lanes *8-11*). A minor 70-kD protein band was also detected. No significant elution of the 140-kD protein occurred when the column was washed with a nonrelated peptide that lacks cell attachment-promoting activity (Fig. 4*A*, lanes *4-7*). Low amounts of the 140-kD protein were detected in column washes obtained before elution with the peptides

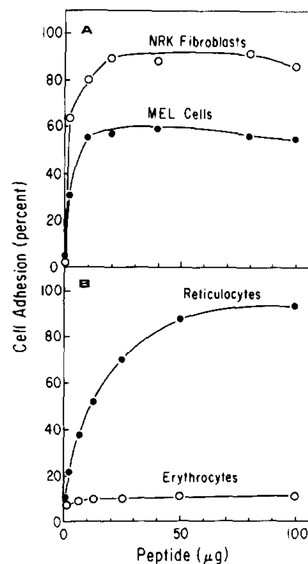

Figure 3. Attachment of cells to microtiter wells coated with synthetic peptide I-albumin complex (Gly-*Arg-Gly-Asp-Ser*-Pro-Ala-Ser). A suspension of 10^4 labeled NRK or MEL cells (*A*) and 3×10^4 rabbit reticulocytes (*B*) in 0.2 ml adhesion medium was incubated in each well for 1 h. Approximately 3×10^4 cell-surface iodinated rabbit erythrocytes (*B*) were also incubated in parallel. Wells coated with albumin or glutaraldehyde-treated albumin alone did not promote significant attachment of cells. Data shown are means of values obtained from two separate experiments. ○, NRK fibroblasts and rabbit erythrocytes; ●, MEL cells and rabbit reticulocytes.

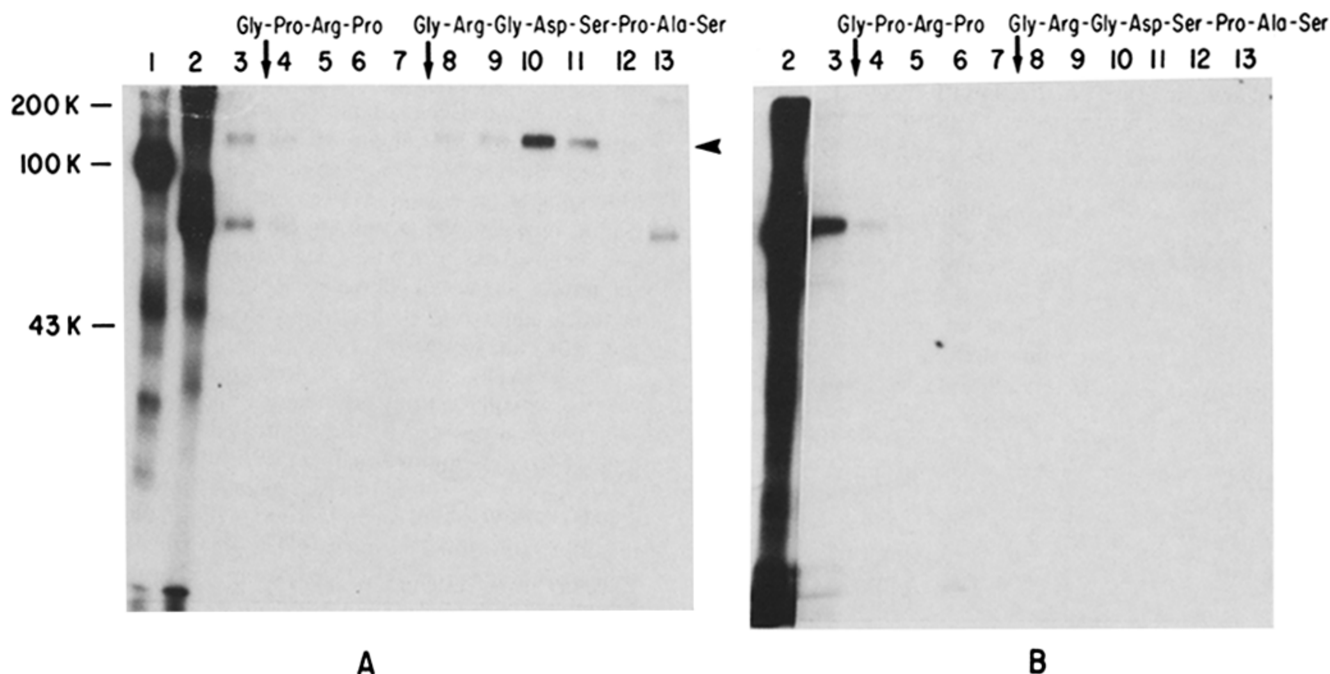

Figure 4. Autoradiogram of an SDS PAGE of components eluted from a Sepharose column to which the 115-kD cell binding fragment of fibronectin was coupled. Affinity chromatography of extracts of ^{125}I-labeled uninduced (*A*) and 4-d induced MEL cells (*B*) was done as described in Materials and Methods. An aliquot of each fraction was subjected to electrophoresis on 10% acrylamide gels under reducing conditions. Arrows indicate initiation of elution with synthetic peptides IV and I. Lane *1*, membranes from surface-labeled erythrocytes used as molecular weight marker; lane *2*, bulk of the unbound components recovered in the column flow-through; lane *3*, fraction obtained by washing with starting buffer just before elution with the peptides; lanes *4–7*, eluted with 1 mg/ml peptide IV; lanes *8–11*, eluted with 1 mg/ml peptide I; and lanes *12* and *13*, 6 M urea eluates. The position of the erythrocyte ankyrin (200 kD) band III protein (100 kD) and actin (43 kD) are shown as visualized by Coomassie Blue staining of the gel. The position of the 140-kD protein band is indicated by an arrowhead.

(Fig. 4*A*, lane *3*), as was observed with fibroblasts by Pytela et al. (33). The 140-kD protein was quantitatively eluted by peptide I, since little was eluted upon subsequent washing of the column with urea (Fig. 4, lanes *12–13*). In other similar experiments, proteins eluted from the column were visualized by silver staining of the gel (not shown). Among other minor, nonradioactive proteins, the 140-kD protein was clearly a major component in peptide I eluates. Since the elution of these minor components was not specifically affected by the peptide I, we conclude that these minor proteins were nonspecifically adsorbed by the column. No specific protein bands were eluted when bovine albumin and the 45-kD gelatin-binding fragment of fibronectin were used as affinity matrices (data not shown).

After 4 d of differentiation induced by dimethyl sulfoxide, ~80% of the MEL cells did not attach to fibronectin-coated dishes (not shown). When extracts of ^{125}I-labeled induced cells were chromatographed on a Sepharose column containing the cell-binding domain of fibronectin, no radioactivity bound that was specifically eluted by peptide I (Fig. 4*B*). In particular, the 140-kD protein was barely detected in any of the column eluates (Fig. 4, lanes *3–13*). Minute amounts of the 140-kD protein can be seen in Fig. 4*B* lane *9*, but only after longer exposures (not shown). Essentially similar results were obtained when detergent extracts of the total membrane fraction of induced cells was iodinated by chloramine-T and used as the starting material for affinity chromatography (data not shown). Thus, the apparent loss of the 140-kD protein in differentiated MEL cell cultures correlated with the loss of cellular adhesion to fibronectin.

Affinity-purified 140-kD protein from uninduced MEL cells was quantitatively bound by WGA-Sepharose and was eluted from this affinity column by *N*-acetylglucosamine (Fig. 5, lanes *1–3*). This was true even for the much smaller amount of protein present in the 4-d induced cells (Fig. 5, lane *7*). No binding to concanavalin A-Sepharose was observed for the 140-kD protein from either uninduced or induced cells (not shown). When the 140-kD protein was subjected to SDS PAGE under nonreducing conditions, a major band at 120 kD and a minor band at 140 kD were resolved (Fig. 6, lane *1*). Thus, the 140-kD protein is a glycoprotein and has properties similar to those described for the fibroblast fibronectin receptor (33).

Discussion

We have shown that the plasma membrane of uninduced MEL cells contains a 140-kD protein that binds specifically to fibronectin and that its loss during erythroid differentiation correlates with a loss of cellular adhesion to fibronectin. The 140-kD protein is similar in size to the putative fibroblast fibronectin receptor, and both erythroid cells and fibroblasts appear to bind to the same small region of fibronectin.

A small segment of fibronectin, called the cell attachment domain, mediates the interaction of fibronectin with fibroblasts (31), platelets (11), and human macrophages (44). Using several approaches we have shown that this segment also promotes adhesion of MEL cells and reticulocytes to plastic surfaces. The adhesion of erythroid cells to fibronectin-coated surfaces was specifically blocked by a monoclonal antibody

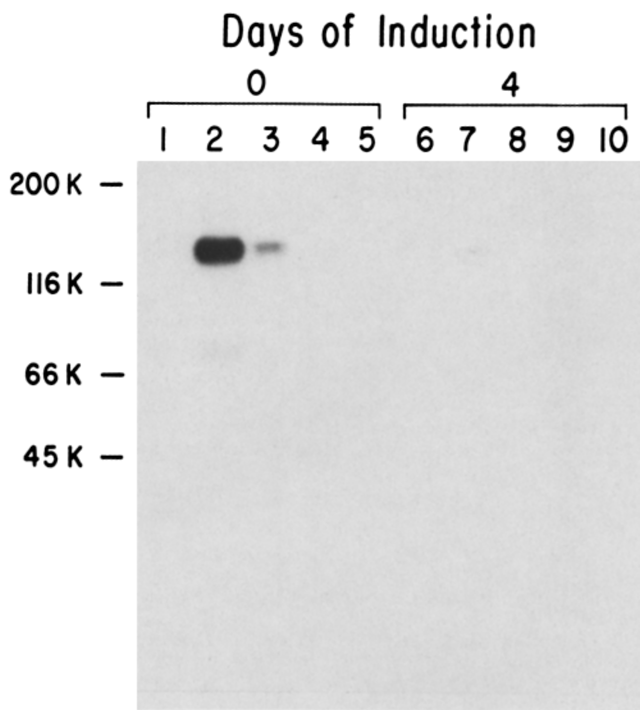

Figure 5. Autoradiogram of a SDS PAGE analysis of fractions eluted from the WGA-Sepharose 4B affinity columns. Synthetic peptide I eluates obtained from the experiment shown in Fig. 4, *A* (0-d induced cells) and *B* (4-d induced) were chromatographed on 0.5-ml (bed volume) WGA-Sepharose affinity column. Bound components were eluted with the column buffer containing 50 mM octylglucoside, 200 mM *N*-acetylglucosamine, 1 mM PMSF, and 1 mM N_α-*p*-tosyl-L-lysine chloromethylketone. 0.25-ml fractions were collected, and 25-μl aliquots of each fraction were subjected to electrophoresis on 10% acrylamide gels under reducing conditions. Lanes *1–3* and *6–8*, *N*-acetylglucosamine eluates; lanes *4, 5, 9*, and *10*, 6 M urea eluates. Molecular weight markers used here were myosin (200 kD), β-galactosidase (116 kD), bovine albumin (66 kD), and ovalbumin (45 kD).

directed against the cell-binding fragment of fibronectin. Erythroid cells attached specifically to dishes coated with pure 115-kD cell-binding fragment of fibronectin, and the attachment of erythroid cells to fibronectin-coated surfaces was specifically inhibited by a synthetic peptide containing the Arg-Gly-Asp-Ser sequence found in the cell-binding fragment of fibronectin. A synthetic peptide, peptide II, containing an Arg-Gly-Asp-Ala sequence was also inhibitory. This sequence exists in a variety of nonrelated proteins and also inhibits fibroblast attachment (29). Thus, we have confirmed that substitution of a serine residue by alanine in the Arg-Gly-Asp-Ser sequence does not significantly diminish its inhibitory effect on cell attachment. As with fibroblasts (46), inhibition of attachment by the peptides appeared to be competitive because the inhibitory effect of the peptide declined as the amount of fibronectin preadsorbed on the plastic surface was increased (data not shown).

MEL cells and reticulocytes attached specifically to dishes coated with peptide I cross-linked to albumin (Fig. 3). In contrast to promoting 90% attachment of NRK fibroblasts and reticulocytes, the immobilized peptide I promoted attachment of only 60% of the MEL cells. Intact fibronectin or its 115-kD cell-binding fragment promoted attachment of >80% of the MEL cells (Table I). Thus, in spite of a large relative molar excess of peptide I as compared with intact fibronectin adsorbed onto the dish, less MEL cell adhesion occurred. This difference in efficiency of cell adhesion might be due to the divalence of intact fibronectin or to the heterogeneity of the MEL cell culture. Alternatively, it may reflect the contribution of cell surface molecules that recognize additional sites on the 115-kD cell-binding fragment of fibronectin. Indeed, a variant of Arg-Gly-Asp-Ser sequence (Arg-Gly-Asp-Val) has been predicted to occur in some fibronectin subunits as a result of alternative RNA splicing (37, 39), and peptides containing such a sequence have cell adhesion–promoting activity (29).

The 140-kD glycoprotein present on the surface of MEL cells exhibited properties required of a fibronectin receptor. It was specifically bound by the affinity matrix that contained the 115-kD cell-binding fragment of fibronectin and specifically eluted by a peptide that has cell attachment–promoting activity. The 140-kD protein binds to WGA. This agrees with the observations of Pytela et al. (33), who showed that liposomes containing the 140-kD WGA-binding protein, purified from fibroblasts, attached specifically to fibronectin. Antibodies raised against WGA-binding components of baby hamster kidney fibroblasts have been shown to inhibit attachment of these cells to fibronectin, and a 48-kD WGA-binding protein has been implicated in the adhesion of these cells to fibronectin (23). However, no cell surface adhesive molecule of this size was found in our studies. Several investigators have shown that antibodies that interfere with the adhesion of various cell types to fibronectin recognize glycoproteins in the 140-kD range (5, 15, 19, 22, 40). The 140-kD protein of MEL cells migrates on SDS PAGE as 120- and 140-kD species under nonreducing conditions (Fig. 6). The reasons for the increased mobility of the nonreduced receptor proteins are not known.

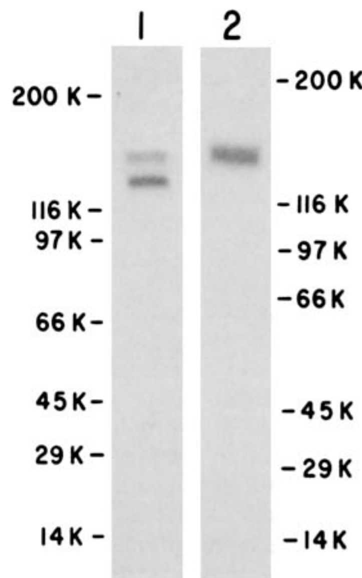

Figure 6. Autoradiogram shows SDS PAGE analysis of the 140-kD protein under nonreducing conditions. ^{125}I-labeled 140-kD proteins eluted from the WGA-Sepharose column (Fig. 5, lane *2*) and molecular weight markers were analyzed by SDS PAGE (8% acrylamide gels) under nonreducing (lane *1*) and reducing (lane *2*) conditions on two separate gels. Molecular weight markers visualized by Coomassie Blue staining of gels were myosin (200 kD), β-galactoside (100 kD), phosphorylase b (97 kD), bovine albumin (66 kD), ovalbumin (45 kD), carbonic anhydrase (29 kD), and lysozyme (14 kD).

It has been suggested that a compact conformation of the native protein stabilized by internal disulfide bonds may be responsible for its increased mobility under nonreducing conditions (33). Similar properties have been described for the fibroblast fibronectin receptor (5, 12, 33, 40). To what extent these receptor molecules are related to the erythroid 140-kD protein described here remains to be determined.

The amount of surface-labeled 140-kD protein that can be specifically bound to the fibronectin affinity column declined during MEL cell differentiation and was barely detectable after 4 d of differentiation. The loss of the 140-kD protein correlated with the loss of cellular adhesion to fibronectin during differentiation. Loss of this protein may occur by an endocytotic mechanism, since spectrin-free surface invaginations and intracellular vesicles have been found in differentiated erythroleukemia cells (21). It is also possible that the 140-kD protein remained on the cell surface but was modified so that it did not bind to fibronectin. If so, this modification also prevented its binding to WGA, since no labeled 140-kD protein was detectable when a detergent extract of surface-iodinated differentiated cells was directly subjected to WGA-affinity chromatography (not shown). Cell surface carbohydrate moieties have been implicated in the interaction of fibronectin with other cell types (18, 45). Thus, it is possible that alterations of carbohydrates bound to the 140-kD protein during differentiation might result in its failure to bind to the cell attachment fragment of fibronectin as well as to WGA.

The physiologic significance of the loss or modification of the 140-kD fibronectin-binding protein during erythroid differentiation remains to be established. The mechanisms involved in the release of the differentiated cells from the interstitial matrix of the bone marrow are complex. We previously suggested that some of the cell surface fibronectin-binding sites that mediate adhesion of erythrocyte precursors to fibronectin are lost in the bone marrow, whereas the rest are lost in the spleen or at some other sites during maturation of reticulocytes to erythrocytes (26). It will be important to characterize the cell surface components involved in the interaction of the erythrocyte precursors of the bone marrow with the fibronectin. These interactions should be studied in detail since the 140-kD protein can be purified.

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References

1. Aplin, J. D., R. C. Hughes, C. L. Jaffe, and N. Sharon. 1981. Reversible cross-linking of cellular components of adherent fibroblasts to fibronectin and lectin-coated substrata. *Exp. Cell Res.* 134:488–494.

2. 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 embryonic 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.

3. Bronner-Fraser, M. 1985. Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. *J. Cell Biol.* 101:610–617.

4. Chang, H., P. J. Langer, and H. F. Lodish. 1976. Asynchronous synthesis of erythrocyte membrane proteins. *Proc. Natl. Acad. Sci. USA.* 73:3206–3210.

5. Chen, W.-T., E. Hasegawa, T. Hasegawa, C. Weinstock, and K. M. Yamada. 1985. Development of cell surface linkage complexes in cultured fibroblasts. *J. Cell Biol.* 100:1103–1114.

6. Damsky, C. H., K. A. Knudsen, and C. A. Buck. 1984. Integral membrane glycoproteins in cell-cell and cell-substratum adhesion. *In* The Biology of Glycoproteins. R. J. Ivatt, editor. Plenum Publishing Corp., New York. 1–63.

7. Dixit, V. M., D. M. Haverstick, K. O. O'Rourke, S. W. Hennessy, T. J. Broekelmann, J. A. McDonald, G. A. Grant, S. A. Santoro, and W. A. Frazier. 1985. Inhibition of platelet aggregation by a monoclonal antibody against human fibronectin. *Proc. Natl. Acad. Sci. USA.* 82:3844–3848.

8. Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* 20:1–5.

9. Gahmberg, C. G., M. Jokinen, and L. C. Andersson. 1979. Expression of major red cell sialoglycoprotein, glycophorin A, in human leukemic cell line K562. *J. Biol. Chem.* 254:7442–7448.

10. Geiduschek, J. B., and S. J. Singer. 1979. Molecular changes in the membrane of mouse erythroid cells accompanying differentiation. *Cell.* 16:149–163.

11. Ginsberg, M., M. D. Pierschbacher, E. Ruoslahti, G. Marguerie, and E. Plow. 1985. Inhibition of fibronectin binding to platelets by proteolytic fragments and synthetic peptides which support fibroblast adhesion. *J. Biol. Chem.* 260:3931–3936.

12. Giancotti, F. G., G. Tarone, K. Knudsen, C. H. Damsky, and P. M. Comoglio. 1985. Cleavage of a 135Kd cell surface glycoprotein correlates with loss of fibroblast adhesion to fibronectin. *Exp. Cell Res.* 156:182–190.

13. Grinnell, F. 1978. Cellular adhesiveness and extracellular substrata. *Int. Rev. Cytol.* 53:65–144.

14. Grinnell, F., and D. Minter. 1978. Attachment and spreading of baby hamster kidney cells to collagen substrata: effects of cold-insoluble globulin. *Proc. Natl. Acad. Sci. USA.* 75:4408–4412.

15. Greve, J. M., and D. I. Gottlieb. 1982. Monoclonal antibodies which alter the morphology of cultured chick myogenic cells. *J. Cell Biochem.* 18:221–229.

16. Hynes, R. O., and K. M. Yamada. 1982. Fibronectin: multifunctional modular glycoproteins. *J. Cell Biol.* 95:369–377.

17. Koch, P. A., J. E. Gartell, F. H. Gardner, and J. R. Carter. 1975. Biogenesis of erythrocyte membrane proteins, *in vivo* studies in anemic rabbits. *Biochem. Biophys. Acta.* 389:162–176.

18. Kleinman, H. K., G. R. Martin, and P. H. Fishman. 1979. Ganglioside inhibition of fibronectin mediated cell adhesion to collagen. *Proc. Natl. Acad. Sci. USA.* 76:3367–3371.

19. Knudsen, K. A., P. E. Rao, C. H. Damsky, and C. A. Buck. 1981. Membrane glycoproteins involved in cell-substratum adhesion. *Proc. Natl. Acad. Sci. USA.* 78:6071–6075.

20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

21. Marshall, L. M., A. Thureson-Klein, and R. C. Hunt. 1984. Exclusion of erythrocyte-specific membrane proteins from clathrin-coated pits during differentiation of human erythroleukemia cells. *J. Cell Biol.* 98:2055–2063.

22. Neff, N. T., C. Lowrey, A. Tovar, C. H. Damsky, and C. A. Buck. 1982. A monoclonal antibody detaches embryonic skeletal muscle from extracellular matrices. *J. Biol. Chem.* 95:654–666.

23. Oppenheimer-Marks, N., and F. Grinnell. 1984. Calcium ions protect cell-substratum adhesion receptors against proteolysis. *Exp. Cell Res.* 152:467–475.

24. Pan, B.-T., and R. M. Johnstone. 1983. Fate of transferrin receptor during maturation of sheep reticulocytes *in vitro*: selective externalization of the receptor. *Cell.* 33:967–977.

25. Patel, V. P., and H. F. Lodish. 1984. Loss of adhesion of murine erythroleukemia cells to fibronectin during erythroid differentiation. *Science (Wash. DC).* 224:996–998.

26. Patel, V. P., A. Ciechanover, O. Platt, and H. F. Lodish. 1985. Mammalian reticulocytes lose adhesion to fibronectin during maturation to erythrocytes. *Proc. Natl. Acad. Sci. USA.* 82:440–444.

27. Perkins, M. E., T. H. Ji, and R. O. Hynes. 1979. Cross-linking of fibronectin to sulfated proteoglycans at the cell surface. *Cell.* 16:941–952.

28. Pierschbacher, M. D., and E. Ruoslahti. 1984. Cell attachment activity of fibronectin can be duplicted by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30–33.

29. Pierschbacher, M. D., and E. Ruoslahti. 1984. Variants of the cell recognition site of fibronectin that retain attachment promoting activity. *Proc. Natl. Acad. Sci. USA.* 81:5985–5988.

30. Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti. 1981. Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. *Cell.* 26:259–267.

31. Pierschbacher, M. D., E. Ruoslahti, J. Sundelin, P. Lind, and P. A. Peterson. 1982. The cell attachment domain of fibronectin: determination of primary structure. *J. Biol. Chem.* 257:9593–9597.

32. Podleski, T. R., I. Greenberg, J. Schlessinger, and K. M. Yamada. 1979. Fibronectin delays the fusion of L6 myoblasts. *Exp. Cell Res.* 122:317–326.

33. Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140kD cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* 40:191–198.

34. Rabinowitz, M., and J. M. Fisher. 1964. Characteristics of the inhibition of hemoglobin synthesis in rabbit reticulocytes by α-amino-β-chlorobutyric acid. *Biochim. Biophys. Acta.* 91:313–322.

35. Rouslahti, E., E. G. Hayman, E. Engvall, W. C. Cothran, and W. T. Butler. 1981. Alignment of biologically active domains in the fibronectin molecule. *J. Biol. Chem.* 256:7277–7281.

36. Ruoslahti, E., E. G. Hayman, M. D. Pierschbacher, and E. Engvall. 1982. Fibronectin: purification, immunochemical properties, and biological activities. *Methods Enzymol.* 82:803–831.

37. Schwarzbauer, J. E., J. W. Tamkun, I. R. Lemischka, and R. O. Hynes. 1983. Three different fibronectin mRNAs arise by alternative splicing within the coding region. *Cell.* 35:421–431.

38. Sefton, B. M., G. G. Wickups, and B. W. Burge. 1973. Enzymatic iodination of sindbis virus proteins. *J. Virol.* 11:730–735.

39. Tamkun, J. W., J. E. Schwarzbauer, and R. O. Hynes. 1984. A single rat fibronectin gene generates three different mRNAs by alternative splicing of a complex exon. *Proc. Natl. Acad. Sci. USA.* 81:5140–5144.

40. Tarone, G., G. Galetto, M. Prat, and P. M. Comoglio. 1982. Cell surface molecules and fibronectin-mediated cell adhesion: effects of proteolytic digestion of membrane proteins. *J. Cell Biol.* 94:179–186.

41. Walter, G., K.-H. Scheidtmann, A. Carbone, A. P. Laudano, and R. F. Doolittle. 1980. Antibodies specific for the carboxy and amino terminal regions of Simian virus 40 large tumor antigen. *Proc. Natl. Acad. Sci. USA.* 77:5197–5200.

42. Weiss, R. E., and A. H. Reddi. 1980. Synthesis and localization of fibronectin during collagenous matrix-mesenchymal cell interaction and differentiation of cartilage and bone in vivo. *Proc. Natl. Acad. Sci. USA.* 77:2074–2078.

43. Weiss, R. E., and A. H. Reddi. 1981. Appearance of fibronectin during the differentiation of cartilage, bone, and bone marrow. *J. Cell Biol.* 88:630–636.

44. Wright, S. D., and B. Christoph Meyer. 1985. Fibronectin receptor of human macrophages recognizes the sequence Arg-Gly-Asp-Ser. *J. Exp. Med.* 162:762–767.

45. Yamada, K. M. 1983. Cell surface interactions with extracellular materials. *Annu. Rev. Biochem.* 52:761–799.

46. Yamada, K. M., and D. W. Kennedy. 1984. Dualistic nature of adhesive protein function: fibronectin and its biologically active peptide fragments can autoinhibit fibronectin function. *J. Cell Biol.* 99:29–36.

47. Yamada, K. M., S. K. Akiyama, T. Hasegawa, E. Hasegawa, M. J. Humphries, D. W. Kennedy, K. Nagata, H. Urshiharda, K. Olden, and W.-T. Chen. 1985. Recent advances in research on fibronectin and other cell attachment proteins. *J. Cell. Biochem.* 28:79–97.

48. Yamada, K. M., D. W. Kennedy, G. R. Grotendorst, and T. Momoi. 1981. Glycolipids: receptors for fibronectin? *J. Cell. Physiol.* 109:343–351.

49. Zuckerman, K. S., and R. K. Rhodes. 1985. The hematopoietic extracellular matrix. *In* The Hematopoietic Stem Cell Physiology. Vol. 184. E. P. Cronkite, N. Dainiak, R. P. McCaffrey, J. Palek, and P. J. Quensberry, editors. Alan R. Liss Inc., New York. 257–266.