

Identification and Characterization of the T Lymphocyte Adhesion Receptor for an Alternative Cell Attachment Domain (CS-1) in Plasma Fibronectin

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Abstract. Using mAb technology (Wayner, E. A., W. G. Carter, R. Piotrowicz, and T. J. Kunicki. 1988. *J. Cell Biol.* 107:1881-1891), we have identified a new fibronectin receptor that is identical to the integrin receptor $\alpha 4\beta 1$. mAbs P3E3, P4C2, and P4G9 recognized epitopes on the $\alpha 4$ subunit and completely inhibited the adhesion of peripheral blood and cultured T lymphocytes to a 38-kD tryptic fragment of plasma fibronectin containing the carboxy-terminal Heparin II domain and part of the type III connecting segment (IIICS). The ligand in IIICS for $\alpha 4\beta 1$ was the CS-1 region previously defined as an adhesion site for melanoma cells. The functionally defined mAbs to $\alpha 4$ partially inhibited T lymphocyte adhesion to intact plasma fibronectin and had no effect on their attachment to an 80-kD tryptic fragment containing the RGD (arg-gly-asp) adhesion sequence. mAbs (PID6 and PIF8) to the previously described fibronectin receptor, $\alpha 5\beta 1$, completely inhibited T lymphocyte adhesion to the 80-kD

fragment but had no effect on their attachment to the 38-kD fragment or to CS-1. Both $\alpha 4\beta 1$ and $\alpha 5\beta 1$ localized to focal adhesions when fibroblasts that express these receptors were grown on fibronectin-coated surfaces. These findings demonstrated a specific interaction of both receptors with fibronectin at focal contacts.

In conclusion, these findings show clearly that cultured T lymphocytes use two independent receptors during attachment to fibronectin and that (a) $\alpha 5\beta 1$ is the receptor for the RGD containing cell adhesion domain, and (b) $\alpha 4\beta 1$ is the receptor for a carboxy-terminal cell adhesion region containing the Heparin II and IIICS domains. Furthermore, these data also show that T lymphocytes express a clear preference for a region of molecular heterogeneity in IIICS (CS-1) generated by alternative splicing of fibronectin pre-mRNA and that $\alpha 4\beta 1$ is the receptor for this adhesion site.

WE and others (reviewed by Hynes, 1987; Hemler, 1988) have described specific cell surface receptors for the extracellular matrix (ECM)¹ components collagen, fibronectin, and laminin. The functions of the extracellular matrix receptors (ECMRs I, II, and VI) we described were defined by affinity chromatography (Wayner and Carter, 1987; Staatz et al., 1989) and by preparing mAbs that specifically inhibited the interaction of cells with purified ligands (Wayner and Carter, 1987) or ECM (Wayner et al., 1988). The ECMRs are members of the integrin

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1. *Abbreviations used in this paper:* BLCL, B lymphocyte cell line; CTL, cytotoxic T lymphocytes; ECM, extracellular matrix; ECMR, extracellular matrix receptor; HBSA, heat-denatured BSA; LAK, lymphokine-activated killer cells; LGL, large granular lymphocyte leukemia; RD, human rhabdomyosarcoma cells; IIICS, type III connecting segment region.

(Hynes, 1987) family of cell adhesion molecules and possess unique α subunits complexed to the integrin $\beta 1$ subunit (Wayner and Carter, 1987; Wayner et al., 1988). ECMR VI is identical to the prototype fibronectin receptor (Pytela et al., 1985), $\alpha 5\beta 1$, platelet glycoprotein (gp) Ic/IIa, and VLA 5; ECMR II is identical to $\alpha 2\beta 1$, platelet gp Ia/IIa and VLA 2 (Hemler et al., 1987b); and ECMR I is identical to $\alpha 3\beta 1$ and VLA 3 (Kunicki et al., 1988; Takada et al., 1988; Wayner et al., 1988). Monoclonal antibodies to $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 5\beta 1$ (PIH5, PID6, and PIB5) inhibit fibroblast or platelet adhesion to collagen, fibronectin and laminin-coated surfaces (Kunicki et al., 1988; Wayner et al., 1988).

The $\beta 1$ integrins are differentially expressed in cultured cells and tissue, and demonstrate clear differences in activation dependent expression. For example, expression of $\alpha 5\beta 1$ in hematopoietic cells is restricted to subpopulations of thymocytes and peripheral blood lymphocytes, monocytes, acute

lymphocytic or myelogenous leukemias, activated T cells, migrating hemopoietic precursor cells, and some cultured T, B, or erythroleukemia cell lines (Bernardi et al., 1987; Cardarelli et al., 1988; Garcia-Pardo et al., 1989; Giancotti et al., 1986; Liao et al., 1987; Savagner et al., 1986; Wayner et al., 1988).

In experiments designed to examine the function of $\alpha 5\beta 1$ in lymphocytes, we observed that resting peripheral blood and cultured T lymphocytes (Molt 4 or Jurkat) expressed an affinity for fibronectin independent of the prototype fibronectin receptor, $\alpha 5\beta 1$. Although these cells attached to fibronectin-coated surfaces (unpublished), they expressed low or undetectable levels of $\alpha 5\beta 1$ recognized by our functionally defined mAb, PID6 (Wayner et al., 1988). Furthermore, T lymphocyte adhesion to fibronectin could only be partially inhibited by PID6- or RGD-containing peptides, suggesting the involvement of other receptors for fibronectin in the adhesion process. Alternatively, adhesion of other cells to fibronectin such as malignant or transformed fibroblasts and activated T lymphocytes (lymphokine-activated killer [LAK] cells) could be completely inhibited by PID6. This suggested that resting peripheral blood T lymphocytes and cultured T cell leukemias express multiple independent and functional fibronectin receptors.

Therefore, we identified an alternative fibronectin receptor by preparing mAbs that specifically inhibited the adhesion of T lymphocytes to fibronectin. This receptor was identical to the integrin receptor $\alpha 4\beta 1$ and mediated the attachment of peripheral blood lymphocytes, cultured T cell lines, and RD cells to plasma fibronectin. Furthermore, as we have shown (Garcia-Pardo, A., and O. C. Ferreira, manuscript submitted for publication) T lymphocytes expressed a clear preference for a 38-kD tryptic fragment of plasma fibronectin (Garcia-Pardo et al., 1987) containing the Heparin II domain and 67 amino acid residues of the type III connecting segment (IIICS) spanning the CS-1, CS-2, and CS-3 regions defined by Humphries et al. (1986, 1987). T lymphocytes attached only to CS-1 and mAbs to $\alpha 4\beta 1$ (P3E3, P4C2, P4G9) completely inhibited T lymphocyte adhesion to the 38-kD fragment and to CS-1. T lymphocytes also attached (with much lower affinity) to a site present in the heparin II domain and mAbs to $\alpha 4\beta 1$ also inhibited this interaction. The functionally defined mAbs to $\alpha 4\beta 1$ did not inhibit T lymphocyte adhesion to an 80-kD tryptic fragment of plasma fibronectin containing the RGD sequence, whereas antibodies to $\alpha 5\beta 1$ completely inhibited this interaction. These data show that T lymphocytes bear at least two receptors for fibronectin and clearly identify $\alpha 4\beta 1$ as the receptor for adhesion site(s) located in the carboxy-terminal region of plasma fibronectin.

Materials and Methods

Materials

PMSF, *N*-ethylmaleimide, leupeptin, diisopropyl fluorophosphate, 2-mercaptoethanol, BSA, Triton X-100, Protein A-agarose, soybean trypsin inhibitor, and V8 protease (from *Staphylococcus aureus*, strain V8, protease type XVII) were purchased from Sigma Chemical Co. (St. Louis, MO). Lactoperoxidase and glucose oxidase were from Calbiochem-Behring Corp. (La Jolla, CA). TPCK-trypsin was from Cooper Biomedicals (Malvern, PA). Fluorescein-conjugated goat anti-mouse IgG and IgM (heavy [H] and light [L] chains) or rhodamine-conjugated goat anti-rabbit IgG and IgM (H and L chains) were obtained from Tago, Inc. (Burlingame, CA). R-phycoerythrin-conjugated streptavidin was from Biomedica (Foster City, CA).

Rabbit anti-mouse IgG (H and L) antiserum was obtained from Cappel Laboratories (Malvern, PA). [^{51}Cr] Sodium chromate was from New England Nuclear (Boston, MA). [^{125}I] was from Amersham Corp. (Arlington Heights, IL). Human recombinant IL 2 was a generous gift from Dr. D. Urdal (Immunex Corp., Seattle, WA). Laminin was purchased from Collaborative Research, Inc. (Bedford, MA) and purified plasma fibronectin and collagen types I and III were prepared as previously described (Wayner and Carter, 1987; Wayner et al., 1988).

Cells and Cell Culture

RD (human rhabdomyosarcoma) and HT1080 (human fibrosarcoma) cells were obtained from the American Type Culture Collection (Rockville, MD). PBMC, platelet, and granulocyte populations from normal human donors were prepared as described (Kunicki et al., 1988; Wayner et al., 1988). Peripheral blood cells from patients with acute lymphocytic, large granular lymphocyte (LGL), or myelogenous leukemia were obtained from Dr. I. Bernstein and Dr. T. Loughran (Fred Hutchinson Cancer Research Center). Human LAK cells (500 U/ml IL 2) and the monoclonal HLA B7-specific human cytotoxic T lymphocyte (CTL) cell line, CIC4, were prepared according to standard protocols (Grimm et al., 1982; Glasebrook and Fitch, 1980; Brooks, 1983; Wayner and Brooks, 1984; Wayner and Brooks, 1985). The EBV-transformed B lymphocyte cell line (BLCL) ST-1, was derived from the donor spleen used in the production of the CIC4 CTL line. All other cell lines and cell culture conditions were as previously described (Wayner and Carter, 1987; Wayner et al., 1988).

Antibodies

A rabbit polyclonal antibody, AB33, prepared against the cytoplasmic domain of the fibronectin receptor, $\alpha 5\beta 1$, (Roman et al., 1988) was used to detect $\alpha 5\beta 1$ in focal adhesions. mAbs A1A5, against the common integrin (Hynes, 1987) $\beta 1$ subunit of the VLA family of receptors (Hemler, 1988) and B5-G10 to the VLA 4 α subunit (Hemler et al., 1987) were obtained from Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA). mAbs to the integrin receptors $\alpha 3\beta 1$ (PIB5), $\alpha 2\beta 1$ (PIH5), and $\alpha 5\beta 1$ (PID6) have been described. PIH5 and PID6 inhibit fibroblast and platelet adhesion to collagen and fibronectin-coated substrates, respectively (Wayner and Carter, 1987; Kunicki et al., 1988; Wayner et al., 1988).

mAbs to lymphocyte adhesion receptors were produced by the methods of Oi and Herzenberg (1980) and Taggart and Samloff (1983) as described (Wayner and Carter, 1987; Wayner et al., 1988). Splens from RBF/Dn mice immunized with 100 μl of packed T lymphocytes were removed and fused with NS-1/FOX-NY myeloma cells. Viable heterokaryons were selected in RPMI 1640 medium supplemented with adenine/aminopterin/thymidine (Taggart and Samloff, 1983). Hybridomas producing antibody directed to lymphocyte adhesion receptors were screened by specific inhibition of lymphocyte adhesion to fibronectin-coated surfaces and cloned by limiting dilution.

Inhibition of Cell Adhesion to Intact Fibronectin and Fibronectin Fragments

Antibodies that would alter cell adhesion to purified plasma fibronectin, tryptic fragments and CS peptides were identified as previously described (Wayner and Carter, 1987). Briefly, 48-well virgin styrene plates were coated with 5 $\mu\text{g}/\text{ml}$ human plasma fibronectin. The plates were blocked with PBS supplemented with 10 mg/ml heat-denatured BSA (HBSA). T lymphocyte or HT1080 cells were labeled with $\text{Na}_2^{51}\text{CrO}_4$ (50 $\mu\text{Ci}/\text{ml}$ for 2–4 h) and washed, and 5×10^4 HT1080 or cultured T cells or 5×10^5 PBL/well were incubated with hybridoma culture supernatants (1:2 dilution in PBS supplemented with 1 mg/ml HBSA) or control myeloma cell culture supernatant for 15 min at room temperature. The cells were allowed to adhere to the protein-coated surfaces in the presence of the hybridoma supernatants for 15–30 min (HT1080) or 2–4 h (lymphocytes) at 37°C. Nonadherent cells were removed by washing with PBS, and the adherent cells were dissolved in SDS/NaOH and bound ^{51}Cr counts per minute were quantitated in a gamma counter.

Immune Precipitation, Sequential Immune Precipitation, V8 Protease Peptide Mapping, and PAGE

Viable cells were surface labeled with [^{125}I] as described (Wayner and Carter, 1987) followed by extraction with 1% vol/vol Triton X-100 deter-

gent or 0.3% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent in 50 mM PBS, pH 7.2. In some cases, 1 mM CaCl_2 was added to the lysis buffer. 1 mM diisopropyl fluorophosphate, 1 mM PMSF, 1 mM *N*-ethylmaleimide, 1 $\mu\text{g/ml}$ leupeptin, and 1 $\mu\text{g/ml}$ soybean trypsin inhibitor were used as protease inhibitors. Immune precipitation and sequential immune precipitations were performed exactly as previously described. Peptide analysis followed the basic procedure of Cleveland et al. (1977) with modifications as described (Wayner and Carter, 1987). Polyacrylamide slab gels containing SDS (SDS-PAGE gels) were prepared following the basic stacking gel system of Laemmli (1970).

Preparation of Tryptic Fragments from Human Plasma Fibronectin and Synthesis of CS Peptides

Human plasma fibronectin was a generous gift from Dr. Horowitz and Dr. R. Schulman (New York Blood Center, New York). Fibronectin was digested with TPCK-trypsin for 90 min at 37°C, and the digest was fractionated by affinity and ion-exchange chromatography as previously described (Garcia-Pardo et al., 1987, 1989). Two overlapping peptides spanning the initial 48 residues of the type III connecting segment (IIICS) region of human fibronectin (CS-1 and CS-2) were synthesized and coupled to rabbit IgG as described (Humphries et al., 1986, 1987).

Fluorescence Analysis of Receptor Expression

Expression of ECMRs on cells in suspension was analyzed by one- or two-color flow cytometry on a dual laser cell sorter (EPICS 750; Coulter Electronics, Hialeah, FL). Positive fluorescence was determined on a three-decade log scale and fluorescence intensity (log FI) was expressed as mean channel number (0–255). Background fluorescence for a nonimmune mouse IgG negative control was determined for each cell population and subtracted. Adherent cells were trypsinized and allowed to recover for 15 min at 37°C in the presence of serum before use for flow cytometry. For one- or two-color fluorescence measurements, 10^6 cells in suspension were incubated for 30 min first with protein G-Sepharose-purified goat IgG (20 $\mu\text{g/ml}$) and then with first-stage antibodies at 4°C for 60 min, washed in HBSS containing 10 mg/ml HBSA and 0.02% sodium azide (HBSS/BSA/SA), and incubated with FITC-conjugated rabbit anti-mouse IgG for 60 min at 4°C in HBSS/BSA/SA. They were washed and fixed in cold 2% paraformaldehyde (prepared fresh) in PBS. For two-color fluorescence, the purified and biotinylated mAb was added to the FITC-stained and fixed cells to a final concentration of 1 $\mu\text{g/ml}$ in HBSS/BSA/SA and incubated at 4°C for 60 min. Prior fixation with 2% paraformaldehyde had little effect on expression of lymphocyte integrin receptors. The fixed cells were washed and incubated in 0.5 ml HBSS/BSA/SA containing phycoerythrin-conjugated streptavidin (Bionetics Laboratory Products, Charleston, SC) at 1/50 for 30 min at 4°C. Finally, the stained cells were washed and fixed again in 2% paraformaldehyde in PBS and held at 4°C in the dark for analysis on the flow cytometer.

Localization of Receptors in Focal Adhesions

Adherent cells were trypsinized, washed in RPMI 1640 supplemented with 1 mg/ml BSA plus 100 $\mu\text{g/ml}$ soybean trypsin inhibitor, and allowed to adhere to acid-washed and silanized glass cover slips coated with fibronectin, laminin, or collagen (20 $\mu\text{g/ml}$) in the absence of serum for 1–4 h as described (Carter, W. G., and E. A. Wayner, manuscript in preparation). At the end of the incubation, nonadherent cells were removed and adherent cells were fixed in 100 mM sodium cacodylate, 100 mM sucrose, 4.5 mM CaCl_2 , 2% formaldehyde for 20 min. They were permeabilized with 0.5% Triton X-100 for 5 min, washed, and blocked with 25% goat serum in PBS. The permeabilized cells were stained with antibodies to specific receptors (60 min at room temperature), washed, incubated with either FITC-conjugated goat anti-mouse or rhodamine-conjugated goat anti-rabbit IgG (45 min at room temperature), and washed again. The cover slips were inverted onto glass slides for fluorescence and interference reflexion microscopy as described (Izzard and Lochner, 1976).

Tissue Staining

The distribution of the integrin receptors in tissue was determined by fluorescence microscopy of cryostat sections. Cryostat sections (6 μm) were prepared from human skin, tonsil, or tumor samples embedded in OCT medium after snap freezing in isopentane/liquid nitrogen. All sections were fixed in 4% paraformaldehyde in PBS before incubation in primary antibodies and secondary fluorescent antibodies as described (Carter and Wayner,

1988). In control experiments, no fluorescence of rhodamine was detected using the fluorescein filters or vice versa.

Results

Identification of an Alternative Fibronectin Receptor

Cultured T lymphocytes (Molt 4), K562, RD (rhabdomyosarcoma), and HT1080 (fibrosarcoma) cells, and freshly derived PBL (not shown) adhered to fibronectin-coated surfaces (Fig. 1, *open bars*). However, Molt 4 and RD cells expressed low or undetectable levels of the prototype fibronectin receptor (integrin $\alpha 5\beta 1$) recognized by monoclonal antibody PID6 (Fig. 1, *striped bars*). Consistent with this, adhesion of Molt 4 and RD cells to fibronectin could not be completely inhibited by PID6 (Fig. 1, *solid bars*). Alternatively, adhesion of cells to fibronectin that expressed abundant $\alpha 5\beta 1$ (HT1080 and K562) could be effectively inhibited by PID6. Furthermore, the synthetic peptide RGDS did not completely inhibit T lymphocyte adhesion to plasma fibronectin (50–70% for Molt 4 or Jurkat cells vs. 80–90% for fibroblasts and 100% for K562-1 cells). Together, these data suggested that some cells, such as T lymphocytes, express fibronectin adhesion receptors other than $\alpha 5\beta 1$.

We attempted to identify other putative fibronectin receptors by preparing mAbs to cultured T lymphocytes and screening them for their ability to specifically inhibit lymphocyte but not fibroblast adhesion to fibronectin-coated surfaces. Using this protocol several mAbs (P4C2, P3E3, P4G9) were identified that inhibited cultured T lymphocyte but not HT1080 cell adhesion to fibronectin (Table I). Immune precipitation from Triton X-100 detergent lysates prepared with ^{125}I -surface-labeled PBL (not shown), Molt 4 or HT1080 (Fig. 2) cells showed that the inhibitory mAbs (data shown for P3E3) reacted with a single protein present in lymphocyte extracts that migrated at M_r 150,000 (p150) in the presence (not shown) or absence (Fig. 2) of reducing agent. Under these immune precipitation conditions p150 lacked an apparent α - β subunit structure and did not co-migrate with either the α or β subunit of the integrin receptors $\alpha 2\beta 1$ or $\alpha 3\beta 1$ (Fig. 2). The antigen immune precipitated from Triton X-100 detergent extracts prepared with chronically activated CD8+ LAK cells or CTL (not shown) contained, in addition to p150, relatively large quantities of two smaller proteins that migrated at M_r 80,000 and 70,000 in the presence (not shown) or absence of reducing agent. V8 protease peptide mapping revealed that p80 and p70 were proteolytic fragments of p150 (not shown). These lower molecular weight forms could be immune precipitated from chronically activated T cells even when detergent extracts were prepared in the presence of multiple protease inhibitors (legend to Fig. 2). p80 and p70 were virtually absent from extracts prepared with resting PBL, cultured T (Molt 4, Jurkat), or B cell leukemias and RD cells (not shown).

The biochemical characteristics of p150 suggested that it might be related to the VLA 4 antigen described by Hemler (Hemler et al., 1987a). This was confirmed by sequential immune precipitation (not shown) with a VLA 4-specific mAb, B5-G10. p150 was established as an α subunit of the integrin super family by its association with $\beta 1$ when immune precipitations were carried out after CHAPS detergent (0.3%) solubilization of ^{125}I -surface-labeled T lymphocytes in the

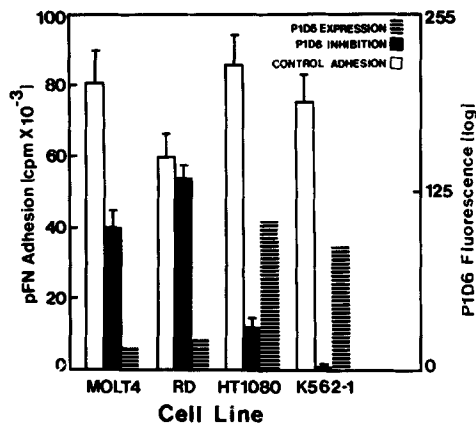


Figure 1. Adhesion of T lymphocytes (Molt 4), K562-1, RD, or HT1080 cells to plasma fibronectin, inhibition with PID6 mAb, and cell surface expression of $\alpha 5\beta 1$. ^{51}Cr -labeled cells (10^5 cells/ml) were incubated with PID6 mAb (50 $\mu\text{g/ml}$) or mouse IgG (50 $\mu\text{g/ml}$) for 60 min at 4°C and allowed to attach to fibronectin-coated (20 $\mu\text{g/ml}$) plastic surfaces in the presence of PID6 (solid bars) or mouse IgG (open bars) for 30 min (HT1080 or RD) or 4 h (Molt 4 or K562) at 37°C. Adhesion to plasma fibronectin (pFN) is expressed as ^{51}Cr bound to the plastic surfaces. Cell surface expression of $\alpha 5\beta 1$ was determined by flow cytometry by staining of cells in suspension with mAb PID6. Log PID6 fluorescence (striped bars) is expressed as mean channel number (0–255) above background.

presence of 1 mM Ca^{2+} (Fig. 3). Under these conditions $\alpha 4$ was precipitated as a heterodimer with $\beta 1$. The identity of $\beta 1$ was confirmed by V8 protease peptide mapping (not shown). The $\alpha 4\beta 1$ heterodimer immune precipitated from T lymphocytes with the inhibitory mAbs (P3E3, P4C2, and P4G9) was shown to be distinct from the prototype fibronectin receptor, $\alpha 5\beta 1$, immune precipitated with PID6 by three criteria. (a) The relative quantities of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ present in detergent extracts of T lymphocytes were distinct with higher levels of $\alpha 4\beta 1$ being present (Fig. 3). This was in agreement with the data we obtained using flow cytometry (Fig. 1). (b) In sequential immune precipitation experiments, mAbs to $\alpha 4\beta 1$

Table I. Specific Inhibition of Lymphocyte Adhesion to Plasma Fibronectin by mAbs P3E3, P4C2, and P4G9

Cells	Fibronectin adhesion				
	SP2	PID6 ($\alpha 5\beta 1$)	P3E3	P4C2	P4G9
	% of control				
PBL	100	43	38	10	52
Jurkat	100	22	33	12	48
Molt 4	100	18	12	8	39
HT1080	100	5	98	93	104

Cells were labeled with ^{51}Cr and allowed to adhere to plasma fibronectin-coated (20 $\mu\text{g/ml}$) plastic surfaces (5×10^5 for PBL or 10^5 for HT1080 or Molt 4 and Jurkat) for 30 min (HT1080 cells) or 2–4 h (Molt 4 or Jurkat) at 37°C in the presence of SP2 myeloma or hybridoma culture supernatants diluted 1:2 with fresh medium. Results are expressed as a percent of the ^{51}Cr counts per minute bound to the SP2-positive control. Data shown are from a single experiment. In general, the inhibition observed for the new inhibitory mAbs ranged from 50 to 80%, with P4C2 always being the most efficient inhibitor of lymphocyte adhesion to plasma fibronectin. The inhibition obtained for PID6 ranged from 10 to 70% for PBL and 50 to 80% for cultured T lymphocyte cell lines. This variability correlated with cell surface expression of $\alpha 5\beta 1$.

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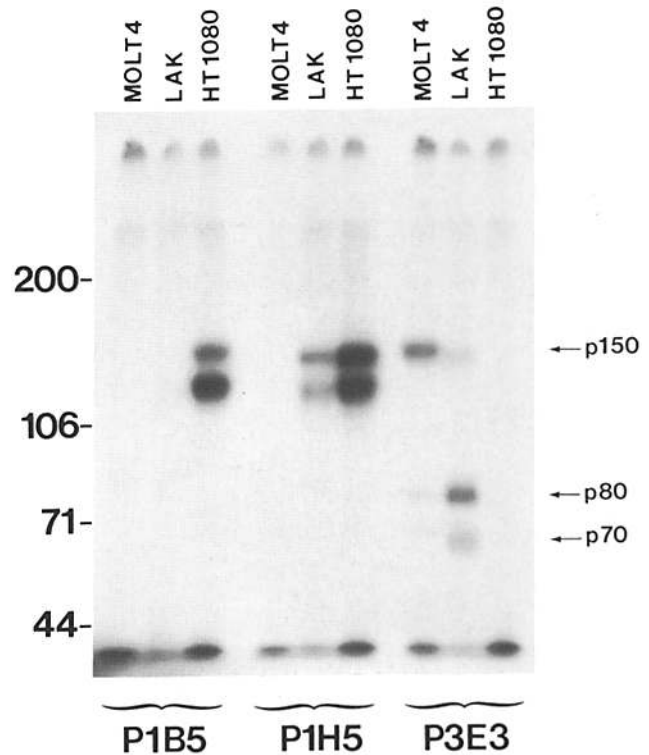


Figure 2. Immune precipitation of lymphocyte fibronectin receptor from HT1080, Molt 4, or chronically activated CD8⁺ T (LAK) cell detergent extracts. ^{125}I -labeled Molt 4, LAK, or HT1080 cells were extracted with 1% Triton X-100 in the presence of 1 mM PMSF, 1 mM *N*-ethylmaleimide, 1 $\mu\text{g/ml}$ leupeptin, and 1 mM diisopropyl fluorophosphate as protease inhibitors. Aliquots of these extracts were immune precipitated with mAbs directed to $\alpha 3\beta 1$ (P1B5), $\alpha 2\beta 1$ (P1H5) and $\alpha 4\beta 1$ (P3E3). The immune precipitated antigens were run on 7.5% SDS-PAGE gels in the absence of 2-mercaptoethanol and visualized by autoradiography. The three bands immune precipitated with P3E3 from T lymphocytes are indicated (arrows).

did not preclear $\alpha 5\beta 1$ (not shown). (c) The V8 protease peptide maps derived from the $\alpha 4$ and $\alpha 5$ subunits precipitated with mAbs P3E3 and PID6 were clearly distinguishable (not shown). Furthermore, under the conditions (0.3% CHAPS and 1 mM CaCl_2) used to solubilize the conjugate of $\alpha 4\beta 1$ from Jurkat cells (Fig. 3) another protein of higher molecular weight (p180) also reacted with the mAbs or coprecipitated with $\alpha 4\beta 1$. p180 was absent from extracts prepared with PID6 mAb (Fig. 3), nonlymphoid cells or Triton X-100 detergent extracts prepared in the absence of Ca^{2+} . The relationship of p180 to other integrins is not known. Because $\alpha 4$ could be immune precipitated without $\beta 1$ after solubilization of T cells with Triton X-100 in the absence of Ca^{2+} this revealed that the inhibitory mAbs recognized epitopes present on the $\alpha 4$ subunit (Fig. 2).

Distribution of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ in Cultured Cells and Tissues

As has been previously reported (Hemler et al., 1987a), $\alpha 4\beta 1$

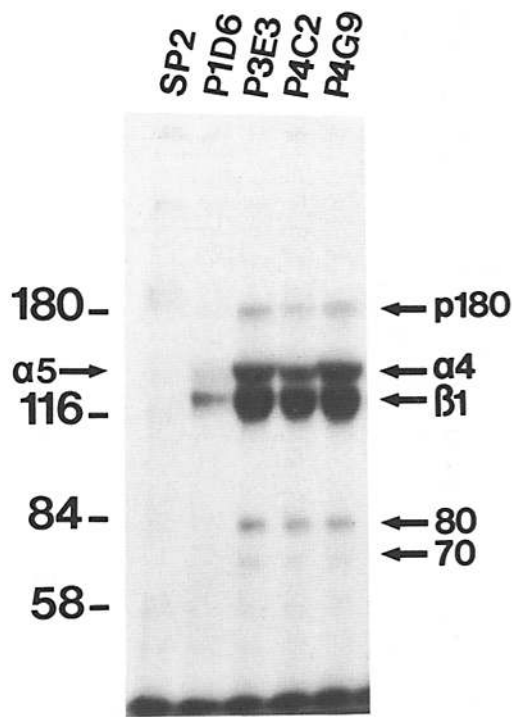


Figure 3. Identification of lymphocyte specific fibronectin receptor as integrin $\alpha 4\beta 1$. ^{125}I -surface-labeled Jurkat cells were extracted with 0.3% CHAPS in the presence of 1 mM CaCl_2 , 1 mM diisopropyl-fluorophosphate, 1 mM PMSF, 1 mM *N*-ethylmaleimide, 1 $\mu\text{g}/\text{ml}$ leupeptin, and 2 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor. Aliquots of the extracts were then immune precipitated with myeloma (SP2) culture supernatant or with mAbs P3E3, P4C2, P4G9, or with P1D6 (anti- $\alpha 5\beta 1$). The immune precipitates were run on 8% SDS-PAGE gels in the absence of reducing agent and visualized by autoradiography. (left) Molecular weight markers. The $\alpha 5$ and $\beta 1$ subunits are indicated as are the bands present in immune precipitates prepared with P3E3, P4C2, and P4G9 (arrows).

was widely distributed on nucleated hematopoietic cells (Table II). Two-color flow cytometry revealed that all lymphocyte subpopulations derived from spleen, tonsil, and peripheral blood expressed abundant $\alpha 4\beta 1$. In addition, peripheral blood monocytes, freshly derived acute lymphocytic (T or B) leukemias, all LGL and myelogenous leukemias, and cultured T and B lymphocyte cell lines we examined expressed abundant $\alpha 4\beta 1$ (Table II). Normal human blood platelets and granulocytes were negative for $\alpha 4\beta 1$. In contrast, the only hematopoietic cell populations that expressed $\alpha 5\beta 1$ were activated T cells, platelets, monocytes, and granulocytes; acute lymphocytic (T or B) and myelogenous leukemias; and cultured K562, HL-60, and U937 cells. Some cultured T (Molt 4 or Jurkat) and B (ST-1) cell lines expressed low levels of $\alpha 5\beta 1$ as detected by P1D6 mAb. In some normal individuals, a subpopulation of PBL were positive for P1D6 fluorescence detected by flow cytometry. We are investigating the nature of this subpopulation of PBL that expresses $\alpha 5\beta 1$. YT cells, a CD3⁻ T cell lymphoma, were completely negative for P1D6 by flow cytometry. These results show that the major fibronectin receptor constitutively expressed by T lymphocytes is $\alpha 4\beta 1$ and as we have previously reported (Wayner

Table II. Distribution of the Fibronectin Receptors $\alpha 4\beta 1$ and $\alpha 5\beta 1$ on Human Cells

Cells	Relative fluorescence intensity	
	$\alpha 4\beta 1$ (P4G9)	$\alpha 5\beta 1$ (P1D6)
Hematopoietic cells*		
PBL	+++	+/- or -
LGL (CD3 ⁻ , CD16 ⁺)	+++	+/- or -
Monocytes (CD16 ⁺)	++	++
Granulocytes	-	+
Platelets	-	+
Spleen	+++	+
Tonsil	+++	+
ALL (T or B)	+++	++
LGL leukemia (CD3 ⁺ , CD16 ⁺)	+++	+/-
AML	+++	++
BLCL	++	+
Molt 4 (CD3 ⁺ , CD4 ⁺)	+++	+
Jurkat (CD3 ⁺ , CD4 ⁺)	+++	++
YT (CD3 ⁻)	++	-
PHA blasts (CD4 ⁺)	++++	++
CTL (CD3 ⁺ , CD8 ⁺)	++++	+++
LAK (CD3 ⁺ , CD8 ⁺)	++++	+++
HL-60	++	+
U937	++	+
K562-1	-	++
Fibroblasts‡		
HFF (p5)	+	+
HT1080	+	++
RD	++	+
VA13	+	++
Epithelial cells		
OC-1§	-	-
OVCAR-4	-	-
T47D	-	-
QG56	-	+
HUVEs (p1)¶		
	-	++

Relative fluorescence intensity was determined as described in Materials and Methods by flow cytometry. Each plus corresponds to 50 channels on a three-decade log scale from 0 to 255. -, no detectable fluorescence above background. +/-, a positive shift in fluorescence above background. mAbs used in all flow cytometry experiments were P4G9 and P1D6 (10 $\mu\text{g}/\text{ml}$). Fc receptors were blocked by preincubation with Protein G-Sepharose-purified goat IgG (20 $\mu\text{g}/\text{ml}$). Protein G-Sepharose-purified mouse IgG was used as a negative control for background staining and detection of possible residual Fc receptor interaction. In general, data obtained with Fc receptor- (CD16) positive cell populations has to be interpreted with caution. In addition, the phenotype of cells in culture can vary from experiment to experiment. The reason for this is unknown. The data presented here are representative of the phenotype expressed most often by cultured cell lines.

* PBL, tonsil or splenic lymphocytes were prepared by standard protocols without enzymatic degradation and stained in suspension for flow cytometry. Peripheral blood subpopulations were distinguished either by two-color flow cytometry analysis or by the use of forward light and high angle (90°) scatter. PBL, resting peripheral blood lymphocytes from normal individuals. CTL, CD8⁺ long-term, antigen-specific cytotoxic T lymphocyte cell line. LAK, CD8⁺ non-antigen-dependent lymphokine-activated killer cells.

‡ Fibroblasts were trypsinized and analyzed for cell surface expression of receptors in suspension. Trypsin was inactivated with soybean trypsin inhibitor and the cells were allowed to recover at 37°C for 10 min before use. HFF, human neonatal foreskin fibroblasts, passage 5 (p5).

§ OC-1 cells were derived from the ascites fluid of a woman with ovarian carcinoma. They were fresh frozen and thawed just before staining. These cells are nonadherent in culture.

|| OVCAR-4 cells are a well-established adherent cell line derived from an ovarian carcinoma.

¶ HUVEs, large vessel endothelial cells obtained from Cell Systems (Seattle, WA) and were used at passage 1. They were derived from human umbilical cords and grown to confluence in serum-free media (Cell Systems). They exhibited typical characteristics of HUVEs.

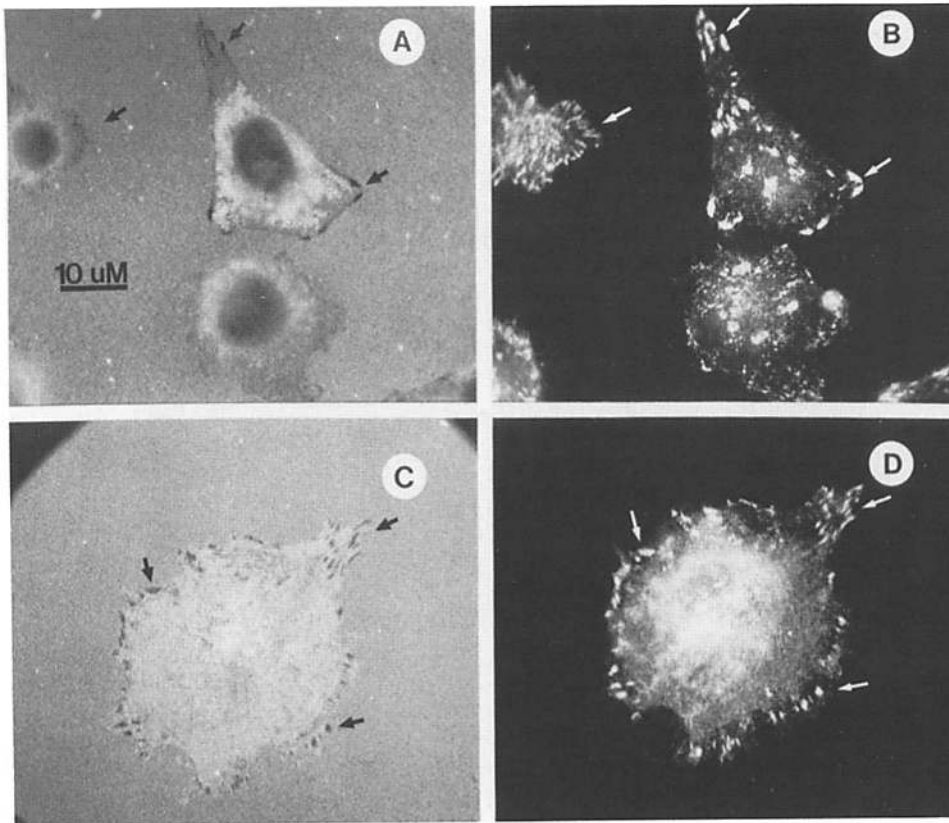


Figure 4. Localization of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ in focal adhesions on fibronectin-coated surfaces. RD cells were trypsinized and allowed to adhere to silanized and fibronectin-coated (20 $\mu\text{g}/\text{ml}$) glass cover slips in the absence of serum for 1 h at 37°C. At the end of this time, the cells were prepared for localization of receptors in focal adhesions as described (Materials and Methods). (A and C) Focal adhesions (arrows) visualized by interference reflexion microscopy when RD cells are adhered to fibronectin. (B) Reorganization of the prototype fibronectin receptor $\alpha 5\beta 1$ stained with antibody AB33 to the focal adhesions (arrows). (D) Reorganization of $\alpha 4\beta 1$ stained with P4G9 (FITC) also to the focal adhesions when RD cells are adhered to fibronectin (arrows). A and B are the same field and C and D are the same field.

et al., 1988), expression of $\alpha 5\beta 1$ in T lymphocytes is restricted to leukemic or activated cultured cells. Interestingly, most fibroblast cell lines expressed low levels of $\alpha 4\beta 1$, whereas large-vessel endothelial cells and cultured epithelial cells were negative for $\alpha 4\beta 1$ by flow cytometry.

In tissue, $\alpha 4\beta 1$ was present in adult spleen, lymph node, and tonsil and essentially absent from all other tissues we examined (not shown). In addition, the relative quantities of the fibronectin adhesion receptors expressed by cells in specific tissue domains varied dramatically. For example, PBL and lymphocytes in tonsil cortex and germinal center areas expressed large quantities of $\alpha 4\beta 1$ but virtually no $\alpha 5\beta 1$. $\alpha 4\beta 1$ was also found in epithelial regions in adult lymphatic tissue, but whether this was the result of lymphocyte infiltration of these areas or expression of $\alpha 4\beta 1$ by lymphatic epithelial cells was unclear.

$\alpha 4\beta 1$ Localizes in Fibronectin-dependent Focal Adhesions

There is a specific reorganization of cell surface adhesion receptors to the focal adhesions when cells are grown on the appropriate ligands in the absence of serum (reviewed by Burridge et al., 1988). As some fibroblasts express $\alpha 4\beta 1$, we investigated whether this receptor would distribute into focal adhesions when fibronectin was used as the adhesion substrate. As can be seen in Fig. 4, A and C, the primary focal contact sites or focal adhesions could be visualized by interference reflexion microscopy (Izzard and Lochner, 1976) when RD cells were grown on fibronectin. As we and others have reported (Roman et al., 1989), in the absence of serum,

$\alpha 5\beta 1$ was concentrated at the focal adhesions when RD cells were grown on fibronectin (Fig. 4 B, arrows) but not laminin-coated surfaces (not shown). Likewise, staining with mAb P4G9 (Fig. 4 D, arrows) revealed that $\alpha 4\beta 1$ was also concentrated in focal adhesions when cells were grown on fibronectin- but not laminin-coated surfaces (not shown). These results demonstrate a specific interaction of $\alpha 4\beta 1$ with fibronectin present in focal adhesions, the primary adhesion structure of cultured cells.

The presence of both receptors in focal contacts suggested the possibility that $\alpha 4\beta 1$ and $\alpha 5\beta 1$ bind to distinct adhesion sequences in fibronectin. Evidence for this was obtained when P4C2 and PID6 were used simultaneously to inhibit cell adhesion to intact plasma fibronectin. PID6 and P4C2 when used together completely inhibited adhesion of T lymphocytes and partially inhibited adhesion of RD cells to intact plasma fibronectin (Table III). Interestingly, unlike T lymphocytes, neither PID6 nor P4C2 alone were good inhibitors of RD cell adhesion to intact plasma fibronectin. RD cell adhesion to fibronectin could be efficiently inhibited by PID6 and P4C2 only when used together.

$\alpha 4\beta 1$ Functions as the Receptor for an RGD-independent Alternative Attachment Site in Fibronectin

The preceding results (Tables I and III, Figs. 1 and 4) clearly indicated that attachment of some cells to plasma fibronectin was mediated by two independent cell surface receptors, $\alpha 4\beta 1$ and $\alpha 5\beta 1$. It has been well documented that the ligand for $\alpha 5\beta 1$ in fibronectin is the 80-kD cell-binding domain that

Table III. Combined Effect of mAbs P1D6 and P4C2 on T Lymphocyte and RD Cell Adhesion to Fibronectin

Cells	Antibody	Specificity	Adhesion
			% of control \pm SD
RD	IGG	—	100
	P1D6	$\alpha 5\beta 1$	81 \pm 11
	P4C2	$\alpha 4\beta 1$	99 \pm 7
	P1D6 + P4C2		36 \pm 8
Jurkat	IGG	—	100
	P1D6	$\alpha 5\beta 1$	26 \pm 9
	P4C2	$\alpha 4\beta 1$	38 \pm 14
	P1D6 + P4C2		0

Cells were labeled with ^{51}Cr and incubated in the presence of the indicated mAbs (50 $\mu\text{g/ml}$) or purified mouse IgG (50 $\mu\text{g/ml}$) for 1 h at 4°C. They were then applied to plasma fibronectin-coated (20 $\mu\text{g/ml}$) surfaces in RPMI 1640/1 mg/ml HBSA and incubated at 37°C for 30 min (RD cells) or 2 h (Jurkat). At the end of this incubation, nonadherent cells were washed off with warm PBS and the bound counts per minute were solubilized and quantitated in a gamma counter. Results from several experiments were pooled and are expressed as mean percent (relative to control) \pm SD.

contains the RGD sequence (Pierschbacher and Ruoslahti, 1984; Pytela et al., 1985). To determine the region of fibronectin that interacts with $\alpha 4\beta 1$, we examined the adhesion of cultured T lymphocytes to various proteolytic fragments of plasma fibronectin (see Fig. 5, A and B), as well as the effect of mAbs P1D6 and P4C2 on lymphocyte adhesion to these fragments. As shown in Fig. 6, Jurkat, YT, and Molt 4 (not shown) cells attach to a 38-kD fragment containing the Heparin (Hep) II domain much more efficiently than to an RGD-containing fragment (80 kD). Jurkat and Molt 4 cells also attach in a dose-dependent manner to another Hep II domain-containing fragment of 58 kD (not shown). Maximum cell

attachment to the 58-kD fragment, however, reached only 30% of that achieved by the 38-kD fibronectin fragment. This suggests that the 38-kD fragment contains a high-affinity attachment site for T lymphocytes. T lymphocytes did not adhere to the NH_2 -terminal 29-kD fragment containing the Hep I domain of plasma fibronectin (not shown). In general, freshly derived PBL showed a similar pattern of attachment as Jurkat or Molt 4 cells and the ability of freshly derived PBL to bind to the 80-kD fragment correlated with expression of $\alpha 5\beta 1$ (not shown). Other hematopoietic cell lines, such as K562 cells (Fig. 6) exhibited a clear preference for the 80-kD fragment of plasma fibronectin, whereas RD cells expressed promiscuous adhesion to all the fragments of plasma fibronectin tested, except the NH_2 -terminal 29-kD fragment (not shown). RGDS (1 mg/ml) partially inhibited (50%) Jurkat cell adhesion to intact fibronectin and completely (100%) inhibited their adhesion to the 80-kD fragment. Jurkat cell adhesion to the 38-kD fragment was unaffected by RGDS (up to 1 mg/ml).

As we have previously shown (Table I and Fig. 1), mAbs to $\alpha 4\beta 1$ and $\alpha 5\beta 1$ partially inhibited T lymphocyte adhesion to intact plasma fibronectin (Fig. 7, top). As expected, P1D6 completely inhibited adhesion of T cells to the 80-kD fragment, which contains the RGD adhesion sequence (Fig. 7, middle). P1D6 did not inhibit T lymphocyte adhesion to the 38- (Fig. 7, bottom) or 58-kD (not shown) fragments. In contrast, P4C2 completely inhibited T lymphocyte adhesion to the 38-kD fragment and had no effect on adhesion to the 80-kD fragment (Fig. 7). Furthermore, adhesion of T lymphocytes to the 58-kD fragment which also contains Hep II could be inhibited by P4C2. In every case other T lymphocyte cell lines which express both $\alpha 4\beta 1$ and $\alpha 5\beta 1$ (such as Jurkat cells) behave exactly as Molt 4 cells (Fig. 7). As seen in Table II, K562 cells express only $\alpha 5\beta 1$. Adhesion of K562 cells to the 38- (Fig. 6) and 58-kD fragments (not shown) was greatly

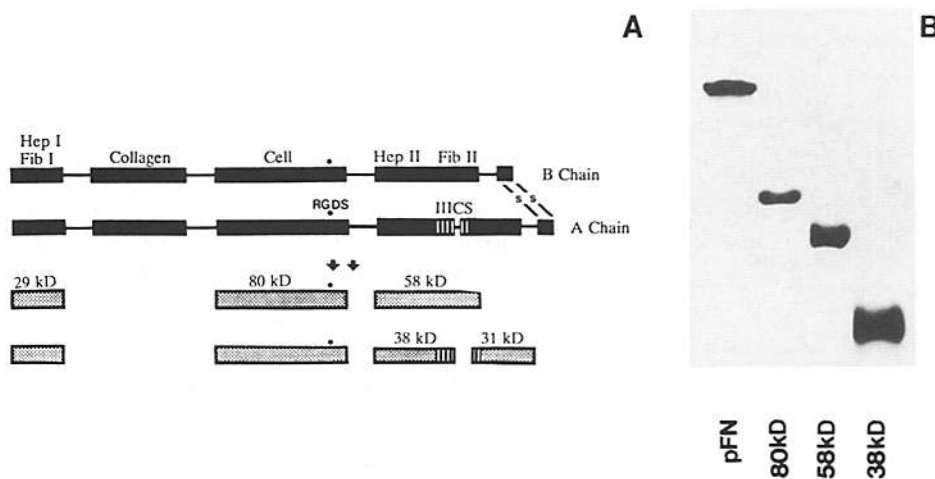


Figure 5. (A) Domain structure of human plasma fibronectin (pFN) showing the origin of the fragments used in this study. **(B)** SDS-PAGE gel analysis (10% acrylamide) demonstrating the purity of the fragments. The 80-kD fragment had the NH_2 -terminal amino acid sequence SD(VPSPR(L)QF, and therefore begins at position 874 of the fibronectin molecule (Kornblihtt et al., 1985). This fragment contains the cell binding domain (Cell) and the RGDS sequence of fibronectin (*). The 58- and 38-kD fragments had the NH_2 -terminal amino acid sequence TAGPDQ-TEMTIEGLQ. Both fragments contain the COOH-terminal heparin

binding domain (Hep II) and result from a different cleavage of the two fibronectin chains by trypsin. The 38-kD fragment comprises the first 67 amino acid residues of the alternatively spliced connecting segment of fibronectin (IIICS) (Garcia-Pardo et al., 1987) and it is therefore derived from the A chain. The 38-kD fragment does not contain the REDV adhesion site recognized by B16-F10 melanoma cells (Humphries et al., 1986, 1987). The 58-kD fragment is derived from the B chain of fibronectin and lacks the IIICS region (Garcia-Pardo, unpublished). The 58-kD fragment also contains the COOH-terminal fibrin binding domain of fibronectin (Fib II), and is similar to previously reported fragments from this region of plasma fibronectin (Click and Balian, 1985; Rogers et al., 1985). The bands are visualized by staining with Coomassie blue.

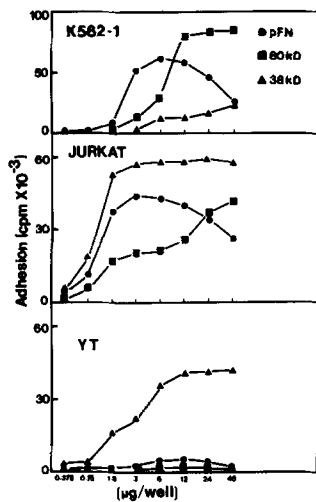


Figure 6. Adhesion of hematopoietic cells to plasma fibronectin and the purified 38- and 80-kD tryptic fragments of plasma fibronectin. ^{51}Cr -labeled K562 (erythroleukemia), Jurkat (CD3+ T lymphocyte), and YT (CD3- T lymphocyte) cells (10^5 /well) were allowed to adhere to plastic surfaces that had been coated with intact plasma fibronectin (pFN) or the purified 80- and 38-kD tryptic fragments at the indicated concentrations (y-axis) for 2 h at 37°C . At the end of this time, nonadherent cells were washed off and the bound cells were solubilized

in SDS/NaOH and quantitated. The results are expressed as bound ^{51}Cr counts per minute.

reduced when compared to their adhesion to the 80-kD fragment (Fig. 6). Adhesion of these cells to intact plasma fibronectin (Fig. 1) or the 80-kD fragment (not shown) could be completely inhibited by PID6. On the other hand, YT cells, which do not express $\alpha 5\beta 1$ (Table II), adhere poorly to intact plasma fibronectin and the 80-kD fragment (Fig. 6). These cells require two to three times longer to adhere to plasma fibronectin-coated surfaces than Jurkat or Molt 4 cells. YT cells, however, adhere efficiently and in a dose-dependent manner to the 38-kD fragment (Fig. 6) and adhesion of these cells to the 38-kD fragment could be completely inhibited by P4C2 (not shown). These data indicate a direct correlation between expression of $\alpha 4\beta 1$ and the ability to attach to fragments of plasma fibronectin containing the Hep II and III CS regions. Furthermore, these data show unequivocally that $\alpha 4\beta 1$ functions as the receptor for this alternative cell adhesion domain.

$\alpha 4\beta 1$ Is the T Lymphocyte Receptor for CS-1

The III CS region present on the A chain of plasma fibronectin (Fig. 5) contains at least two sites responsible for mediating cell adhesion to fibronectin (Humphries et al., 1986, 1987, 1988). Using a series of overlapping synthetic peptides spanning the entire III CS region (CS peptides), Humphries and co-workers showed that the CS-1 (NH₂-terminal) and CS-5 (COOH-terminal) peptides contained adhesion sequences recognized by mouse melanoma cells (Humphries et al., 1986, 1987). We have shown here that the 38-kD fragment contains a high-affinity adhesion site recognized by T lymphocytes and that $\alpha 4\beta 1$ is the receptor that mediates T lymphocyte adhesion to 38 kD. This fragment does not contain the CS-5 site but it does contain the entire CS-1 region (Garcia-Pardo et al., 1987), which was defined as a high-affinity adhesion site for melanoma cells (Humphries et al., 1987). Therefore, it was of interest to determine if T lymphocytes would recognize and bind to CS-1 and if $\alpha 4\beta 1$ was the receptor involved in this interaction.

T lymphocytes (Jurkat or Molt 4 cells) recognize and at-

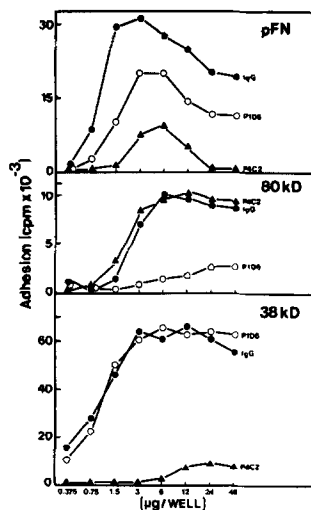


Figure 7. Effect of the mAbs PID6 and P4C2 to the integrin receptors $\alpha 5\beta 1$ and $\alpha 4\beta 1$, respectively, on adhesion of T lymphocytes to intact plasma fibronectin (pFN) or the purified 80- and 38-kD tryptic fragments. ^{51}Cr -labeled Molt 4 cells were incubated with purified PID6 or P4C2 mAbs ($50 \mu\text{g/ml}$) or purified mouse IgG ($50 \mu\text{g/ml}$) for 1 h at 4°C . They were then allowed to adhere to plastic surfaces that had been coated with intact plasma fibronectin, or the 80- and 38-kD tryptic fragments at the indicated concentrations (y-axis) for 1 h. At the end of this time, the nonadherent

cells were washed off and the adherent cells were solubilized and bound ^{51}Cr counts per minute were quantitated in a gamma counter. The results are expressed as bound counts per minute.

tach to CS-1 (rabbit IgG conjugate) -coated plastic surfaces (Table IV). Jurkat cells do not attach to CS-2 (rabbit IgG conjugate) -coated surfaces or to plastic surfaces coated with rabbit IgG alone (not shown). Furthermore, mAbs to $\alpha 4\beta 1$ (P4C2) completely inhibited T lymphocyte adhesion to CS-1, whereas antibodies to $\alpha 5\beta 1$ (PID6) had absolutely no effect (Table IV). As we have previously shown, antibodies to $\alpha 4\beta 1$ completely and specifically inhibited T lymphocyte adhesion to the 38-kD fragment (Table IV), whereas antibodies to $\alpha 5\beta 1$ specifically inhibited adhesion to the RGD containing 80-kD fragment.

Discussion

In this study, we have described a new fibronectin receptor, identical to the integrin receptor $\alpha 4\beta 1$ (Hemler et al., 1987a), preferentially expressed by nucleated hematopoietic cells. Identification of $\alpha 4\beta 1$ as a specific fibronectin receptor was based on (a) inhibition of cell adhesion to fibronectin by mAbs P4C2, P3E3, and P4G9, and (b) specific reorganization and concentration of $\alpha 4\beta 1$ into fibronectin-dependent focal adhesions. These findings suggest that $\alpha 4\beta 1$ and $\alpha 5\beta 1$ function together as primary mediators of cell adhesion to fibronectin.

mAbs to either $\alpha 5\beta 1$ or $\alpha 4\beta 1$ partially inhibited T lymphocyte adhesion to intact fibronectin. However, inhibition of T lymphocyte or RD cell adhesion to fibronectin was most efficient when the inhibitory antibodies to $\alpha 4\beta 1$ and $\alpha 5\beta 1$ were used together. This suggested that these receptors recognized independent sites on intact fibronectin. It has been well documented that the ligand for $\alpha 5\beta 1$ is the cell binding domain containing the RGD sequence (Pytela et al., 1985). Analysis of the region of fibronectin recognized by $\alpha 4\beta 1$ was accomplished by examining the ability of the functionally defined mAbs to inhibit lymphocyte adhesion to purified tryptic fragments of plasma fibronectin. Although T lymphocytes attached to the 80-kD cell binding domain (Fig. 5 A) they demonstrated a clear preference for a non-RGD-

Table IV. Inhibition of T Lymphocyte Adhesion to CS-1 Peptide with mAbs to $\alpha 4\beta 1$

Ligand	IgG	Antibody P4C2	P1D6
<i>⁵¹Cr cpm bound to the adhesion surface \pm SD</i>			
80 kD	8,580 \pm 214	7,154 \pm 398	202 \pm 105
38 kD	22,680 \pm 1,014	114 \pm 78	24,917 \pm 352
CS-1	44,339 \pm 513	841 \pm 555	42,897 \pm 728
CS-2	2,576 \pm 214	535 \pm 258	435 \pm 168

Jurkat cells were labeled with ⁵¹Cr and incubated in the presence of the indicated mAbs (50 μ g/ml) or purified mouse IgG (50 μ g/ml) for 1 h at 4°C. They were then applied to plastic surfaces coated with the 80- and 38-kD tryptic plasma fibronectin fragments (10 μ g/ml) or the CS-1 and CS-2 rabbit IgG conjugates (1:100). After a 2-h incubation at 37°C, nonadherent cells were washed off with warm PBS and the bound counts per minute were solubilized and quantitated in a gamma counter. Results from several experiments were pooled and are expressed as ⁵¹Cr counts per minute bound to the adhesion surface \pm SD.

containing region located on a 38-kD tryptic fragment derived from the A (or heavy) chain of plasma fibronectin. In the present study we have shown that T lymphocytes also recognize and bind to another Hep II-containing 58-kD fragment. However, the high-affinity lymphocyte binding site was located on the 38-kD fragment. On a molar basis, the 38-kD fragment was three times more efficient than the 58-kD fragment in mediating T lymphocyte adhesion. As shown in Fig. 5 A, the 38- and 58-kD fragments were derived from the A and B chains of plasma fibronectin, respectively. They therefore differ in the presence or absence of IIICS (Kornblihtt et al., 1985; Garcia-Pardo et al., 1987). Thus, it is possible that the 38- and 58-kD fragments used here share a common low affinity T lymphocyte binding site, located in the Hep II domain, and that additional high-affinity T lymphocyte adhesion sites are present in the IIICS region unique to the 38-kD fragment. We show here that T lymphocytes specifically recognize and bind to CS-1, which has been defined as a high-affinity adhesion site for B16 melanoma cells and avian neural crest cells (Humphries et al., 1987, 1988; Dufour et al., 1988). Furthermore, as we have reported (Garcia-Pardo, A., and O. C. Ferreira, manuscript submitted for publication) soluble CS-1 (500 μ g/ml), however, did not completely inhibit T lymphocyte adhesion to the 38-kD fragment. This supports the notion that T lymphocytes may use other sites (possibly in Hep II) during their adhesion to fibronectin.

T lymphocyte adhesion to the 38- and 58-kD fragments could be completely inhibited by functionally defined mAbs to $\alpha 4\beta 1$. Furthermore, T lymphocyte adhesion to CS-1- (IgG conjugate) coated surfaces could also be completely inhibited by P4C2, P3E3, or P4G9. These data show clearly that $\alpha 4\beta 1$ is the T lymphocyte receptor for CS-1. In contrast, these antibodies failed to inhibit adhesion of T cells to the 80-kD fragment containing the prototype adhesion sequence arg-gly-asp (RGD). Adhesion of T cells to the 80-kD fragment could be completely inhibited by an mAb to $\alpha 5\beta 1$ (PID6) or by RGDS. PID6 and RGDS failed to inhibit T lymphocyte adhesion to the 38- and 58-kD fragments or to CS-1. Together, these data show that $\alpha 4\beta 1$ functions as the receptor for the carboxy-terminal adhesion domain of plasma fibronectin. $\alpha 4\beta 1$ is, therefore, clearly a new and RGD independent fibronectin receptor for alternative adhesion sequences in IIICS (CS-1) and possibly Hep II.

Recently, Bernardi et al. (1987) and Liao et al. (1987, 1989) reported that some B lymphocyte cell lines bind to a region of plasma fibronectin located within the carboxy-terminal Hep II domain. Liao et al. (1987) identified an integrin-like receptor on B cells. However, it is not clear whether the protein they described was $\alpha 4\beta 1$ or $\alpha 5\beta 1$. Bernardi et al. (1987) also identified fibronectin receptors expressed by B lymphocytes. Interestingly, in this study, B cells that attached to fragments containing Hep II expressed a receptor similar to $\alpha 4\beta 1$, whereas cells that attached to the RGD-containing cell adhesion domain expressed a receptor similar to $\alpha 5\beta 1$. Together, the results of these previous reports and the present findings provide clear evidence in support of (a) the existence of an alternative adhesion domain present in the carboxy-terminal region of plasma fibronectin and (b) a role for $\alpha 4\beta 1$ as the receptor for this alternative adhesion site. It will be interesting to determine the precise amino acid sequences responsible for $\alpha 4\beta 1$ interaction with fibronectin. As neither of the 38- or 58-kD fragments nor CS-1 contains an RGD sequence (Kornblihtt et al., 1985; Garcia-Pardo et al., 1987; Humphries et al., 1986, 1987), it is clear that characterization of the ligand for $\alpha 4\beta 1$ will identify a new amino acid sequence important for cell adhesion to fibronectin. Because the 38-kD fragment does not contain CS-5 (Garcia-Pardo et al., 1987), the minimal amino acid sequence responsible for T lymphocyte adhesion to 38 kD and therefore the ligand for $\alpha 4\beta 1$ in these cells is not arg-glu-asp-val or REDV (Humphries et al., 1986).

Like $\alpha 2\beta 1$, the $\alpha 4$ subunit is weakly associated with the $\beta 1$ subunit. The data presented here (Fig. 2) and our previous findings (Wayner and Carter, 1987; Wayner et al., 1988) show that the functionally defined mAbs to $\alpha 2\beta 1$ and $\alpha 4\beta 1$ selectively interact with epitopes present on the α subunits, based on immune precipitation of $\alpha 2$ or $\alpha 4$ without $\beta 1$ after subunit dissociation. These results suggest that the unique α subunit is responsible for determining the ligand-binding specificity of each α - β complex. This concept is now further supported by the observations presented here that $\alpha 5$ and $\alpha 4$, which are both complexed with $\beta 1$, mediate adhesion to distinct sites on fibronectin. This is not to suggest that the β subunit is not important in binding, but that the specificity of receptor-ligand interactions is determined by α or a unique α - β complex.

It is interesting that although LAK cells expressed abundant cell surface $\alpha 4\beta 1$, it did not appear to be a functional receptor; PID6 completely inhibited LAK cell adhesion to fibronectin (not shown). The reason for this could be that LAK cells express a degraded form of $\alpha 4$ (see Fig. 2). In addition, because they are activated, LAK cells over express $\alpha 5\beta 1$ when compared with resting peripheral blood or leukemic T cells (Table II). In other cells that express larger quantities of $\alpha 5\beta 1$ relative to $\alpha 4\beta 1$ (K562-1 and HT1080) adhesion to the 80-kD RGD-containing domain via $\alpha 5\beta 1$ is dominant (see K562-1 cells, Fig. 6). This implies that regulation of receptor expression determines the ability of a cell to recognize and bind to different sites on fibronectin. Furthermore, it is also possible that coexpression of the two receptors for fibronectin could increase the avidity of cell binding, for example Jurkat and RD cells, which express relatively promiscuous adhesion to fibronectin when compared with YT cells, which express only $\alpha 4\beta 1$.

The regulation of cell adhesion to fibronectin is potentially

complex even under the simplest possible conditions, which assume that $\alpha 5\beta 1$ and $\alpha 4\beta 1$ function independently of each other and do not overlap during interaction with the two binding sites on fibronectin. Variation from this simple state provides opportunities for exquisitely sensitive regulation of cell adhesion. At the least complex level, this regulation can be roughly categorized as (a) processes that control the synthesis and/or exposure of the binding sites on the ligand and (b) regulation of functional expression of the receptors. Examples of regulation at both levels are currently available and include, the observation that lymphokines and specific antigen induce $\alpha 5\beta 1$ expression on T lymphocytes followed by increased cell adhesion to fibronectin (Wayner et al., 1988, and unpublished). In addition, the control of mRNA splicing in the IIIc region of fibronectin (Kornbliht et al., 1985) during wound healing or inflammation may dictate the specificity of receptor-ligand binding in resting or activated T cells. Variations from the simple state are intriguing but require additional experimentation to even begin to identify the multitude of potential mechanisms.

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Note added in proof. We have identified a minimal peptide derived from the carboxy terminal portion of CS-1, LHGPEILDVPST, which inhibits T lymphocyte adhesion to plasma fibronectin, 38 kDa, and CS-1.

References

- Bernardi, P., V. P. Patel, and H. F. Lodish. 1987. Lymphoid precursor cells adhere to two different sites on fibronectin. *J. Cell Biol.* 105:489-498.
- Brooks, C. G. 1983. Reversible induction of natural killer cell activity in cloned murine cytotoxic T lymphocytes. *Nature (Lond.)* 305:155-158.
- Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487-525.
- Cardarelli, P. M., I. N. Crispe, and M. D. Pierschbacher. 1988. Preferential expression of fibronectin receptors on immature thymocytes. *J. Cell Biol.* 106:2183-2190.
- Carter, W. G., and E. A. Wayner. 1988. Characterization of a collagen-binding, phosphorylated, transmembrane glycoprotein expressed in nucleated human cells. *J. Biol. Chem.* 263:4193-4201.
- Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102-1106.
- Click, E. M., and G. Balian. 1985. Domain structure of human plasma and cellular fibronectin. Use of a monoclonal antibody and heparin affinity to identify three different subunit chains. *Biochemistry* 24:6685-6696.
- Durfour, S., J. L. Duband, M. Humphries, M. Obara, K. Yamada, and J. P. Thiery. 1988. Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2661-2671.
- Garcia-Pardo, A., A. Rostagno, and B. Frangione. 1987. Primary structure of human plasma fibronectin. Characterization of a 38 kDa domain containing the C-terminal heparin-binding site (Hep III site) and a region of molecular heterogeneity. *Biochem. J.* 241:923-928.
- Garcia-Pardo, A., O. C. Ferreira, J. Valinsky, and C. Bianco. 1989. Fibronectin receptors of mononuclear phagocytes: binding characteristics and biochemical isolation. *Exp. Cell Res.* 181:420-431.
- Giancotti, F. G., P. M. Comoglio, and G. Tarone. 1986. Fibronectin-plasma membrane interaction in the adhesion of hemopoietic cells. *J. Cell Biol.* 103:429-437.
- Glasebrook, A. L., and F. W. Fitch. 1980. Alloreactive cloned T cell lines. *J. Exp. Med.* 151:876-895.
- Grimm, E. A., A. Mazumder, H. Z. Zhang, and S. A. Rosenberg. 1982. Lymphokine activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J. Exp. Med.* 155:1923-1941.
- Hemler, M. E., C. Huang, and L. Schwarz. 1987a. The VLA protein family: characterization of five distinct cell surface heterodimers each with a common 130,000 molecular weight β subunit. *J. Biol. Chem.* 262:3300-3309.
- Hemler, M. E., C. Huang, Y. Takada, L. Schwarz, J. L. Strominger, and M. L. Clabby. 1987b. Characterization of the cell surface heterodimer VLA-4 and related peptides. *J. Biol. Chem.* 262:11478-11485.
- Hemler, M. E. 1988. Adhesive protein receptors on hematopoietic cells. *Immunol. Today* 9:109-113.
- Humphries, M. J., S. K. Akiyama, A. Komoriya, K. Olden, and K. M. Yamada. 1986. Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion. *J. Cell Biol.* 103:2637-2647.
- Humphries, M. J., A. Komoriya, S. K. Akiyama, K. Olden, and K. M. Yamada. 1987. Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. *J. Biol. Chem.* 262:6886-6892.
- Humphries, M. J., S. K. Akiyama, A. Komoriya, K. Olden, and K. M. Yamada. 1988. Neurite extension of chicken peripheral nervous system neurons on fibronectin: relative importance of specific adhesion sites in the central cell-binding domain and the alternatively spliced type III connecting segment. *J. Cell Biol.* 106:1289-1297.
- Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell* 48:549-554.
- Izzard, C. S., and L. R. Lochner. 1976. Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J. Cell Sci.* 21:129-159.
- Kornbliht, A. R., K. Umezawa, K. Vibe-Pedersen, and F. Baralle. 1985. Primary structure of human plasma fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1755-1759.
- Kunicki, T. J., D. J. Nugent, S. J. Staats, R. P. Orzechowski, E. A. Wayner, and W. G. Carter. 1988. The human fibroblast class II extracellular matrix receptor mediates platelet adhesion to collagen and is identical to the platelet Ia/IIa complex. *J. Biol. Chem.* 263:4516-4519.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Liao, N. S., J. St. John, Z. J. Du, and H. T. Cheung. 1987. Adhesion of lymphoid cell lines to fibronectin-coated substratum: biochemical and physiological characterization and the identification of a 140-kDa fibronectin receptor. *Exp. Cell Res.* 171:306-320.
- Liao, N. S., J. St. John, J. B. McCarthy, L. T. Furcht, and H. T. Cheung. 1989. Adhesion of lymphoid cells to the carboxy-terminal heparin-binding domains of fibronectin. *Exp. Cell Res.* 181:348-361.
- Oi, V. T., and L. A. Herzenberg. 1980. Immunoglobulin producing hybrid cell lines. In *Selected Methods in Cellular Immunology*. B. B. Mishell and S. M. Shiigi, editors. W. H. Freeman & Co., San Francisco, CA. 351-373.
- Pierschbacher, M. D., and E. Ruoslahti. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.)* 309:30-33.
- Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell* 40:191-198.
- Rogers, S. L., J. McCarthy, S. L. Palm, L. T. Furcht, and P. C. Letourneau. 1985. Neuron-specific interactions with two neurite-promoting fragments of fibronectin. *J. Neurosci.* 5:369-378.
- Roman, J., R. LaChance, T. J. Broekelmann, C. J. Roberts, E. A. Wayner, W. G. Carter, and J. McDonald. 1988. 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.
- Savagner, P., B. A. Imhof, K. M. Yamada, and J. P. Thiery. 1986. Homing of hemopoietic precursor cells to the embryonic thymus: characterization of an invasive mechanism induced by chemotactic peptides. *J. Cell Biol.* 103:2715-2727.
- Staatz, W. D., S. M. Rajpara, E. A. Wayner, W. G. Carter, and S. A. Santoro. 1989. The membrane glycoprotein Ia-IIa (VLA 2) complex mediates the Mg^{++} -dependent adhesion of platelets to collagen. *J. Cell Biol.* 108:1917-1924.
- Taggart, R. T., and I. M. Samloff. 1983. Stable antibody-producing murine hybridomas. *Science (Wash. DC)* 219:1228-1230.
- Takada, Y., E. A. Wayner, W. G. Carter, and M. E. Hemler. 1988. Extracellular matrix receptors, ECMR II and ECMR I, for collagen and fibronectin correspond to VLA-2 and VLA-3 in the VLA family of heterodimers. *J. Cell. Biochem.* 37:385-393.
- Wayner, E. A., and C. G. Brooks. 1984. Induction of NKCF-like activity in mixed lymphocyte-tumor cell culture: direct involvement of mycoplasma infection of tumor cells. *J. Immunol.* 132:2135-2142.
- Wayner, E. A., and G. Brooks. 1985. An investigation of the role of soluble cytotoxic factors and reactive oxygen intermediates in lysis by NK cells. *Adv. Exp. Med. Biol.* 184:221-238.
- Wayner, E. A., and W. G. Carter. 1987. Identification of multiple cell adhesion receptors for type VI collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. *J. Cell Biol.* 105:1873-1884.
- Wayner, E. A., W. G. Carter, R. Piotrowicz, and T. J. Kunicki. 1988. The function of multiple extracellular matrix receptors (ECMRs) in mediating cell adhesion to ECM: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic/IIa. *J. Cell Biol.* 107:1881-1891.