Activation of the $\alpha 4\beta 1$ Integrin through the $\beta 1$ Subunit Induces Recognition of the RGDS Sequence in Fibronectin

Paloma Sánchez-Aparicio, Carmen Dominguez-Jiménez, and Angeles Garcia-Pardo

Departamento de Inmunología, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain

Abstract. Lymphocyte attachment to fibronectin is mainly mediated by the interaction of $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins with the RGD and CS-1/Hep II sites, respectively. We have recently shown that the anti-$\beta 1$ mAb TS2/16 can convert the partly active $\alpha 4\beta 1$ present on certain hemopoietic cells that recognizes CS-1 but not Hep II, to a high avidity form that binds both ligands. In this report we have studied whether mAb TS2/16 also affects $\alpha 4\beta 1$ ligand specificity. Incubation of the B cell lines Ramos and Daudi (which lack $\alpha 5\beta 1$) with mAb TS2/16 induced specific attachment to an 80-kD fragment which lacks CS-1 and Hep II and contains the RGD sequence. mAbs anti-$\alpha 4$ and the synthetic peptides CS-1 and IDAPS inhibited adhesion to the 80-kD fragment thus implying $\alpha 4\beta 1$ as the receptor for this fragment. Interestingly, the synthetic peptide GRGDSPC and a 15-kD peptic fibronectin fragment containing the RGD sequence also inhibited B cell adhesion to the 80-kD fragment. Because we have previously shown that RGD peptides do not affect the constitutive function of $\alpha 4\beta 1$, we tested whether TS2/16-activated $\alpha 4\beta 1$ acquired the capacity to recognize RGD. Indeed RGD peptides inhibited TS2/16-treated B cell adhesion to a 38-kD fragment containing CS-1 and Hep II but did not affect binding of untreated cells to this fragment. An anti-fibronectin mAb reactive with an epitope on or near the RGD sequence also efficiently inhibited cell adhesion to the 80-kD fragment, indicating that the RGD sequence is a novel adhesive ligand for activated $\alpha 4\beta 1$. These results emphasize the role of $\alpha 4\beta 1$ as a receptor with different ligand specificities according to the activation state, a fact that may be important for lymphocyte migration, localization, and function.

LYMPHOCYTE interactions with fibronectin (Fn)[1] are important for their differentiation, migration, activation, and biological function (reviewed in 20, 46). The specific sequences in Fn that mediate cell attachment are located within two main regions of the molecule: the central cell-binding domain contains the RGD site which acts in synergy with at least two other regions within this domain (56). The carboxy-terminal region of Fn comprises the Hep II domain and the IIICS region. Within the Hep II domain the following sites have been shown to support melanoma cell adhesion: HI (35), FNC/H I, and FNC/H II (21, 31). Two active sites have been identified within IIICS, namely CS-1 (residues 1–25) and CS-5 (residues 90–109) (19). Both CS-1 and CS-5 are regulated by alternative splicing and are not present in all Fn isoforms. While CS-1 and the entire Hep II domain clearly mediate lymphocyte adhesion to Fn (15, 16, 52) it is not known if these cells recognize the specific sequences contained in HI, FNC/H I, FNC/H II, and CS-5.

Address all correspondence to Dr. Angeles Garcia-Pardo, Centro de Investigaciones Biológicas, Velázquez 144, 28006 Madrid, Spain.

1. *Abbreviations used in this paper*: ECM, extracellular matrix; Fn, fibronectin; PMA, phorbol myristate acetate; VCAM-1, vascular cell adhesion molecule-1.

Lymphocytes interact with Fn mainly via two main receptors which belong to the integrin family: the $\alpha 5\beta 1$ integrin is the receptor for the RGD and synergistic sites (42). The $\alpha 4\beta 1$ integrin is the receptor for HI (35), Hep II, and CS-1 (16, 18, 33, 52) and CS-5 (34).

The functional activity of most integrins can be regulated intracellularly by phorbol esters (45, 54) or by the cytoplasmic domain of some α and β chains (22, 39) as well as extracellularly. Among these external factors certain mAb directed to the β subunit have been shown to upregulate the function of most $\beta 1$, $\beta 2$, and $\beta 3$ integrins (reviewed in 20). These mAb most likely induce a conformational change on the receptor which increases the affinity/avidity for its ligands. Indeed, we and others have been able to measure this increase in the affinity of $\alpha 5\beta 1$ for an 80-kD Fn fragment containing the RGD sequence (2) or for intact Fn (11). The high avidity state can also be induced on solubilized integrins and thus acquisition of the activated form is an intrinsic property of the receptor (2, 6, 38). The existence of low and high affinity interactions with the extracellular matrix may play a crucial role for the migration and localization of lymphocytes.

Using one of these activating anti-$\beta 1$ mAb (TS2/16), we have recently shown that the $\alpha 4\beta 1$ integrin also exist on several states of activation among different hemopoietic cells

(44). The more active form, present on cultured lymphoid cells, was able to bind two fragments of 38 kD (contains CS-1 and Hep II) (13) and 58 kD (contains Hep II) (15–17) derived from the A and B chains of Fn, respectively. The less active form, present on monocytic cells and peripheral blood T lymphocytes, bound only the 38-kD fragment and thus recognized CS-1 but not Hep II. mAb TS2/16 converted this $\alpha4\beta1$ form to a more active one able to recognize both sites. These previous observations therefore indicate that there are cell populations which constitutively express a low or intermediate avidity $\alpha4\beta1$ form unable to recognize some of its ligands. In view of these results it was conceivable that activation of the receptor would lead to the recognition of new low affinity ligands not previously identified.

In the present report we have studied whether $\alpha4\beta1$ activation via the $\beta1$ subunit affects the ligand specificity of this receptor. We show that $\alpha5\beta1$-negative B lymphoid cells that do not bind constitutively to the 80-kD Fn fragment, efficiently attach to this fragment upon incubation with mAb TS2/16. We have identified the $\alpha4\beta1$ integrin as the receptor that mediates adhesion to the 80-kD fragment and we show that the RGD sequence in Fn is a novel ligand for activated $\alpha4\beta1$.

Materials and Methods

ECM Proteins, Fragments, and Synthetic Peptides

Human plasma Fn was the generous gift of Drs. B. Horowitz and R. Shulman (New York Blood Center, New York). Fragments of 80, 58, 38, 31, and 29 kD (see Fig. 1) were obtained from tryptic digestions of Fn and purified exactly as previously described (13–15). Purity of the 80-kD fragment was assessed by SDS-PAGE, Western blots, ELISA, and NH_2-terminal amino acid sequencing. Amino acid sequence analyses were performed by Mr. Javier Varela at the Protein Chemistry Laboratory, Centro de Investigaciones Biológicas (Madrid, Spain), using a 477A liquid-phase sequencer (Applied Biosystems, Inc., Foster City, CA). The 80-kD fragment was cleaved with pepsin (1:100 wt/wt, 37°C, 1 h) as described (40). Peptic digests were resolved by fast protein liquid chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden) using a Mono Q ion exchange column (Pharmacia LKB Biotechnology) equilibrated in 10 mM Tris pH 7.0, buffer (A). Bound fragments were eluted by applying a 45-min gradient from 100% A to 40% A–60% B (10 mM Tris, 500 mM NaCl, pH 7.0). Characterization of peptic fragments was achieved by SDS-PAGE, Western blots, ELISA, and NH_2-terminal amino acid sequencing. Purified fragments were dialyzed versus PBS and stored at −70°C. The following synthetic peptides were purchased from Bio-Synthesis D&I (Madrid, Spain): GRGDSPC and GRGES, containing sequences from the central cell-binding domain; DELPQLVTLPHPNLHGPEILDVPSTC (CS-1), PSTVQKTPFVTHPGYDTGNGIQLPG (CS-2), and, CIQLPGTSGQQPSVGQQMIFEEHGFR (CS-3), containing sequences from the IIICS region, and IDAPS containing an active sequence present in the Hep II domain (35). Laminin and collagen type I were purchased from Sigma Chemical Co. (Saint Louis, MO).

Monoclonal Antibodies

Fn-specific mAbs P1F11 (anti-CS-1) and P3D4 (anti-Hep II domain) were produced as reported (17). mAbs N-295 and N-296 were originally purchased from Mallinckrodt Specialty Chemicals (Maryland Heights, MO) and are now available through Chemicon International, Inc. (MAB1934 and MAB1935, respectively; Temecula, CA). mAb N-295 reacts with the 11-kD fragment that contains the RGD sequence of fibronectin (40) and partially inhibits cell adhesion (32). mAb N-296 reacts with a carboxy-terminal fragment that contains the Fib II domain and does not affect cell adhesion (32). mAbs to the $\alpha4$ integrin subunit HP1/1 (epitope A), HP2/1 (epitope B1), and mAbs Alex 1/4 and TS2/16 specific for the $\beta1$ subunit were obtained from Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). mAbs P1D6 (anti-$\alpha5$) and P1B5 (anti-$\alpha3$) were obtained from Dr. E. A. Wayner (University of Minnesota, Minneapolis, MN). mAb LM609 (anti-$\alpha V\beta3$) was obtained from Dr. D. Cheresh (Scripps Clinic, San Diego, CA). mAb IIF5 (anti-$\alpha3$) was obtained from Dr. M. Hemler (Dana Farber Cancer Institute, Boston, MA). mAb D3/9 (anti-CD45) was obtained from Dr. C. Bernabeu (Centro de Investigaciones Biólogicas, Madrid, Spain).

Cells and Cell Culture

The human cell lines Daudi, RPMI 8866, JY (B lymphoblastoid), and K562 (erythroleukemia) were obtained from Drs. F. Sánchez-Madrid and C. Bernabeu. The melanoma cell line A375 was obtained from Dr. J. Teixidó (Centro de Investigaciones Biológicas). The B cell line Ramos and the monocytic cell line U937 were obtained from the American Type Culture Collection (Rockville, MD). Cells were expanded in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Middlesex, UK), 2 mM L-glutamine, 100 U/ml penicillin (Antibióticos, Madrid, Spain), and 24 μg/ml gentamycin (Llorente, Madrid, Spain). In general, three/four-day cultures were used for assays.

Cell Attachment Assays

These assays were performed exactly as described (44) using flat bottom, high binding, 96-well plates (Costar Corp., Cambridge, MA). Quantitation of cell attachment was done by determining the absorbance at 620 nm on a Multiskan Bichromatic plate reader (Labsystems, Helsinki, Finland) and using calibration curves as described (44). Integrin activation through the $\beta1$ subunit was performed by incubating the cells with a 1:10 dilution of supernatant containing anti-$\beta1$ mAbs for 15–20 min at 37°C prior to the attachment assay. For inhibition experiments cells were incubated with anti-integrin antibodies (15 min, 37°C) or synthetic peptides or fragments (30 min, room temperature) prior to adding to substrate-coated wells. Inhibition of cellular attachment by antibodies to Fn was determined after incubation of 80- or 38-kD–coated wells with 50 μl of appropriate antibody dilutions for 30 min at room temperature; 50 μl of cell suspension was then added and the assay continued as described above.

Immunofluorescence Analyses

Cells (5×10^5) were incubated for 30 min at 4°C with 100 μl of culture supernatants (1:2 dilution) or ascites (1:500 dilution) containing the appropriate mAb. Cells were washed twice with cold PBS-1% BSA and resuspended in 100 μl of a 1/100 dilution of fluorescein-conjugated $F(ab')_2$ fragments of rabbit antibodies to mouse IgG (Dakopatts, Glostrop, Denmark). After 30 min at 4°C cells were washed twice, resuspended in PBS, and analyzed by flow cytometry on an EPICS-CS (Coulter Cientifica, Móstoles, Spain).

ELISA Assays

These assays were performed as described (17) with the following modifications to improve background: Fn fragments used for coating wells were in 0.1 M sodium borate, pH 8.5 (instead of PBS); plates were washed with 0.1% Tween 20 (instead of 0.05%); incubation with antibodies was done at room temperature (instead of 37°C). Absorbance at 492 nm was determined after 10 and 30 min using a microplate reader.

Results

Fibronectin Fragments That Contain the Central (RGD-dependent) or Carboxy-terminal (RGD-independent) Cell-binding Domains

The Fn tryptic fragments of 38 and 80 kD used in the present study have been previously characterized and are schematically shown in Fig. 1. The 38-kD fragment, derived from the carboxy-terminal region of the A chain of Fn, contains the Hep II domain and part of the IIICS region including the CS-1 site (13, 15, 17). The 80-kD fragment, derived from the central region of both chains of Fn, contains a low-affinity heparin and DNA-binding domain, the RGD sequence and the RGD-dependent synergistic sites (56). Both fragments are contiguous in the Fn sequence.

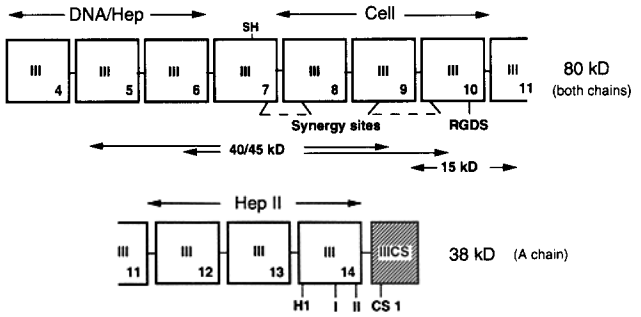

Figure 1. Schematic drawing of the 38- and 80-kD Fn tryptic fragments used in this study. Fibronectin type III homology repeats contained in these fragments are indicated and numbered. The 80-kD fragment contains a DNA/heparin-binding domain and a cell-binding domain composed of the RGD sequence and the synergistic regions. The location of the peptic fragments 40/45 and 15 kD is indicated. The 38-kD fragment is derived from the A chain of Fn and contains the HepII domain and part of the IIICS region. The location of the active sites within these regions is indicated. The CS-5 site (see Introduction) is not contained in the 38-kD fragment. Both fragments are contiguous in the Fn sequence.

Analysis of the 80-kD fragment rendered the NH_2-terminal sequence SDVPTS. Further characterization of this fragment was achieved using two recently developed mAbs with specificity for the Hep II (P3D4) and CS-1 (P1F11) regions, respectively (17) as well as a commercial mAb (N-295) which reacts with a site near the RGD sequence in Fn (32). As shown in Fig. 2, using an ELISA assay, mAb N-295 reacted strongly with the 80-kD fragment while mAbs P3D4 and P1F11 were negative. These two mAbs also failed to recognize the 80-kD fragment on Western blots (17). mAbs P3D4 and P1F11 did react with the 38-kD fragment used as control while mAb N-295 did not (Fig. 2). Altogether these results clearly show that the 80-kD fragment contains the RGD sequence and that there are no contaminants containing the $\alpha 4\beta 1$ ligands Hep II and CS-1 in the 80-kD fragment preparation.

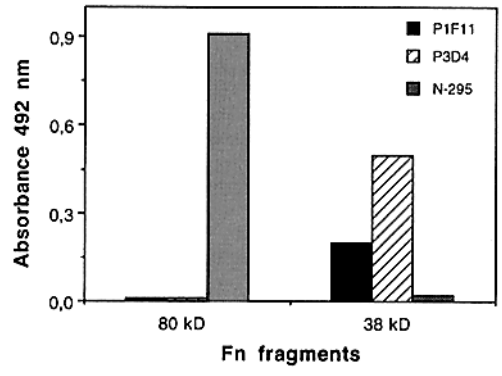

Figure 2. ELISA analysis of the reactivity of mAb P1F11 (anti-CS-1), P3D4 (anti-HepII), and N-295 (anti-RGD) with purified 80- and 38-kD Fn fragments. Wells were coated with 80 (4 μg) or 38 kD (2 μg) overnight and incubated with mAb P1F11 (no dilution), P3D4 (1:5 dilution), or N-295 (1:250 dilution). Quantitation of the reaction was done by measuring the absorbance at 492 nm. All determinations were done in duplicates.

Pepsin digestion of the 80-kD fragment rendered two fragments of interest (Fig. 1): a 15-kD fragment that was positive on Western blots and ELISA with mAb N-295 (not shown) and thus contained the RGD sequence. The 15-kD fragment had the NH_2-terminal sequence: IGQQ (end of repeat III-9); a 40/45-kD fragment that rendered two sequences: VLV(R)WTPP (beginning at residue 18 in repeat III-5) and IQVLRDGQ (end of repeat III-6). The 40/45-kD fragment did not react with mAb N-295 (not shown) and thus did not contain the RGD sequence.

Adhesion of $\alpha 5\beta 1$-negative B Cell Lines to the 80-kD Fn Fragment Following Incubation with Anti-$\beta 1$ mAb TS2/16

The following experiments were undertaken to determine whether mAb TS2/16 affects not only the affinity of the receptor(s) but also its specificity thus resulting in the recognition of novel ligands. To this purpose we performed attachment assays using several Fn fragments not previously shown to support B lymphoid cell adhesion. These include the 80-kD fragment shown in Fig. 1 and two fragments of 29 and 31 kD derived from the amino- and carboxy-terminal regions of Fn, respectively (not shown in Fig. 1). The various cell populations used in these studies and their pattern of integrin expression are listed in Table I. As can be observed none of the four B cell lines studied expressed the $\alpha 5$ integrin subunit on their surface in agreement with previous reports (5, 16, 47). As shown in Fig. 3, resting Ramos or Daudi cells or cells incubated with the control anti-$\beta 1$ mAb Alex1/4 did not attach to the 80-kD fragment. However, preincubation of Ramos or Daudi cells with mAb TS2/16 resulted in a dose-response attachment to the 80-kD fragment (Fig. 3). Incubation of Ramos cells with mAb TS2/16 always resulted in a more efficient adhesion (60%) to the 80-kD fragment than incubation of Daudi cells (40%), a result consistent with the higher $\beta 1$ expression on Ramos cells (Table I). The surface expression of $\beta 1$ integrins did not change upon treatment with mAb TS2/16 (1, 2, and this report, not shown). mAb TS2/16 did not induce binding of Ramos or Daudi cells to

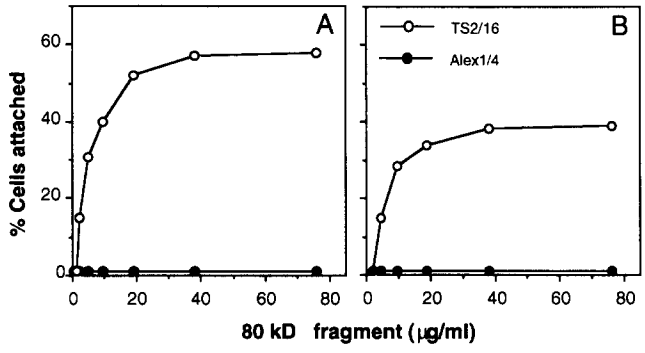

Figure 3. Adhesion of Ramos (*A*) and Daudi (*B*) B lymphoid cells to the 80-kD fragment. Cells were preincubated with either mAb Alex1/4 (*closed circles*) or mAb TS2/16 (*open circles*) and added to wells coated with the indicated concentrations of 80-kD fragment (5×10^4 cells/well). After 30 min at 37°C, attached cells were stained with 0.1% toluidine blue and quantitated as described in Materials and Methods. Each determination was done in duplicate and values represent the average of three different experiments.

Table I. Expression of Integrin Subunits on the Cell Lines Studied

Cells	Mean fluorescence intensity						
	Control	α2	α3	α4	α5	αVβ3	β1
Ramos	35	35	45	95	35	36	113
Daudi	40	ND	39	96	40	43	76
JY	49	48	47	103	46	71	51
RPMI 8866	43	43	42	107	41	61	50
K562	47	ND	45	46	78	56	100
A375	43	89	82	80	68	102	123

the 29- or 31-kD fragments at any of the concentrations tested (not shown).

To confirm the specificity of the effect of mAb TS2/16 and the involvement of β1 integrins on the recognition of the 80-kD fragment, we studied the adhesion of the B cell lines JY and RPMI 8866 to this fragment. These cells were chosen because they do not express surface β1 integrin subunit (Table I and reference 5, 47, 48). Instead JY and RPMI 8866 cells express an alternative β chain called β7, which is associated with the α4 subunit (5, 41). As shown in Fig. 4, mAb TS2/16 did not induce adhesion of JY or RPMI 8866 cells to the 80-kD fragment. JY cells constitutively attached to the 80-kD fragment and mAb TS2/16 did not affect this constitutive binding (Fig. 4).

Effect of PMA on Adhesion of B Cells to the 80-kD Fragment: Involvement of Two Different Receptors

To determine whether phorbol myristate acetate (PMA) could reproduce the effect of mAb TS2/16, cells were incubated with 50 ng/ml of PMA for 20 min and tested for adhesion to 80-kD coated wells. As shown in Fig. 4, PMA did not induce attachment of either Ramos or Daudi cells to the 80-kD fragment while on the same experiment mAb TS2/16 was an effective inducer (Fig. 4). PMA did not affect either the constitutive adhesion of these cells to the 38-kD fragment (not shown). In contrast to these results, PMA was an effective activator of JY and RPMI 8866 cells. As shown in Fig. 4, PMA induced de novo adhesion of RPMI 8866 cells to the 80-kD fragment and increased the constitutive binding of JY cells to this fragment. No changes on integrin surface expression were detected after PMA treatment (not shown).

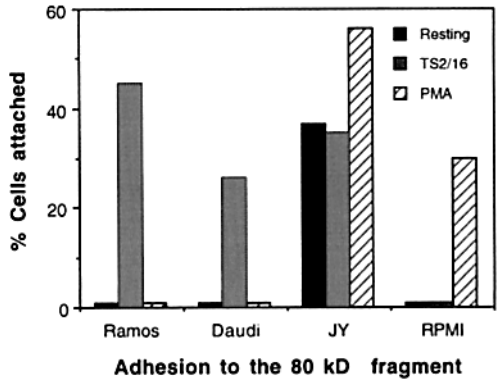

Figure 4. Effect of PMA and mAb TS2/16 on the attachment of several B cell lines to the 80-kD fragment. Cells were incubated with either cell culture medium (*resting*), PMA (50 ng/ml), or mAb TS2/16 (1:10 dilution of supernatant) for 15 min at 37°C and added to 80-kD coated wells (38 μg/ml, 5 × 10^4 cells/well). After 30 min at 37°C, attached cells were quantitated as described. Values are the average of three different experiments.

These results suggested the involvement of two different B cell receptors for the 80-kD fragment, one of them being regulated by mAb TS2/16 and the other by PMA. It was recently demonstrated that the αVβ3 integrin functions as a Fn receptor on some B lymphoid cells (48). To establish whether αVβ3 was the receptor for the 80-kD fragment on JY and RPMI 8866 cells, we performed attachment assays to this fragment in the presence of anti-αVβ3 mAb LM609. In results not shown, mAb LM609 completely inhibited (100%) the adhesion of resting JY and PMA-treated RPMI 8866 cells to the 80-kD fragment, and partially inhibited (45%) the attachment of PMA-treated JY cells to this fragment. These results therefore confirm the involvement of αVβ3 on adhesion of β1-negative B cells to the 80-kD fragment and suggest a role for yet another nonidentified receptor on PMA-stimulated JY cells. mAb LM609 had no effect on the adhesion of Ramos or Daudi cells to the 80-kD fragment (Fig. 5) in agreement with the lack of expression of αVβ3 on these cells (Table I).

Identification of the α4β1 Integrin as a New Receptor for the 80-kD Fragment

The preceding results clearly indicate that adhesion of Ramos and Daudi cells to the 80-kD fragment is regulated through the β1 subunit. To identify the receptor involved in adhesion to this fragment, cell attachment assays were carried out in the presence of several anti-integrin mAbs. As shown in Fig. 5, two anti-α4 mAbs, HP2/1 and HP1/1, inhibited TS2/16-treated Ramos (and TS2/16-treated Daudi, not shown) cell adhesion to the 80-kD fragment. mAbs P1D6

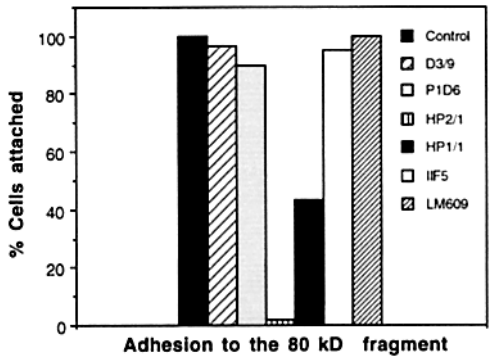

Figure 5. Effect of several anti-integrin or control mAb on attachment of TS2/16-treated Ramos cells to the 80-kD fragment. Cells were incubated with mAb TS2/16 (15 min at 37°C) followed by incubation with the indicated mAb for another 15 min. The dilutions used were 1:10 for mAb used as culture supernatants (*D3/9, PID6, HP2/1,* and *HP1/1*) and 1:500 for those used as ascitic fluid (IIF5 and LM609). Cells (5 × 10^4/well) were added to 80-kD coated wells (38 μg/ml) and after 30 min at 37°C, attached cells were quantitated as described. Values on the ordinate represent the percentage of attached cells relative to the number of cells on control wells (TS2/16, no inhibitor). Values are the means of two different experiments.

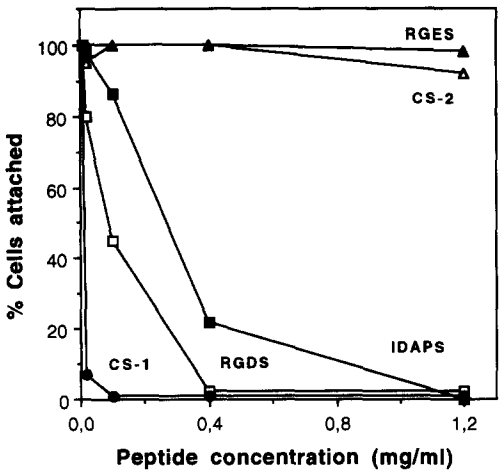

Figure 6. Inhibition of Ramos cell attachment to the 80-kD fragment by synthetic peptides. Cells were incubated with mAb TS2/16 15 min at 37°C followed by 30-min incubation at room temperature with the indicated dilutions of synthetic peptides. Cells (5×10^4/well) were added to 80-kD coated wells (19 μg/well) and attached cells were quantitated after 30 min at 37°C. Values on the ordinate are expressed as percentage of attached cells relative to the number of cells on control wells (TS2/16, no inhibitor). Values are the means of three different experiments.

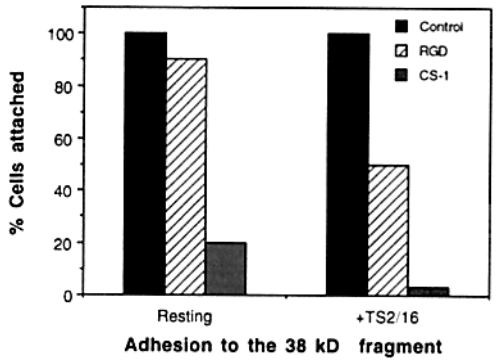

Figure 7. Effect of the GRGDSPC synthetic peptide on Ramos cell attachment to the 38-kD fragment. Cells were either untreated (*Resting*) or treated with mAb TS2/16 (15 min, 37°C) and incubated 30 min at room temperature with 0.5 mg/ml of GRGDSPC or CS-1 peptides. Cells were added to wells coated with 0.8 μg/ml (*Resting*) or 0.6 μg/ml (TS2/16 activated) of 38-kD fragment. The number of cells on control wells (no inhibitor) were 35.000 (*Resting*) and 29.000 (TS2/16 treated). The number of attached cells relative to control wells was quantitated as described. Values are the means of two different experiments.

(anti-α5), IIF5 (anti-α3), LM609 (anti-αVβ3), or the control D3/9 (anti-CD45) had no effect on these assays (Fig. 5).

The effect of the synthetic peptides CS-1 and IDAPS on TS2/16-treated Ramos cell adhesion to the 80-kD fragment was also tested. As shown in Fig. 6, CS-1 or IDAPS peptides produced a dose-dependent inhibition of cell attachment to the 80-kD fragment, while the control peptides CS-2 (Fig. 6) and CS-3 (not shown) had no effect. Interestingly, the synthetic peptide GRGDSPC was also completely inhibitory in these assays while the control GRGES peptide had no effect (Fig. 6). The calculated concentration (nM) of peptide required to produce 50% inhibition of cell attachment to the 80-kD fragment was 5.45 (CS-1, 15 μg/ml), 104 (GRGDSPC, 80 μg/ml), and 400 (IDAPS, 220 μg/ml). Thus, CS-1 was the most effective inhibitor and the RGD-containing peptide was a better inhibitor than the reported α4β1 ligand IDAPS.

The RGD Sequence is a Ligand for TS2/16-activated α4β1 Integrin

The above results therefore suggested that the conformational change induced in α4β1 by mAb TS2/16 results in the ability to recognize the RGD sequence. To prove this, we tested the capacity of the GRGDSPC synthetic peptide to inhibit the adhesion of resting and TS2/16-activated Ramos cells to the 38-kD fragment. As shown in Fig. 7, resting Ramos cell adhesion to the 38-kD fragment was not affected (90% specific adhesion) by the GRGDSPC peptide (0.5 mg/ml) in agreement with our previous report (16). However upon incubation with mAb TS2/16, the GRGDSPC peptide (0.5 mg/ml) inhibited cell adhesion to the 38-kD fragment by 50% (Fig. 7). As expected, the CS-1 peptide used as control was a good inhibitor in both cases. These results clearly show that soluble RGD-containing peptides can effectively inhibit the function of activated α4β1 and that activated α4β1 (but not resting α4β1) acquires the capacity to recognize the RGD sequence.

As shown in Table II, adhesion of TS2/16-treated Ramos cells to the 80-kD fragment was also inhibited by soluble 80-, 38-, and 15-kD (RGD site) fragments. It was poorly inhibited by the 40/45-kD fragment (no RGD). Direct binding to the 15-kD fragment however could not be demonstrated probably due to the low affinity displayed by this fragment when used as substrate. To further confirm that there was a specific ligand for α4β1 within the 80-kD fragment, we carried out cell attachment assays in the presence of a panel of anti-Fn mAbs. As shown in Fig. 8, mAb N-295 which recognizes an epitope near the RGD sequence, inhibited (80%) Ramos cell adhesion to the 80-kD fragment while mAb N-296 (anti-Fib II) and P3D4 (anti-Hep II) had no effect. These results together with the data shown in Fig. 7, indicate that α4β1 interaction with the 80-kD fragment is primarily mediated by the RGD sequence. Because inhibition by mAb N-295 was not complete (Fig. 8), a small contribution from other sites in the 80-kD fragment cannot be completely ruled out.

mAb TS2/16-induced Recognition of New Ligands May Be Specific for Fn and for the α4β1 Integrin

We next tested whether mAb TS2/16 could induce the recog-

Table II. Effect of Fn Fragments on Adhesion of TS2/16-treated Ramos Cells to the 80-kD Fragment

	% Inhibition				
80 kD Coating concentration μg/ml	80 kD 4.5*	38 kD 3	15 kD 4.5	45 kD 1.8	31 kD 3
19	80	100	85	10	2
38	70	100	50	0	0

* Nanomoles of fragments used in the incubation mixture.

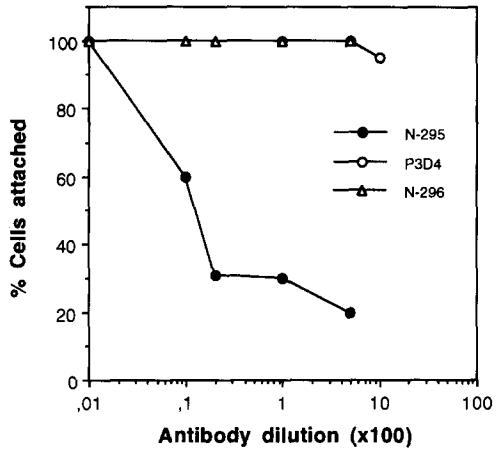

Figure 8. Effect of anti-Fn antibodies on TS2/16-treated Ramos cell adhesion to the 80-kD fragment. The indicated dilutions of N-295 (anti-RGD), P3D4 (anti-Hep II), or N-296 (anti-Fib II site, control) were added to 80-kD fragment coated wells (38μg/ml) and incubated for 30 min at room temperature. Cells previously incubated with mAb TS2/16 (15 min at 37°C) were added and attachment was quantitated as described. Values are expressed as percentage relative to the number of cells on control wells (no inhibitor) and are the average of two different experiments.

nition of other extracellular matrix (ECM) proteins not previously known to interact with α4β1. To this purpose, we performed cell attachment assays using collagen and laminin as substrata. These two proteins are known ligands for other β1 integrins including α2β1, α3β1, and α6β1 (20). As shown in Fig. 9, incubation of Ramos cells with mAb TS2/16 did not result in adhesion to either laminin or collagen. Because α4β1 is the main integrin expressed on Ramos cells (Table I) these results indicate that mAb TS2/16 does not induce α4β1 recognition of laminin or collagen. To confirm that it was enough substrata on the plates to support cell attachment we used the melanoma cell line A375 as control. A375 cells express several β1 integrins (Table I) including the laminin receptor α6β1 (1). As shown in Fig. 9, A375 cells constitutively bound to the 80-kD fragment and showed low constitutive binding to laminin and collagen. Preincubation with mAb TS2/16 effectively increased A375 cell adhesion to all three substrata (Fig. 9) in agreement with previous reports (1, 2). This effect was not due to cross-linking of cells to ECM proteins via the TS2/16 antibody because in control experiments mAb TS2/16 did not bind to either Fn, laminin, or collagen (results not shown). Increased adhesion of A375 cells to ECM proteins was rather due to the upregulation of α5β1, α2β1, and α6β1 integrins which are receptors for the 80-kD Fn fragment, collagen, and laminin, respectively (1, 2, 6). As expected, RPMI 8866 and JY cells, which lack β1, did not bind to laminin or collagen in response to incubation with mAb TS2/16 (not shown).

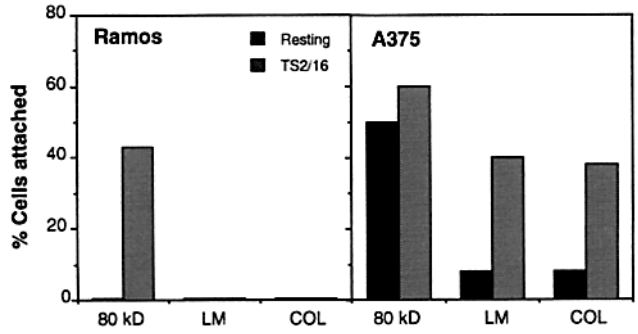

Figure 9. Effect of mAb TS2/16 on cell adhesion to laminin and collagen type I. Ramos or control A375 melanoma cells were incubated with mAb TS2/16 prior to adding to 80 kD (38 μg/ml), laminin (*LM*, 150 μg/ml), or type I collagen (*COL*, 150 μg/ml) coated wells. After 30 min at 37°C attached cells were quantitated as described. Values on the ordinate represent the number of attached cells relative to the total cell input (5×10^4/well) and are the means of two different experiments.

To determine whether the effect of mAb TS2/16 on α4β1 was also observed on other integrins such as α5β1, K562 cells were incubated with this mAb and tested for adhesion to the 38-kD fragment. K562 cells do not express α4β1 (Table I) and consequently they do not bind to the 38-kD fragment constitutively. mAb TS2/16 did not induce adhesion of K562 cells to the 38-kD fragment although this mAb effectively enhanced their constitutive binding to the 80-kD fragment via α5β1 (results not shown). Thus the capacity to recognize new ligands upon activation via β1 may be an intrinsic property of the α4β1 integrin.

Discussion

The major conclusions of this report are: (*a*) α5β1-negative human B lymphoid cells, which do not bind constitutively to the 80-kD fragment of Fn, acquire the capacity to attach to this fragment upon incubation with anti-β1 mAb TS2/16; (*b*) the B lymphoid cell receptor that interacts with the 80-kD fragment is the α4β1 integrin; (*c*) the RGDS sequence is a ligand for TS2/16-activated α4β1 integrin, this indicates that the conformational change induced on α4β1 by mAb TS2/16 results in the ability to recognize RGD; and (*d*) α4β1 on B lymphoid cells can be induced to recognize novel ligands in Fn but not in laminin or type I collagen.

Our previous work has definitely established that some B lymphoid cells do not express the α5β1 integrin and are unable to attach to the 80-kD fragment containing the RGD site (16). In the present study we show that incubation of the α5β1-negative B cell lines Ramos and Daudi with anti-β1 mAb TS2/16 results in an efficient and specific attachment to the 80-kD fragment. Our results clearly show that adhesion of B cells to the 80-kD fragment is mediated by the α4β1 integrin. This was demonstrated by the ability of mAbs anti-α4 and the synthetic peptides CS-1 and IDAPS to inhibit cell attachment to the 80-kD fragment. Interestingly, the GRGDSPC synthetic peptide was also a good inhibitor of TS2/16-treated α4β1 function. We have conclusively shown in previous work that the interaction of α4β1 with its ligands, the 38- and 58-kD Fn fragments, is not affected by RGD-containing peptides (12, 15, 16, 52). Consistent with this Ramos (untreated or treated with a control mAb) cell attachment to the 38-kD fragment was not affected by the GRGDSPC peptide (this report, Fig. 7). As shown in the present studies, the RGD sequence not only inhibited TS2/16-treated α4β1 function in soluble form but was also the primary site that mediated attachment to the 80-kD fragment, as suggested by the good inhibition attained by N-295

mAb. However the contribution of other regions of the 80-kD which may act synergistically with RGD cannot be completely disregarded.

In a previous report (34) Mould et al., showed that $\alpha4\beta1$-mediated spreading of melanoma cells can be inhibited by RGD synthetic peptides and that these peptides may elute the $\alpha4\beta1$ integrin complex from a CS-1 affinity column. In contrast with these findings, we did not observe $\alpha4\beta1$ recognition of RGD-synthetic peptides unless previous incubation of B cells with mAb TS2/16. Although we cannot explain this discrepancy with the available data, it should be considered that a direct comparison between the work by Mould et al., and ours is difficult because of the different cell types and phenomena studied (spreading versus adhesion). Alternatively, it is possible that the melanoma cells used in reference 34 bear a constitutively activated form of $\alpha4\beta1$ sensitive to RGD-containing peptides.

Several studies have shown that certain anti-$\beta1$ mAbs enhance the function of some $\beta1$ integrins by inducing a conformational change which results in an activated form of the receptor with higher avidity/affinity for its ligands (1, 27, 49, 53). The activated form of the receptor may display broader ligand specificity than the resting form. For example, $\alpha2\beta1$ is a collagen receptor on platelets and fibroblasts and a collagen and laminin receptor on other cell types (8, 23, 29). A recent report has shown that, on the same cell, mAb TS2/16 can convert the partly active form of $\alpha2\beta1$ which recognizes only collagen to a fully active form that also binds laminin (6).

We and others have recently shown that $\alpha4\beta1$ also exists in several states of activation among different hemopoietic cells. Monocytic cells and PBL bear a partly active form of $\alpha4\beta1$ which recognizes CS-1 but not Hep II; mAb TS2/16 converted $\alpha4\beta1$ to a more active form (constitutively present on cultured lymphoid cells) able to recognize both ligands in Fn (44). In another study that compares the binding to CS-1 and vascular cell adhesion molecule-1 (VCAM-1), the less active form of $\alpha4\beta1$ bound only VCAM-1 while the TS2/16-activated form bound VCAM-1 and CS-1 (30). The phorbol ester PMA can also activate $\alpha4\beta1$ on U937 cells or PBL (44). It is interesting that in the present studies PMA did not induce Ramos or Daudi cell attachment to the 80-kD fragment. PMA did not enhance either the constitutive binding of these cells to the 38-kD fragment thus suggesting that the $\alpha4\beta1$ form present on Ramos and Daudi cells cannot be further activated by phorbol esters. A different regulation of $\alpha4\beta1$ function by anti-$\beta1$ mAbs and Ca^{2+} has already been observed (30). It has also been reported that PMA has no (or minimal) effect on the binding of $\alpha2\beta1$ to collagen or laminin while mAb TS2/16 was highly stimulatory (6). The fact that mAb TS2/16 can also activate solubilized $\beta1$ integrins (2, 6) is in accordance with the lack of requirement for an intracellular activation pathway. These observations support the existence of distinct mechanistic ways of regulating integrin function. Because PMA does increase $\alpha4\beta1$ function on some cells, it is possible that the sensitivity to a particular stimulus is determined by the activation state of the integrin or by the cell type or both.

These previous studies have analyzed the effect of anti-$\beta1$ mAbs or PMA on the interaction of $\alpha4\beta1$ with its already identified ligands (CS-1, Hep II, and VCAM-1). Similarly, although the study by Chan and Hemler (6) clearly shows changes in specificity according to the activation state of $\alpha2\beta1$, there are cell populations that bind laminin or/and collagen constitutively. The novelty of the present results is that mAb TS2/16 induces binding to a region of Fn (80-kD fragment) not previously identified as an $\alpha4\beta1$ ligand. In fact, the 80-kD fragment is a well described ligand for the $\alpha5\beta1$ integrin and we have extensively documented that the constitutive function of $\alpha4\beta1$ on leukocytes is RGD independent (12, 15, 16, 52). The activated form of $\alpha4\beta1$ described here not only binds CS-1 and Hep II with high avidity, but also recognizes RGD.

Although in general a single integrin binds several ligands, $\alpha4\beta1$ can bind multiple and apparently quite distinct sequences within the same or different molecules. Within Fn the minimal sequences shown to be $\alpha4\beta1$ ligands are LDV (contained in CS-1 [26, 53]), REDV (contained in CS-5 [34]), and IDAPS (contained in H1 [35]). It has been proposed that the common aspartate residue on these sequences is important for $\alpha4\beta1$ recognition (35). While this may be true, it is clear that other sequences may also be specific $\alpha4\beta1$ ligands. There is indirect evidence that the FNC/H I (YIKPGSPPREVVPRPRPGV) and FNC/H II (KNNQKSEPLIGRKKT) peptides derived from the Hep II Fn domain (31) may also interact with $\alpha4\beta1$ (21). All these sequences (CS1, CS5, H1, FNC/H I, and FNC/H II) are located in close proximity within the type III-14 repeat and the contiguous IIICS region of Fn (see Fig. 1). Besides Fn, $\alpha4\beta1$ is also the receptor for VCAM-1 (9), the bacterial protein invasin (10), and thrombospondin (55). Although the cell-binding site(s) for some of these proteins has not been identified, it seems clear that the interaction of $\alpha4\beta1$ with VCAM-1 does not involve the LDV motif present on this protein (37, 51).

The present results show that the conformational change induced in $\alpha4\beta1$ through the $\beta1$ subunit allows recognition of the Fn RGD sequence which is located in repeat III-10 and thus distant from the previously described $\alpha4\beta1$ ligands. Interestingly, Koivunen et al. (24, 25), using a phage display library, have recently identified several cyclic synthetic peptides which bind the $\alpha5\beta1$ integrin and do not contain the RGD motif. One of these peptides corresponds to the sequence STSDVGG which is homologous to the TVSDVPR sequence present in repeat III-10 of Fn. As noticed in that report, there is also some degree of homology with EILDVPST, a sequence present in CS-1 which we and others have shown to be a high affinity ligand for $\alpha4\beta1$ (16, 18, 33, 52). Although these authors did not show direct interaction of $\alpha5\beta1$ with either of the two homologous sequences present in Fn, their work suggests that $\alpha5\beta1$ has the potential to recognize other ligands in Fn besides RGD. Our present results are in agreement with a certain degree of overlapping in ligand recognition by the $\alpha4\beta1$ and $\alpha5\beta1$ integrins. This is also supported by another report that shows that a synthetic cyclic peptide, distinct from RGD, can inhibit the function of $\alpha4\beta1$ and $\alpha5\beta1$ integrins (36). While peptide cyclation may provide an optimal ligand conformation for high affinity interactions with either $\alpha4\beta1$ or $\alpha5\beta1$, it is clear from our present study that mAb TS2/16 does not induce $\alpha5\beta1$ binding to the CS-1 sequence of Fn. Therefore the ability to recognize new ligands upon conformational activation may be intrinsic to the $\alpha4\beta1$ structure.

The present report therefore describes a novel role for $\alpha4\beta1$ as a receptor for the RGD site in Fn. Because $\alpha5\beta1$

is the classical receptor for this site we have attempted to study the contribution of $\alpha4\beta1$ to cell adhesion to the 80-kD Fn fragment when both integrins are expressed together. In experiments not shown, we tested whether anti-$\alpha4$ mAbs could partially inhibit the binding of TS2/16-treated U937 cells to the 80-kD fragment. However, because mAb TS2/16 also effectively upregulates the adhesion mediated by $\alpha5\beta1$ (1, 2, and this study), we could not detect any inhibition with mAbs anti-$\alpha4$. It is possible that when $\alpha5\beta1$ and $\alpha4\beta1$ are present together, $\alpha5\beta1$ function predominates over $\alpha4\beta1$ function in mediating adhesion to Fn. In support of this, the 80-kD fragment or the GRGD synthetic peptide completely inhibit U937 cell adhesion to Fn while the 38-kD fragment or the CS-1 peptide produce partial or no inhibition (12). These results do not rule out a cooperation of both receptors for an efficient attachment to Fn as we have previously proposed for T lymphoid cells (15). The results reported here are obviously important for cells which express $\alpha4\beta1$ as the major surface integrin. These include B cells (16, 47), some B cell progenitors (43), and probably some tumor cells. Furthermore, there are reports showing different interactions with Fn during pre-B cell development (3, 50). On cells that express $\alpha5\beta1$ besides $\alpha4\beta1$, properly activated $\alpha4\beta1$ may serve to cooperate with $\alpha5\beta1$ or to functionally replace it when $\alpha5\beta1$ is already engaged.

It is now well documented that integrins undergo transitions among different states of activation (20) and that these transitions are induced by several agents including some mAbs as shown in the present report. The fact that the various integrin activation forms are also found in vivo, as clearly shown for $\alpha4\beta1$ for example (28), suggests that activating anti-$\beta1$ mAbs (TS2/16 and others) are mimicking the effect of physiological ligands not yet identified. On the other hand, the multiplicity of sequences recognized by $\alpha4\beta1$ must have physiological relevance. For example, it may serve to drive synergistic interactions with the receptor resulting in high affinity binding. Such synergy has already been shown for $\alpha5\beta1$ interaction with the RGD sequence in Fn (56). Similarly, activation of the platelet integrin $\alpha IIb\beta3$ with thrombin results in recognition of an additional Fn region (besides RGD) comprising part of homology repeats III-9 and III-10 (4, see Fig. 1). An interesting possibility is that some of the sequences that interact with $\alpha4\beta1$ may regulate $\alpha4\beta1$ binding to other ligands, as already shown for $\alpha IIb\beta3$ (7). The $\alpha4\beta1$ ligands described so far, including the RGD site identified in this report, apparently bind to identical or overlapping sites on $\alpha4\beta1$ because they are cross-inhibitory. However, it seems possible that other not yet identified ligands bind $\alpha4\beta1$ with low affinity or at a different site, and upregulate rather than inhibit the function of $\alpha4\beta1$. In support of this is the fact that two Fn sequences shown to interact with $\alpha4\beta1$, FNC/H I, and FNC/H II, do not (or slightly) interfere with $\alpha4\beta1$ adhesive function (21, 31). The phage display library approach used for identification of ligands for $\alpha5\beta1$ (24, 25) may be a useful one to identify new potential ligands for $\alpha4\beta1$. This would also open the possibility of using these modulatory sequences for therapeutic purposes. Moreover, because the avidity of $\alpha4\beta1$ for CS-1, Hep II, and RGD is different, binding to each of these sites may have different cellular consequences. Another implication of the present study is that physiologically stimulated $\alpha4\beta1$ may play an important role for the recruitment of lymphocytes at inflammatory sites and tissues where Fn fragments containing CS-1, Hep II, or/and RGD are produced. While these possibilities require further study, our results highlight the role of $\alpha4\beta1$ as a flexible receptor able to recognize multiple ligands and support the importance of the extracellular matrix, particularly Fn, on lymphocyte development and function.

We thank Drs. C. Bernabeu, D. Cheresh, M. Hemler, F. Sánchez-Madrid, J. Teixidó, and E. A. Wayner, for providing cells and mAbs; Ana Gutiérrez for excellent technical assistance; and Pedro Lastres for performing the flow cytometry analyses.

This work was supported by Grants SAL91-0785 from Comisión Interministerial de Ciencia y Tecnología and 133/92 from Comunidad Autónoma de Madrid, Madrid, Spain, and BMH1-CT92-0376 from the European Economic Community, Biomed 1 Program. P. Sánchez-Aparicio is the recipient of a fellowship from the Ministerio de Educación y Ciencia, Madrid; C. Dominguez-Jiménez is the recipient of a fellowship from Comisión Interministerial de Ciencia y Tecnología.

Received for publication 28 January 1994 and in revised form 25 March 1994.

References

1. Arroyo, A. G., P. Sánchez-Mateos, M. R. Campanero, J. Martin-Padura, E. Dejana, and F. Sanchez-Madrid. 1992. Regulation of the VLA integrin-ligand interaction through the β_1 subunit. *J. Cell Biol.* 117: 659–670.
2. Arroyo, A. G., A. Garcia-Pardo, and F. Sánchez-Madrid. 1993. A high affinity conformational state on VLA integrin heterodimers induced by an anti-$\beta1$ chain monoclonal antibody. *J. Biol. Chem.* 268:9863–9868.
3. Bernardi, P., V. P. Patel, and H. Lodish. 1987. Lymphoid precursor cells adhere to two different sites on fibronectin. *J. Cell Biol.* 105:489–498.
4. Bowditch, R. D., C. E. Halloran, S.-I. Aota, M. Obara, E. F. Plow, K. M. Yamada, and M. H. Ginsberg. 1991. Integrin $\alpha IIb\beta3$ (platelet GPIIb-IIIa) recognizes multiple sites in fibronectin. *J. Biol. Chem.* 266:23323–23328.
5. Chan, B. M. C., M. J. Elices, E. Murphy, and M. E. Hemler. 1992. Adhesion to vascular cell adhesion molecule 1 and fibronectin. Comparison of $\alpha4\beta1$ and $\alpha4\beta7$ on the human B cell line JY. *J. Biol. Chem.* 267: 8366–8370.
6. Chan, B. M. C., and M. E. Hemler. 1993. Multiple functional forms of the integrin VLA-2 can be derived from a single α^2 cDNA clone: interconversion of forms induced by an anti-β_1 antibody. *J. Cell Biol.* 120: 537–543.
7. Du, X., E. F. Plow, A. L. Frelinger, III, T. E. O'Toole, J. C. Loftus, and M. H. Ginsberg. 1991. Ligands "activate" integrin $\alpha IIb\beta3$ (platelet GPIIb-IIIa). *Cell.* 65:409–416.
8. Elices, M. J., and M. E. Hemler. 1989. The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. *Proc. Natl. Acad. Sci. USA.* 86:9906–9910.
9. Elices, M. J., L. Osborn, Y. Takada, C. Crouse, S. Luhowsky, M. E. Hemler, and R. R. Lobb. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell.* 60:577–584.
10. Ennis, E., R. R. Isberg, and Y. Shimizu. 1993. Very late antigen-4-dependent adhesion and costimulation of resting human T cells by the bacterial $\beta1$ integrin ligand invasin. *J. Exp. Med.* 177:207–212.
11. Faull, R. J., N. L. Kovach, J. M. Harlan, and M. H. Ginsberg. 1993. Affinity modulation of integrin $\alpha5\beta1$: regulation of the functional response by soluble fibronectin. *J. Cell Biol.* 121:155–162.
12. Ferreira, O. C. Jr., A. Garcia-Pardo, and C. Bianco. 1990. Specific binding of the human monocytic cell line U937 to the alternatively spliced connecting segment (IIICS) of fibronectin. *J. Exp. Med.* 171:351–356.
13. 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.
14. Garcia-Pardo, A., O. C. Ferreira, J. E. Valinsky, and C. Bianco. 1989. Fibronectin receptors of mononuclear phagocytes: binding characteristics and biochemical isolation. *Exp. Cell Res.* 181:420–431.
15. Garcia-Pardo, A., and O. C. Ferreira, Jr. 1990. Adhesion of human T lymphoid cells to fibronectin is mediated by two different fibronectin domains. *Immunology.* 69:121–126.
16. Garcia-Pardo, A., E. A. Wayner, W. G. Carter, and O. C. Ferreira. 1990. Human B lymphocytes define an alternative mechanism of adhesion to fibronectin: the interaction of the $\alpha4\beta1$ integrin with the LHGPEILDV-

PST sequence of the type III connecting segment is sufficient to promote cell attachment. *J. Immunol.* 144:3361–3366.
17. Garcia-Pardo, A., P. Sánchez-Aparicio, and E. A. Wayner. 1992. Two novel monoclonal antibodies to fibronectin that recognize the Hep II and CS-1 regions respectively: their differential effect on lymphocyte adhesion. *Biochem. Biophys. Res. Commun.* 186:135–142.
18. Guan, J.-L., and R. O. Hynes. 1990. Lymphoid cells recognize an alternative spliced segment of fibronectin via the integrin receptor $\alpha4\beta1$. *Cell.* 60:53–61.
19. Humphries, M. J., A. Komoriya, S. K. Akiyama, K. Olden, 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.
20. Hynes, R. O. 1992. Integrins: versatility, modulation and signaling in cell adhesion. *Cell.* 69:11–25.
21. Iida, J., A. P. N. Skubitz, L. T. Furcht, E. A. Wayner, and J. B. McCarthy. 1992. Coordinate role for cell surface chondroitin sulfate proteoglycan and $\alpha4\beta1$ integrin in mediating melanoma cell adhesion to fibronectin. *J. Cell Biol.* 118:431–444.
22. Kassner, P. D., and M. E. Hemler. 1993. Interchangeable α chain cytoplasmic domains play a positive role in control of cell adhesion mediated by VLA-4, a β_1 integrin. *J. Exp. Med.* 178:649–660.
23. Kirchhofer, D., L. R. Languino, E. Ruoslahti, and M. D. Pierschbacher. 1990. $\alpha2\beta1$ integrins from different cell types show different binding specificities. *J. Biol. Chem.* 265:615–618.
24. Koivunen, E., D. A. Gay, and E. Ruoslahti. 1993. Selection of peptides binding to the $\alpha5\beta1$ integrin from phage display library. *J. Biol. Chem.* 268:20205–20210.
25. Koivunen, E., B. Wang, and E. Ruoslahti. 1994. Isolation of a highly specific ligand for the $\alpha_5\beta_1$ integrin from a phage display library. *J. Cell Biol.* 124:373–380.
26. Komoriya, A., L. J. Green, M. Mervic, S. S. Yamada, K. M. Yamada, and M. J. Humphries. 1991. The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. *J. Biol. Chem.* 266:15075–15079.
27. Kovach, N. L., T. M. Carlos, E. Yee, and J. M. Harlan. 1992. A monoclonal antibody to β_1 integrin (CD29) stimulates VLA-4-dependent adherence of leukocytes to human umbilical vein endothelial cells and matrix components. *J. Cell Biol.* 116:499–509.
28. Laffón, A., R. Garcia-Vicuña, A. Humbría, A. A. Postigo, A. L. Corbí, M. O. de Landázuri, and F. Sánchez-Madrid. 1991. Upregulated expression and function of VLA-4 fibronectin receptor on human activated T cells in rheumatoid arthritis. *J. Clin. Invest.* 88:546–552.
29. Languino, L. R., K. R. Gehlsen, E. A. Wayner, W. G. Carter, E. Engvall, and E. Ruoslahti. 1989. Endothelial cells use $\alpha2\beta1$ integrin as a laminin receptor. *J. Cell Biol.* 109:2455–2462.
30. Masumoto, A., and M. E. Hemler. 1993. Multiple activation states of VLA-4. Mechanistic differences between adhesion to CS1/fibronectin and to vascular adhesion molecule-1. *J. Biol. Chem.* 268:228–234.
31. McCarthy, J. B., M. K. Chelberg, D. J. Mikelson, and L. T. Furcht. 1988. Localization and chemical synthesis of fibronectin peptides with melanoma adhesion and heparin binding activities. *Biochemistry.* 27:1380–1388.
32. McDonald, J. A., B. J. Quade, T. J. Broekelmann, R. LaChance, K. Forsman, E. Hasegawa, and S. Akiyama. 1987. Fibronectin's cell-adhesive domain and an amino-terminal matrix assembly domain participate in its assembly into fibroblast pericellular matrix. *J. Biol. Chem.* 262: 2957–2967.
33. Mould, A. P., L. A. Wheldon, A. Komoriya, E. A. Wayner, K. M. Yamada, and M. J. Humphries. 1990. Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin $\alpha4\beta1$. *J. Biol. Chem.* 265:4020–4024.
34. Mould, A. P., A. Komoriya, K. M. Yamada, and M. J. Humphries. 1991. The CS5 peptide is a second site in the IIICS region of fibronectin recognized by the integrin $\alpha4\beta1$. Inhibition of $\alpha4\beta1$ function by RGD peptide homologues. *J. Biol. Chem.* 266:3579–3585.
35. Mould, A. P., and M. J. Humphries. 1991. Identification of a novel recognition sequence for the integrin $\alpha4\beta1$ in the COOH-terminal heparin-binding domain of fibronectin. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:4089–4095.
36. Nowlin, D. M., F. Gorcsan, M. Moscinski, S.-L. Chiang, T. J. Lobl, and P. M. Cardarelli. 1993. A novel cyclic pentapeptide inhibits $\alpha4\beta1$ and $\alpha5\beta1$ integrin-mediated cell adhesion. *J. Biol. Chem.* 268:20352–20359.
37. Osborn, L., C. Vasallo, and C. D. Benjamin. 1992. Activated endothelium binds lymphocytes through a novel binding site in the alternatively spliced domain of vascular cell adhesion molecule-1. *J. Exp. Med.* 176: 99–107.
38. O'Toole, T. E., J. C. Loftus, X. P. Du, A. A. Glass, Z. M. Ruggeri, S. J. Shattil, E. F. Plow, and M. H. Ginsberg. 1991b. Affinity modulation of the αIIb$\beta3$ (platelet GPIIb-IIIa) is an intrinsic property of the receptor. *Cell Regul.* 1:883–893.
39. O'Toole, T. E., D. Mandelman, J. Forsyth, S. J. Shattil, E. F. Plow, and M. H. Ginsberg. 1991. Modulation of the affinity of integrin αIIb$\beta3$ (GPIIb-IIIa) by the cytoplasmic domain of αIIb. *Science (Wash. DC).* 254:845–847.
40. 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.
41. Postigo, A. A., P. Sánchez-Mateos, A. I. Lazarovits, F. Sánchez-Madrid, and M. O. de Landázuri. 1993. $\alpha4\beta7$ integrin mediates B cell binding to fibronectin and vascular cell adhesion molecule-1. Expression and function of $\alpha4$ integrins on human B lymphocytes. *J. Immunol.* 151: 2471–2483.
42. 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.
43. Roldán, E., A. Garcia-Pardo, and J. A. Brieva. 1992. VLA-4-fibronectin interaction is required for the terminal differentiation of human bone marrow cells capable of spontaneous and high rate immunoglobulin secretion. *J. Exp. Med.* 175:1739–1747.
44. Sánchez-Aparicio, P., O. C. Ferreira, and A. Garcia-Pardo. 1993. $\alpha4\beta1$ recognition of the Hep II domain of fibronectin is constitutive on some hematopoietic cells but requires activation on others. *J. Immunol.* 150: 3506–3514.
45. Shimizu, Y., G. A. van Seventer, K. J. Horgan, and S. Shaw. 1990. Regulated expression and binding of three VLA ($\beta1$) integrin receptors on T cells. *Nature (Lond.).* 345:250–253.
46. Shimizu, Y., and S. Shaw. 1991. Lymphocyte interactions with extracellular matrix. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:2292–2299.
47. Stupack, D. G., S. Stewart, W. G. Carter, E. A. Wayner, and J. A. Wilkins. 1991. B lymphocyte fibronectin receptors: expression and utilization. *Scand. J. Immunol.* 34:761–769.
48. Stupack, D. G., C. Shen, and J. A. Wilkins. 1992. Induction of $\alpha v\beta3$ integrin-mediated attachment to extracellular matrix in $\beta1$ integrin (CD-29) negative B cell lines. *Exp. Cell Res.* 203:443–448.
49. Van de Wiel-van Kemenade, E., Y. van Kooyk, A. J. de Boer, R. J. F. Huijbens, P. Weder, W. Van de Kasteele, C. J. M. Melief, and C. G. Figdor. 1992. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. *J. Cell Biol.* 117:461–470.
50. Verfaillie, C. M., J. B. McCarthy, and P. B. McGlave. 1991. Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interaction with fibronectin. *J. Exp. Med.* 174:693–703.
51. Vonderheide, R. H., and T. A. Springer. 1992. Lymphocyte adhesion through very late antigen 4: evidence for a novel binding site in the alternatively spliced domain of vascular cell adhesion molecule 1 and an additional $\alpha4$ integrin counter-receptor on stimulated endothelium. *J. Exp. Med.* 175:1433–1442.
52. Wayner, E. A., A. Garcia-Pardo, M. E. Humphries, J. A. McDonald, and W. G. Carter. 1989. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* 109:1321–1330.
53. Wayner, E. A., and N. L. Kovach. 1992. Activation-dependent recognition by hematopoietic cells of the LDV sequence in the V region of fibronectin. *J. Cell Biol.* 116:489–497.
54. Wilkins, J. A., D. Stupack, S. Stewart, and S. Caixia. 1991. $\beta1$ integrin-mediated lymphocyte adherence to extracellular matrix is enhanced by phorbol ester treatment. *Eur. J. Immunol.* 21:517–522.
55. Yabkowitz, R., V. M. Dixit, N. Guo, D. D. Roberts, and Y. Shimizu. 1993. Activated T-cell adhesion to thrombospondin is mediated by the $\alpha4\beta1$ (VLA-4) and $\alpha5\beta1$ (VLA-5) integrins. *J. Immunol.* 151:149–158.
56. Yamada, K. M. 1991. Adhesive recognition sequences. *J. Biol. Chem.* 266:12809–12812.