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 Domínguez-Jiménez, and Angeles García-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 RGDS 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.

Lymphocytes interact with fibronectin (Fn) 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.

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 \( \alpha \) and \( \beta \) chains (22, 39) as well as extracellularly. Among these external factors certain mAb directed to the \( \beta \) 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.
do not bind constitutively to the 80-kD Fn fragment, that the RGD sequence in Fn is a novel ligand for activated .

In the present report we have studied whether α4β1 activation via the β1 subunit affects the ligand specificity of this receptor. We show that α5β1-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 α4β1 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 α4β1.

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. I) were obtained from tryptic digests 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 NH2-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 pepstatin (1:100 wt/wt, 37°C, 1 h) as described (40). Peptic digestions 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 peptide fragments was achieved by SDSPAGE, Western blots, ELISA, and NH2-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; DELPQLVTLHPNHLQPEILDVPSTC (CS-1), PSTVQKTPFIDAPS (CS-2), and CQQLPTSVGQPSGQQMIFEEHGFR (CS-3), containing sequences from the IIICS region, and IDAP containing an active sequence present in the Hep II domain (35). Laminin and collagen type I were purchased from Sigma Chemical Co. (St. 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 purified from a culture supernatant of Drs. B. Horowitz and R. Shulman, and are now available through Chemicon International, Inc. (MAB1934 and MAB1935, respectively; Temecula, CA). mAbs N-295 reacts with the 11-kD fragment that contains 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 (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).

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 substrate-coated wells. Inhibition of cellular attachment to 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 × 104) 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 Fab' fragments of rabbit antibodies to mouse IgG (Dakopatts, Glostrup, 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). Incorporation of 3H-nitau amine 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.
Analysis of the 80-kD fragment rendered the NH2-terminal sequence SDVPTS. Further characterization of this fragment was achieved using two recently developed mAbs with specificity for the Hep II (P3D4) and CS-I (PIF11) 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 PIF11 were negative. These two mAbs also failed to recognize the 80-kD fragment on Western blots (17). mAbs P3D4 and PIF11 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 α5β1 ligands Hep II and CS-I in the 80-kD fragment preparation.

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 NH2-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 α5β1-negative B Cell Lines to the 80-kD Fn Fragment Following Incubation with Anti-β1 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 β1 integrin subunit higher on Ramos cells (Table I). The surface expression of β1 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 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.

Figure 2. ELISA analysis of the reactivity of mAb PIF11 (anti-CS-I), 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 PIF11 (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.

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 x 106 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 1. Expression of Integrin Subunits on the Cell Lines Studied

| Cells    | 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).

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 cells to the 80-kD fragment and 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 x 10^4 cells/well). After 30 min at 37°C, attached cells were quantitated as described. Values are the average of three different experiments.

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 x 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.
The RGD Sequence is a Ligand for TS2/16-activated αβ1 Integrin

The above results therefore suggested that the conformational change induced in αβ1 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 80-kD fragment was inhibited by 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 αβ1 and that activated αβ1 (but not resting αβ1) 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 αβ1 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 P3IM (anti-Hep II) had no effect. These results together with the data shown in Fig. 7, indicate that αβ1 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 αβ1 Integrin

We next tested whether mAb TS2/16 could induce the recogni-
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 (38 µg/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.

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 x 10^4/well) and are the means of two different experiments.

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 Rams (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 α4β1-mediated spreading of melanoma cells can be inhibited by RGD synthetic peptides and that these peptides may elute the α4β1 integrin complex from a CS-1 affinity column. In contrast with these findings, we did not observe α4β1 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 α4β1 sensitive to RGD-containing peptides.

Several studies have shown that certain anti-β1 mAbs enhance the function of some β1 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, α2β1 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 α2β1 which recognizes only collagen to a fully active form that also binds laminin (6).

We and others have recently shown that α4β1 also exists in several states of activation among different hemopoietic cells. Monocytic cells and PBL bear a partly active form of α4β1 which recognizes CS-1 but not Hepl; mAb TS2/16 converted α4β1 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 α4β1 bound only VCAM-1 while the TS2/16-activated form bound VCAM-1 and CS-1 (30). The phorbol ester PMA can also activate α4β1 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 the constitutive binding of these cells to the 38-kD fragment thus suggesting that the α4β1 form present on Ramos and Daudi cells cannot be further activated by phorbol esters. A different regulation of α4β1 function by anti-β1 mAbs and Ca2+ has already been observed (30). It has also been reported that PMA has no (or minimal) effect on the binding of α2β1 to collagen or laminin while mAb TS2/16 was highly stimulatory (6). The fact that mAb TS2/16 can also activate solubilized β1 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 α4β1 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-β1 mAbs or PMA on the interaction of α4β1 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 α2β1, there are cell populations that bind laminin or 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 α4β1 ligand. In fact, the 80-kD fragment is a well described ligand for the α5β1 integrin and we have extensively documented that the constitutive function of α4β1 on leukocytes is RGD independent (12, 15, 16, 52). The activated form of α4β1 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, α4β1 can bind multiple and apparently quite distinct sequences within the same or different molecules. Within Fn the minimal sequences shown to be α4β1 ligands are LDV (contained in CS-1 [26, 53]), RGDV (contained in CS-5 [34]), and IDAPS (contained in HI [35]). It has been proposed that the common aspartate residue on these sequences is important for α4β1 recognition (35). While this may be true, it is clear that other sequences may also be specific α4β1 ligands. There is indirect evidence that the FNC/H I (YIKPGSPPEVPPRPGV) and FNC/H II (KNNKQSEPLIGERKKT) peptides derived from the Hep II Fn domain (31) may also interact with α4β1 (21). All these sequences (CSI, CS5, HI, 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, α4β1 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 α4β1 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 α4β1 through the β1 subunit allows recognition of the Fn RGD sequence which is located in repeat III-10 and thus distant from the previously described α4β1 ligands. Interestingly, Koivunen et al. (24, 25), using a phage display library, have recently identified several cyclic synthetic peptides which bind the α5β1 integrin and do not contain the RGD motif. One of these peptides corresponds to the sequence STSDVGG which is homologous to the TVSDVPR sequence within the same or different molecules. Within Fn sequences within the same or different molecules.

The present report therefore describes a novel role for α4β1 as a receptor for the RGD site in Fn. Because α5β1...
is the classical receptor for this site we have attempted to study the contribution of α4β1 to cell adhesion to the 80-kD Fn fragment when both integrins are expressed together. In experiments not shown, we tested whether anti-α4 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 α5β1 (1,2, and this study), we could not detect any inhibition with mAbs anti-α4. It is possible that when α5β1 and α4β1 are present together, α5β1 function predominates over α4β1 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 α4β1 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 α5β1 besides α4β1, properly activated α4β1 may cooperate with α5β1 in order to functionally replace it when α5β1 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 α4β1 for example (28), suggests that activating anti-β1 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 α4β1 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 α5β1 interaction with the RGD sequence in Fn (56). Similarly, activation of the platelet integrin αIIbβ3 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 α4β1 may regulate α4β1 binding to other ligands, as already shown for αIIbβ3 (7). The α4β1 ligands described so far, including the RGD site identified in this report, apparently bind to identical or overlapping sites on α4β1 because they are cross-inhibitory. However, it seems possible that other not yet identified ligands bind α4β1 with low affinity or at a different site, and upregulate rather than inhibit the function of α4β1. In support of this is the fact that two Fn sequences shown to interact with α4β1, FNC/H I, and FNC/H II, do not (or slightly) interfere with α4β1 adhesive function (21,31). The phage display library approach used for identification of ligands for α5β1 (24,25) may be a useful one to identify new potential ligands for α4β1. This would also open the possibility of using these modulatory sequences for therapeutic purposes. Moreover, because the avidity of α4β1 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 α4β1 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 α4β1 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. Chereau, M. Hemler, F. Sánchez-Madrid, J. Teixíó, 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. Domínguez-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. Sánchez-Madrid. 1992. Regulation of the VLA integrin-ligand interaction through the β, subunit. J. Cell Biol. 117: 659-670.
2. Arroyo, A. G., A. García-Pardo, and F. Sánchez-Madrid. 1993. A high affinity conformational state on VLA integrin heterodimers induced by an anti-β1 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. J. Acta, M. Obara, E. F. Plow, K. M. Yamada, and M. H. Ginsberg. 1991. Integrin αIIbβ3 (platelet GPIIIa-IIIb) 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 I and fibronectin. Comparison of α4β1 and α4β7 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 α2 cDNA clone: interconversion of forms induced by an anti-β, 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 αIIbβ3 (platelet GPIIIa-IIIb). Cell. 65:409-416.
8. Elices, M. J., and M. E. Hemler. 1989. The human integrin VLA-2 is a collagen receptor on eosinophils 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. Lobb. 1990. VCAM-1 on activated endothelium interacts with the leucocyte 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 cotumulation of resting human T cells by the bacterial β1 integrin ligand invasion. 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 α5β1: regulation of the functional response by soluble fibronectin. J. Cell Biol. 121:155-162.
12. Ferreira, O. C. Jr., A. García-Pardo, and C. Bianco. 1990. Specific binding of the human monocytic cell line U937 to the alternatively spliced con-
PST sequence of the type III connecting segment is sufficient to promote cell attachment. J. Immunol. 144:3361–3366.

17. García-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 α4β1. Cell. 60:53–61.

19. Humphries, M. J., A. Komoriya, S. K. Akiva, 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. Biochem. 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. Skdhitz, L. T. Furcht, E. A. Wayner, and J. B. McCarthy. 1992. Coordinate role for cell surface chondroitin sulfate proteoglycan and α4β1 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 control mediated by VLA-4, a β integrin. J. Exp. Med. 178:649–660.

23. Kirchhofer, D., L. R. Languino, E. Ruoslahti, and M. D. Pierschbacher. 1990. α2β1 integrins from different cell types show different binding specificities. J. Biol. Chem. 265:615–618.

24. Kovacs, N. L., T. M. Carlos, E. Yee, and J. M. Harlan. 1992. A monoclonal antibody to α5β1 integrin (CD29) stimulates VLA-4-dependent adhesion of leukaemic to human umbilical vein endothelial cells and matrix components. J. Cell Biol. 116:499–509.

25. Laffon, A., R. García-Vicuña, A. Humbria, 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.

26. Languino, L. R., K. R. Gehlsen, E. A. Wayner, W. G. Carter, E. Engvall, and E. Ruoslahti. 1989. Endothelial cells use α2β1 integrin as a laminin receptor. J. Cell Biol. 109:2455–2462.

27. Masumoto, A., and M. E. Hemler. 1993. Multiple activation states of VLA-4. Mechanistic differences in cell adhesion between α5β1 integrin and vascular adhesion molecule-1. J. Biol. Chem. 268:228–234.

28. McCarthy, J. B., M. K. Chelberg, D. J. Mikelson, and L. T. Furcht. 1988. Localization and chemical synthesis of fibronectin peptides with melanoma adhesion receptor for the HICS region of fibronectin and its identification as the integrin α4β1. J. Biol. Chem. 263:20523–20532.

29. Mould, A. P., and M. J. Humphries. 1991. Identification of a novel recognition sequence for the integrin α4β1 in the COOH-terminal heparin-binding domain of fibronectin. EMBO (Eur. Mol. Biol. Organ.) J. 10:4089–4095.

30. Nowlin, D. M., F. Gorcsan, M. Moscinski, S.-L. Chiang, T. J. Lobb, and P. M. Cardarelli. 1993. A novel cyclic pentapeptide inhibits α4β1 and α5β1 integrin-mediated cell adhesion. J. Biol. Chem. 268:20352–20359.

31. 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.

32. O'Toole, T. E., J. C. Lothux, X. P. Du, A. A. Glass, Z. M. Raggeri, S. J. Shattil, E. F. Plow, and M. H. Ginsberg. 1991. Affinity modulation of the αIβ3 (platelet GP IIb-IIIa) is an intrinsic property of the receptor. J. Biol. Chem. 10:883–893.

33. 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 αIβ3 (GP IIb-IIIa) by the cytoplasmic domain of αIb. Science (Washington, DC) 254:845–847.

34. Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti. 1981. Location of the cell adhesion site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. Cell. 26:259–257.

35. Postigo, A. A., P. Sánchez-Matoos, A. I. Lazarovits, F. Sánchez-Madrid, and M. O. de Landázuri. 1993. α4β7 integrin mediates B cell binding to fibronectin and vascular cell adhesion molecule-1. Expression and function of α4 integrins on human B lymphocytes. J. Immunol. 151:2465–2483.

36. Pyzel, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 kD cell surface glycoprotein with properties expected of a β integrin receptor. Cell. 40:191–198.

37. Roldán, E., A. García-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.

38. Sánchez-Aparicio, P., O. C. Ferreira, and A. García-Pardo. 1993. α4β1 recognition of the Hep II domain of fibronectin is constitutive on some hematopoietic cells but requires activation on others. J. Immunol. 150:3506–3514.

39. Shimizu, Y., G. A. van Sendert, J. K. Horgan, and S. Shaw. 1990. Regulated expression and binding of three VLA (β1) integrin receptors on T cells. Nature (London) 345:250–253.

40. Shimizu, Y., and S. Shawa. 1991. Lymphocyte interactions with extracellular matrix. FASEB (Fed. Proc. Am. Soc. Exp. Biol.) J. 5:2292–2299.

41. 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.

42. Stupack, D. G., C. Shen, and J. A. Wilkins. 1992. Induction of αvβ3 integrin-mediated attachment to extracellular matrix in β1 integrin (CD-29) negative B cells line. Exp. Cell Res. 203:444–448.

43. Van den Vlier-Manenade, E., Y. van Kooyk, A. J. de Boer, R. J. F. Huijben, P. Weser, W. Van de Kasteele, C. J. M. Meijer, 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-1. J. Cell Biol. 117:461–470.

44. Verfaille, C. M., J. B. McCarthy, and P. B. McGlave. 1991. Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clongogenic progenitors is accompanied by alterations in their interaction with fibronectin. J. Exp. Med. 174:693–703.

45. Vonderheide, R. H., and T. A. Springer. 1992. Lymphocyte adhesion through very late antigen 4: evidence for a novel binding site in the selectively spliced domain of vascular cell adhesion molecule 1 and an additional α4 integrin counter-receptor on stimulated endothelium. J. Exp. Med. 175:1433–1442.

46. Wayner, E. A., A. García-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.

47. Wayner, E. A., and N. L. Kovach. 1992. Activation-dependent recognition by hematopoietic cells of the LDL sequence in the V region of fibronectin. J. Cell Biol. 116:489–497.

48. Wilkins, J. A., D. Stupack, S. Stewart, and S. Caixia. 1991. β1 integrin-mediated lymphocyte adherence to extracellular matrix is enhanced by phorbol ester treatment. Eur. J. Immunol. 21:517–522.

49. Yakobowitz, R., V. M. Dixit, N. Guo, D. B. Roberts, and Y. Shimizu. 1993. Activated T-cell adhesion to thrombospondin is mediated by the α4 (VLA-4) and α5β1 (VLA-5) integrins. J. Immunol. 151:149–158.

50. Yamada, K. M. 1991. Adhesive recognition sequences. J. Biol. Chem. 266:12809–12812.