**αβ1** Integrin-dependent Cell Adhesion Is Regulated by a Low Affinity Receptor Pool That Is Conformationally Responsive to Ligand*

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αβ1 integrin (VLA-4) appears to be unique among the leukocyte integrins in that it can initiate the adhesion of circulating lymphocytes without cellular activation. It is not known how lymphocytes or other cell types maintain constitutive levels of αβ1 integrin activity. The current report describes a monoclonal antibody, 15/7, that recognizes a high affinity or ligand-occupied conformation of β1 integrin. Studies with 15/7 revealed that αβ1 integrin-dependent adhesion of leukocytic cell lines is mediated by a population of low affinity receptors that is conformationally responsive to ligand; the 15/7 epitope could be induced by nanomolar concentrations of soluble VCAM-1 or by micromolar concentrations of a peptide derived from the type III connecting segment domain of fibronectin (as ligands for αβ1 integrin). The same receptors were also responsive to adhesion activating reagents, such as Mn²⁺, activating anti-β1 integrin antibodies, and phorbol myristate acetate, which induced the 15/7 epitope directly and/or decreased the concentration of ligand required for epitope induction. In addition to the responsive receptor pool, cells expressed a second population of αβ1 integrin that was conformationally restrained, failing to respond to ligand or to any of the activating reagents. The relative size of the responsive and inactive receptor pools, as well as the affinity of the responsive receptors, represented a stable phenotype of different cell types and played important roles in defining the cells' adhesive capacity and ligand specificity. Similar receptor populations were measured on lymphocyte subsets in whole blood. These studies provide insight into how cells maintain different constitutive levels of αβ1 integrin activity, and how the activity of β1 integrin can be modulated by activators of cell adhesion.

Integrins are heterodimeric adhesion molecules that contribute to the specificity of cellular interactions through the recognition of numerous matrix and cell-associated ligands (1). Importantly, the ligand binding activity of integrins can be modulated rapidly, allowing cells to specify the timing and location of integrin-mediated adhesive interactions. On circulating leukocytes, for example, the β2 integrins LFA-1 and Mac-1 are thought to be activated by site-specific factors (cyto-}

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activators; in some systems PMA stimulation enhances integrin affinity (25, 26), while in others, it causes cell spreading without inducing receptor activation (27). Changes in receptor conformation have been characterized by anti-integrin antibodies that recognize activation-dependent epitopes. These antibodies can either inhibit adhesive function by engaging the active binding site of the receptor (18, 28, 29), or promote adhesion by stabilizing an active integrin conformation (30–32). Another class of antibodies recognizes ligand-induced binding sites associated with α1β2 integrin (33), and document changes that occur in receptor conformation upon ligand occupation.

Although much is known about integrin activity and changes in receptor conformation, there is no clear understanding of how cells modulate both integrin activity and ligand specificity, and how cells maintain stable differences in integrin activation states. In this report we describe a monoclonal antibody, 15/7, against an activation/ligand-induced epitope on β1 integrin. Studies with 15/7 provided a model for the regulation of αβ integrin activity that differs from the conventional view of integrin-mediated cell adhesion. In this model, receptors that mediate cell adhesion under resting conditions undergo a change in conformation in response to ligand, rather than a change in conformation that triggers ligand binding. The ligand-responsive receptors were also sensitive to Mn2+ and to activating antibodies against β1 integrin, as well as to PMA. These receptors generally exhibited a low affinity conformation (interacting reversibly with soluble ligand), but supported cell adhesion through a multivalent interaction with immobilized ligand. However, not all surface β1 integrin could respond to ligand or to activating agents, and this subset constituted an inactive receptor pool. The size of the inactive pool was found to be a stable phenotype of different cells and played an important role in determining the capacity of the cells to bind ligand, as well as the specificity of ligands that the cells could bind.

MATERIALS AND METHODS

Monoclonal Antibodies—The monoclonal antibodies 15/1, 15/7, and 15/10 (all mouse IgG1) were raised against immunopurified αβ integrin. A lysate of 10^6 U937 cells (in 1% triton X-100, with 1 mM phenylmethylsulfonyl fluoride, 3.6 μg/ml E-64, and 0.5 μg/ml leupeptin) was exposed to 0.3 ml of Sepharose conjugated with the anti-α4 integrin antibody, HP2/1 (Immunotech, Inc.; 3 mg of antibody/ml gel). αβ integrin was released from the column at pH 3 in 1% octyl glucoside. The eluate, containing approximately 10^6 integrin was released from the column at pH 3 in 1% octyl glucoside. The purified antibodies contained less than 1 unit of endotoxin/mg of protein. 15/10 was used as a hybridoma supernatant diluted 1:4. For some experiments, 15/7 was conjugated to fluorescein isothiocyanate (FITC; Sigma) according to the manufacturer’s instructions and used within 30 days. FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rat IgG, and FITC-conjugated mouse IgG (MOPC-21; negative control) were obtained from Sigma. FITC-conjugated goat anti-human IgG was obtained from Vector Laboratories, Inc. (Burlingame, CA), and PE-conjugated goat F(ab')2 anti-mouse IgG Fc was obtained from Immunotech (Westbrook, ME). FITC-conjugated CD45RO was obtained from DAKO (Denmark).

Reagents—Human VCAM-1 was obtained from the American Type Culture Collection (Rockville, MD). A stable mouse L cell line that expressed human VCAM-1 was obtained as follows: human VCAM-1 cDNA was obtained by polymerase chain reaction, based on the published sequence (36–38) from tumor necrosis factor-stimulated human umbilical vein endothelial cells (Clonetech, San Diego, CA). The cDNA was cloned into the pR2 expression vector (39), and transfected into mouse L cells by calcium phosphate precipitation. Stable L cell line clones expressing high levels of human VCAM-1 were selected by FACS, and maintained in culture in 600 μg/ml G418. All cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine.

Recombinant soluble VCAM-1 was expressed as a fusion protein with the heavy chain of human IgG1. The construct carried the seven immuno-noglobulin domains of VCAM-1 on the N terminus (to phenylalanine 697) and the Fc domain of human IgG1 at the C terminus (including the hinge, CH2, and CH3 regions; the hinge region cysteine, normally disulfide-bonded to the light chains, was mutagenized to serine). The cDNA for the soluble constant domains of α4 integrin was ligated into the MCS of pVL1939 (40), and the plasmid was designated pVLsVCAM.Fc. Recombinant baculovirus was generated by co-transfecting Sf9 cells with 3 μg of pVLsvCAM.Fc plasmid DNA and 1 μg of BaculoGold vector DNA (Pharmingen) and plaque-purified by visual screening. High titer virus (10^9 plaque-forming units/ml) was used to infect Trichoplusa ni High Five cells (Invitrogen, San Diego, CA). The supernatant 72 h post-infection was collected and sVCAM-1-IgG was affinity-purified by Protein A-Sepharose chromatography. Its purity was established by SDS-PAGE and silver staining.

Whole human fibrinogen, rat laminin, and the peptides GRGDSP and GRGESP were purchased from Life Technologies, Inc. An eight amino acid Lys-flanked peptide (EILDERP) from plasminogen, derived from fibrinogen, was prepared (430 ABI peptide synthesizer) and purified by high performance liquid chromatography. For adhesion studies, the same peptide was synthesized with a linker sequence on the N terminus (CGGG) and was conjugated to rabbit serum albumin as described by Wayner and Kovach (41). Recombinant soluble human ICAM-1 was a generous gift from Dr. Mary Perez (Wyeth-Ayerst, Princeton, NJ). Cell Adhesion Assays—Purified membrane VCAM-1 was plated onto wells of a 96-well RIA plate (#3590 Costar; Cambridge, MA) in 50 μl of phosphate-buffered saline with 0.1% dextran glusclde (Figs. 5, 8, and 9). Adhesion to immobilized recombinant soluble VCAM-1 was examined in Fig. 1; in this instance wells of the RIA plate were coated with Protein A (Calbiochem) at 0.5 μg/ml, blocked with 1% bovine serum
Identification of an Antibody Against an Activation Epitope Associated with $\beta_1$ Integrin—Jurkat and THP-1 cells expressed similar levels of $\alpha_4\beta_1$ integrin (see Fig. 2), but exhibited marked differences in $\alpha_4\beta_1$ integrin activity. The experiments shown in Fig. 1 compared the ability of Jurkat and THP-1 cells to bind to purified VCAM-1 that had been coated on assay wells over a range of densities. A 10-fold higher level of VCAM-1 was required to support THP-1 cell adhesion at a level that was comparable to that of Jurkat cells (half-maximal binding occurred at 20 and 2 ng of VCAM-1/well respectively; Fig. 1A). Binding of both cell lines was increased by adhesion activating reagents. As shown in Fig. 1B, THP-1 cells exhibited the highest levels of adhesion following exposure to $\beta_2$ A2, an activating antibody against $\beta_1$ integrin. Adhesion was also strongly stimulated by either PMA or Mn$^{2+}$. Under all conditions VCAM-1 binding was completely inhibited by anti-$\alpha_4$ integrin (not shown). Comparable results were obtained with Jurkat cells, although the activating reagents did not produce as great of a change in the already high levels of Jurkat cell adhesion (not shown).

THP-1 and Jurkat cells were used as the basis of a screen to identify potential activation epitopes associated with $\alpha_4\beta_1$ integrin; it was expected that such an epitope would be expressed at higher levels on Jurkat cells in correspondence to their higher level of VCAM-1 binding activity (both cell types expressed similar levels of $\alpha_4\beta_1$ integrin). Antibodies were raised against purified $\alpha_4\beta_1$ integrin (see "Materials and Methods") and their ability to recognize THP-1 and Jurkat cells was examined by FACS analysis; the reactivity of three of these antibodies is shown in Fig. 2 (15/1, 15/7, and 15/10) compared to that of control reagents against $\alpha_4$ and $\beta_1$ integrin (all antibodies were mouse IgG). Jurkat and THP-1 cells expressed similar levels of $\alpha_4$ integrin, while THP-1 cells expressed higher levels of the $\beta_1$ integrin subunit. Of the 70 reactive antibodies raised against purified $\alpha_4\beta_1$ integrin, only 15/7 produced a staining profile that was markedly different than that of the control reagents. 15/7 reacted at low levels with Jurkat cells, and even lower levels with THP-1 (fluorescence intensity of 24 and 2 units above control IgG background, respectively). Interestingly, addition of the integrin activating reagent Mn$^{2+}$ (1.5 mM) caused a 10-fold increase in the expression of the 15/7 epitope on both cell types (fluorescence intensity of 253 and 22 for Jurkat and THP-1 cells, respectively), but did not affect the reactivity of the other antibodies (not shown). Western analysis indicated that 15/7 recognized the $\beta_1$ integrin subunit (Fig. 2B). Even though THP-1 cells expressed higher levels of $\beta_1$ integrin than Jurkat, they expressed much lower levels of the 15/7 epitope (in the presence or absence of Mn$^{2+}$); this difference could not be explained by expression of different $\alpha$-chain subunits, since both cell types expressed similar levels of $\alpha_4$ integrin, and expression of $\alpha_4$ integrin could account for the majority of $\beta_1$ integrin on both cell types (see Fig. 2, legend). Neither THP-1 nor Jurkat cells expressed $\beta_7$ integrin (not shown).

The sensitivity of the 15/7 epitope to Mn$^{2+}$, and the higher expression levels of the epitope on Jurkat than on THP-1 cells...
A. Reactivity of Antibodies Against α4 and β1 Integrin with THP-1 and Jurkat Cells

B. Western Analysis

Fig. 2. Antibody reactivity by FACS and Western analysis. A, FACS analysis: Jurkat and THP-1 cells were exposed to the indicated antibody for 30 min at room temperature, washed, and exposed to PE-conjugated goat anti-mouse IgG Fc for FACS analysis ("Materials and Methods"). In a separate experiment it was found that α4 integrin accounts for the majority of β1 integrin expressed on both THP-1 and Jurkat cells. The cells were exposed to α-chain-specific monoclonal antibodies (see "Materials and Methods") and then to FITC-conjugated anti-mouse IgG for FACS analysis. Fluorescence intensity Jurkat/THP-1: IgG, 3/5; anti-α4, 3/3; anti-α5, 5/6; anti-α6, 9/25; anti-α2, 20/193; anti-α3/4, 14/62; anti-α5, 25/49; anti-β1, integrin, 200/276. B, THP-1 cells. B, Western analysis. αβ4 integrin was isolated from a lysate of U937 cells with an anti-α4 integrin affinity column, subjected to SDS-PAGE (non-reduced), and transferred to Immobilon. A separate sample of αβ4 integrin was isolated from a lysate of U937 cells that had been surface biotinylated. Individual strips of the blot were probed with control IgG, 15/1, 15/7, and TS2/16 (control anti-β1 integrin) at 10 μg/ml. Bound antibody was detected with sheep anti-mouse horseradish peroxidase ("Materials and Methods"). The strip derived from the biotinylated cells was probed with streptavidin-horseradish peroxidase (SA) to visualize the separate α4 and β1 integrin bands. On U937 cells α4 integrin is expressed as the cleaved form of the molecule, as described (48), migrating as two bands at 70 and 80 kDa. A small amount of uncleaved α4 integrin (150 kDa), as well as a 180-kDa form described (49) is also present. β1 integrin runs as a 130-kDa protein.

suggested that 15/7 recognized an activation epitope associated with β1 integrin. In order to examine this possibility further, the antibody was conjugated to FITC and incubated with Jurkat and THP-1 cells in the presence of different activators of β1 integrin (Fig. 3). Consistent with the results above, 15/7-FITC reacted with resting Jurkat cells better than with THP-1, although reactivity with both cell types was low. Treatment of the cells with PMA produced a small increase in expression of the 15/7 epitope, while higher expression levels were induced by MnCl2 (1.5 mM), and by two activating antibodies against β1 integrin, TS2/16 and 8A2 (tested at saturating concentrations). 8A2 induced the highest expression levels of the 15/7 epitope, representing a 10-fold increase above resting levels for both cell types. The inductive effects of MnCl2 and TS2/16 were additive, resulting in an expression level of the 15/7 epitope equivalent to that produced by 8A2 alone. MnCl2 did not affect the already high expression levels of the epitope induced by 8A2. In contrast to the activating antibodies, the inhibitory antibody against β1 integrin, AIIB2, completely eliminated the 15/7 epitope. A control antibody against β1 integrin (K20), that has no effect on β1 integrin adhesive function, did not significantly affect expression of the 15/7 epitope. 15/7-FITC reactivity with the cells was specific since it was effectively competed by excess unlabeled 15/7. MnCl2, PMA, and the activating antibodies against β1 integrin influence receptor activity through distinct mechanisms; therefore, these results suggest that 15/7 recognizes an epitope associated with the activation state of β1 integrin, rather than a conformation particular to a given activating agent.

Regardless of how the cells were stimulated, THP-1 cells exhibited 3–10-fold lower levels of the 15/7 epitope than Jurkat cells, and even though THP-1 cells expressed higher levels of β1 integrin (Fig. 2). This result was not due to limiting quantities of the activating reagents since the stimulatory antibodies were tested at concentrations above saturation. Furthermore, the responsive receptors on the two cell types demonstrated nearly identical sensitivity to MnCl2 (excitatory concentration for half maximal expression, or EC50, 0.7 mM); higher concentrations of MnCl2 could not compensate for low expression of the 15/7 epitope on THP-1 cells (Fig. 3, inset). These results indicate that THP-1 cells exhibited a lower number of activation-responsive receptors than Jurkat cells.

Expression of the 15/7 Epitope Is Correlated with the Activity of αβ4 Integrin—In addition to VCAM-1, αβ4 integrin binds to the CS1 domain of fibronectin (11, 12); however, cell adhesion to the fibronectin domain requires higher levels of αβ4 integrin activity (14). As shown in Fig. 4, THP-1 cells failed to bind to a peptide derived from FN CS1, even though the peptide was plated at a saturating density. Jurkat cells bound to the peptide at a high level, consistent with their higher level of αβ4 integrin activity. Adhesion of THP-1 cells could be induced by the activating agents described above, and the degree of adhesion reflected closely the degree to which the activators induced the 15/7 epitope (Fig. 3). Thus, the effects of MnCl2 and TS2/16 were additive, and together they produced a level of adhesion comparable to that of 8A2. The degree of enhancement produced by PMA was similar to that of MnCl2. The high level of Jurkat cell adhesion was not significantly affected by any of the activating reagents; however, when the CS1 peptide was plated at lower densities (conditions where THP-1 cells failed to bind
Ligand-responsive β₁ Integrin Receptor Pool

**Fig. 4.** THP-1 and Jurkat cell adhesion to FN CS-1 peptide. THP-1 and Jurkat cells were pre-exposed to the indicated reagent for 5 min at 37°C and then added to wells coated with a saturating level of FN CS1 peptide-albumin conjugate (3 μg/ml). Adhesion was allowed to occur for 30 min at room temperature. □, Jurkat; ▣, THP-1 cells.

Even with activation, Jurkat cells responded to Mn²⁺ and the activating antibodies in a manner identical to that of THP-1 (not shown). Thus, expression of the 15/7 epitope (as shown in Fig. 3) correlates strongly with the adhesive activity of α₄β₁ integrin.

**15/7 Promotes Cell Adhesion—** In addition to recognizing an activation epitope associated with β₁ integrin, 15/7 also promoted β₁ integrin-dependent cell adhesion (Fig. 5). 15/7 enhanced THP-1 adhesion to VCAM-1 through α₄β₁ integrin, to whole FN through α₄β₁ and α₄β₃ integrin, and to laminin through α₄β₃ integrin. Comparable results were obtained with monovalent Fab fragments of 15/7 (not shown), indicating that the enhancing effects did not require antibody Fc and were not the result of receptor cross-linking. The degree of enhanced binding to each of the ligands was equivalent to that induced by Mn²⁺. 15/7 did not promote THP-1 cell adhesion to ICAM-1, whereas Mn²⁺ induced ICAM-1 adhesion that was mediated by β₂ integrin. Thus, 15/7 selectively enhanced β₁ integrin-dependent cell adhesion. As shown below, 15/7 promoted cell adhesion by stabilizing an active conformation of β₁ integrin.

Expression of the 15/7 Epitope Is Enhanced in the Presence of Ligand—The degree to which 15/7 enhanced THP-1 cell adhesion was surprising since the antibody reacted with unstimulated THP-1 cells at very low levels (Fig. 2), yet promoted adhesion as strongly as Mn²⁺. This discrepancy may be explained if expression of the 15/7 epitope was enhanced by ligand encountered during the adhesion assay. In order to address this possibility THP-1 and Jurkat cells were exposed to 15/7 in the presence of recombinant soluble VCAM-1. Fig. 6 illustrates that soluble VCAM-1 enhanced expression of the 15/7 epitope in a dose-dependent fashion on both cell types, and the induction was completely prevented by an antibody against α₄ integrin. The conformational response of the receptors was rapid, occurring within 1 min of VCAM-1 addition, and occurred to the same extent at 4, 25, and 37°C (although longer incubation times were required at 4°C; not shown). Even though THP-1 and Jurkat cells expressed similar levels of α₄β₁ integrin (Fig. 2), they differed in both the number of receptors responsive to sVCAM-1 (3-fold difference in fluorescence at saturation) and in the concentration of ligand required to induce the epitope (EC₅₀ for Jurkat = 20 nM; sVCAM-1; THP-1 = 180 nM).

Expression of the 15/7 Epitope Is Induced by Peptide Ligand and Potentiated by Activators of Cell Adhesion—The expression of the 15/7 epitope could also be enhanced on Jurkat and THP-1 cells by a peptide derived from the CS1 domain of fibronectin, as a soluble ligand for α₄β₁ integrin (Fig. 7). This peptide was an unconjugated, monovalent form of the peptide used for the adhesion assay presented above (Fig. 4), and contained the activity of α₄β₁ integrin.
Concentration Soluble VCAM-1 (µM)

Fig. 6. Induction of the 15/7 epitope by soluble VCAM-1. Jurkat and THP-1 cells were treated with F(ab)\(_2\) fragments of anti-α\(_4\) integrin (AN100226m: 10 µg/ml), or with no additions for 30 min on ice. The cells were then exposed to 15/7 (10 µg/ml) in the presence of recombinant soluble VCAM-1 (at the indicated concentration) for 30 min at room temperature, washed, and exposed to PE-conjugated goat anti-mouse Fc (in the presence of human serum) to detect 15/7 by FACS analysis.

-□-, Jurkat; -○-, Jurkat + anti-α\(_4\) integrin; -○-, THP-1; -□-, THP-1 + anti-α\(_4\) integrin.

Concentration LDV Peptide (µM)

Fig. 7. Induction of the 15/7 epitope by LDV peptide. THP-1 (○) and Jurkat (□) cells were treated with 15/7 (10 µg/ml) and LDV peptide (at the indicated concentration) for 30 min at room temperature in the presence of F(ab)\(_2\), fragments of 8A2 (---, 5 µg/ml), Mn\(^{2+}\) (---, 1 mM), PMA (-----, 50 nM), or no additions (--). The cells with PMA were preincubated for 5 min at 37 °C. The cells were washed and exposed to PE-conjugated goat anti-mouse Ig Fc for FACS analysis.

population. In contrast, 8A2 increased both the sensitivity of receptors to LDV peptide, as well as the number of receptors that expressed the 15/7 epitope at peptide saturation; in the presence of 8A2, receptors on both Jurkat and THP-1 cells exhibited an EC\(_{50}\) of 2 µM for the peptide, while fluorescence at saturation increased 30 and 100% on the two cell types, respectively. PMA increased the apparent affinity of receptors on THP-1 cells by 5-fold, but did not affect receptor affinity on Jurkat cells. Thus, in the presence of PMA, both cell types exhibited the same apparent affinity for the peptide ligand (EC\(_{50}\) = 20 µM). PMA did not significantly increase the number of responsive receptors on either cell type; Jurkat cells continued to express 4-fold higher levels of the 15/7 epitope than THP-1 cells in the presence of PMA. Table I summarizes the

EC\(_{50}\) for induction of the 15/7 epitope on Jurkat and THP-1 cells in the presence of the activating reagents.

15/7 Locks β\(_1\) Integrin in an Active Conformation Induced by Mn\(^{2+}\) or Peptide Ligand—Since THP-1 cells express the 15/7 epitope in the presence of ligand, 15/7 may promote THP-1 cell adhesion (as in Fig. 5) by stabilizing the active or ligand-occupied conformation of β\(_1\) integrin. This possibility was examined by taking advantage of the reversible nature of the interactions of α\(_4\)β\(_1\) integrin with LDV peptide (as a ligand) and with Mn\(^{2+}\) (as an activating reagent). Fig. 8A demonstrates that while LDV peptide and Mn\(^{2+}\) both induced the 15/7 epitope on THP-1 cells, neither reagent produced a lasting effect on epitope expression if they were removed from the cells prior to 15/7 exposure. In contrast, if THP-1 cells were exposed to the reagents in the presence of 15/7, then the antibody remained bound to β\(_1\) integrin when the reagents were removed. Fig. 8B demonstrates that 15/7 stabilized an active conformation of α\(_4\)β\(_1\) integrin, induced by either Mn\(^{2+}\) or LDV peptide, to promote THP-1 cell adhesion to VCAM-1. Exposure of THP-1 cells to 15/7, by itself, did not promote adhesion if the antibody was washed away prior to the binding assay. This result is consistent with the low expression level of the 15/7 epitope on resting THP-1 cells (Fig. 3). Likewise, Mn\(^{2+}\), by itself, induced cell adhesion only when it was present during the adhesion assay. Pretreatment of THP-1 cells with the combination of 15/7 and Mn\(^{2+}\), followed by washing, promoted cell adhesion to the same extent as if Mn\(^{2+}\) or 15/7 were present during the adhesion incubation. This result indicates that 15/7 stabilized the active conformation of α\(_4\)β\(_1\) integrin that had been induced by Mn\(^{2+}\). Pretreatment of THP-1 cells with 15/7 and the LDV peptide also induced strong adhesion to VCAM-1; however, if the reagents were left present throughout the assay, the LDV peptide completely inhibited adhesion. These results demonstrate that 15/7 recognized and stabilized the “ligand-occupied” conformation of α\(_4\)β\(_1\) integrin as an “active” conformation to promote cell adhesion to VCAM-1 when the low affinity peptide was washed away. The fact that 15/7, by itself, promoted adhesion when present throughout the assay supports the idea that THP-1 cells express the 15/7 epitope when they encounter immobilized VCAM-1 during the adhesion incubation. Treatment of THP-1 cells with TS2/16 promoted cell adhesion to the same extent regardless of whether unbound antibody was left present during or removed prior to the adhesion assay. This result indicates that, unlike 15/7, TS2/16 reacted with β\(_1\) integrin to directly induce an active conformation of the receptor.

Mn\(^{2+}\) Activates Receptors That Normally Mediate Cell Adhesion—Resting THP-1 cells did not express the 15/7 epitope, yet bound to VCAM-1 when the ligand was plated at sufficient densities (as shown in Fig. 1). Since a subpopulation of receptors on THP-1 cells were responsive to Mn\(^{2+}\) or to ligand for expression of the 15/7 epitope, we wanted to determine if these responsive receptors normally mediated cell adhesion under resting conditions. In the experiment shown in Fig. 9, THP-1 cells were left untreated or were treated with 15/7 and Mn\(^{2+}\) to stabilize the Mn\(^{2+}\)-responsive β\(_1\) integrin receptors in an active conformation (as in Fig. 8). The cells were then examined for their ability to bind to immobilized VCAM-1 in the presence or absence of soluble VCAM-1 (30 nM). Resting THP-1 cells bound to high densities of VCAM-1 as expected, and adhesion was not significantly affected by the presence of soluble ligand. These results suggest that the receptors on THP-1 cells that mediate adhesion to VCAM-1 exhibit a low affinity, and are unable to establish a stable association with the soluble molecule. Presumably, the low affinity receptors can support adhesion through multivalent (high avidity) interactions with immobi-
Expression of the 15/7 epitope on THP-1, Jurkat cells, and whole blood lymphocyte subsets was measured in the presence of LDV peptide (over a range of concentrations) following treatment with the indicating activating reagent (as described in Figs. 7 and 10). Lymphocyte subsets were detected by double color FACS analysis, as in Fig. 10, using FITC-conjugated anti-CD45Ro. Values are based on three independent experiments.

**TABLE I**

| Cell type        | No additions | PMA       | Mn^{2+}   | BA2       |
|------------------|--------------|-----------|-----------|-----------|
| THP-1 cells      | 120 ± 10 μM  | 24 ± 2 μM | 5 ± 2 μM  | 2 ± 0.5 μM|
| Jurkat cells     | 25 ± 4 μM    | 18 ± 2 μM | 2 ± 0.5 μM| 2 ± 0.5 μM|
| CD45^+ lymphocytes | 148 ± 46 μM | 15 ± 4 μM | ND*       | ND        |
| CD45^− lymphocytes | 222 ± 74 μM | 25 ± 9 μM | ND        | ND        |

*ND*, not determined.

**FIG. 9.** Mn^{2+} activates receptors that normally mediate cell adhesion. THP-1 cells were treated with a combination of 15/7 (10 μg/ml) and Mn^{2+} (1.5 mM), or with no additions for 15 min at room temperature. The cells were washed rapidly, resuspended in buffer with or without recombinant soluble VCAM-1 (30 μg/ml), and tested immediately for their ability to bind to purified membrane VCAM-1 (plated at the indicated concentration). The adhesion incubation was allowed to occur for 15 min at room temperature. ○, no treatment; ∆, rsVCAM-1; □, 15/7 + Mn^{2+}; ○, 15/7 + Mn^{2+} with rsVCAM-1.

Ligand-responsive αβ1 Integrin Receptors on Circulating Lymphocytes—Since it has been reported that lymphocytes in the circulation can bind to VCAM-1 without activation (7), 15/7 was used to probe the state of αβ1 integrin activity on lymphocytes and the responsiveness of lymphocyte receptors to LDV peptide. The measurements were performed in freshly drawn whole blood to minimize the potential for receptor activation through cell handling: heparin, as an anticoagulant, did not affect expression of the 15/7 epitope, nor its ability to be induced by LDV peptide (as determined by control measurements with THP-1 and Jurkat cells added to whole blood; not shown). CD45^+ (memory) and CD45^− (naive) lymphocyte subsets were examined independently by double color FACS analysis. As shown in Fig. 10, both exhibited low resting levels of the 15/7 epitope and the epitope was induced by LDV peptide in a dose-dependent fashion (reaching saturation at 600 μM). Perhaps reflecting higher expression levels of αβ1 integrin, CD45^+ cells exhibited a higher level of peptide-responsive receptors than CD45^− cells (mean fluorescence 75 ± 9 and 32 ± 7, respectively, at peptide saturation). Furthermore, the CD45^− cells exhibited slightly higher affinity for the LDV peptide than the Ro^− cells (EC_{50} = 148 ± 46 and 222 ± 74, respectively). These results suggest that lymphocytes in the circulation exhibit a level of αβ1 integrin activity that is similar to that of THP-1 cells, expressing low resting levels of the 15/7 epitope and exhibiting receptors with similar responsive-

![Fig. 8.](image_url)

**Fig. 8.** 15/7 stabilizes an active conformation of αβ1 integrin induced by Mn^{2+} or by LDV peptide. A, FACS analysis. THP-1 cells were treated with Mn^{2+} (1.5 mM), LDV peptide (500 μg/ml), or no additions for 30 min on ice. Half of the cells in each sample were washed (□) free of the reagents, while the remaining cells were left un washed (■). Both sets of cells were exposed to 15/7 (10 μg/ml) for 30 min on ice, and then to PE-conjugated goat anti-mouse Ig Fc for FACS analysis. B, cell adhesion. THP-1 cells were treated with the following reagents either individually or in combination (as indicated in the figure) for 30 min on ice: 15/7 (10 μg/ml), Mn^{2+} (1.5 mM), LDV peptide (500 μg/ml), anti-α4 integrin (AN100226m; 5 μg/ml), or TS2/16 (5 μg/ml). The cells were washed quickly or left un washed, as in A, and immediately tested for their ability to bind to purified membrane VCAM-1 (3 ng/well) as described under "Materials and Methods."
Fig. 10. **Induction of the 15/7 epitope on whole blood lymphocytes by LDV peptide.** Samples of freshly isolated whole human blood (containing heparin as an anticoagulant) were incubated for 5 min at 37°C with or without PMA (50 nM). Samples of the blood were then exposed to 15/7 (10 µg/ml) in the presence of LDV peptide (at the indicated concentration) for 30 min at room temperature and processed for double color FACS analysis with anti-CD45Ro ("Materials and Methods"). —C—, CD45Ro−; —o—, CD45Ro+; —>, PMA, CD45Ro−; —v—, PMA, CD45Ro+.

ness to peptide ligand. The effect of PMA on the lymphocytes was also similar to that with THP-1 cells; treatment with PMA for 5 min increased the apparent affinity of the lymphocyte receptors for LDV peptide by 10-fold (EC50 CD45Ro+ = 15 ± 4 µM; CD45Ro− = 25 ± 9 µM peptide). While PMA induced low levels of the 15/7 epitope in the absence of ligand, it had little impact on the number of receptors that expressed the 15/7 epitope at peptide saturation.

**Discussion**

Several conclusions can be drawn from the studies with 15/7, and are illustrated in the model shown in Fig. 11. 1) 15/7 recognized an activation epitope on β1 integrin that was strongly associated with the adhesive capacity of cells. The antibody promoted β1 integrin-dependent cell adhesion by recognizing and directly stabilizing an active conformation of the receptor. 2) 15/7 identified both high affinity and ligand-occupied β1 integrin, and provided evidence that these receptor populations share a similar conformation. 3) Studies with 15/7 indicated that there are at least three subsets of β1 integrin on the surface of resting cells: constitutively active or high affinity, transiently active or low affinity, and receptors that are inactive or resistant to activation. 4) The low affinity receptors were available for interaction with ligand, were responsive to Mn2+ and activating antibodies, and were the receptors that normally mediated cell adhesion when ligand was present at sufficient density. 5) The size of the low affinity receptor pool, and its relative affinity for ligand, was a distinct phenotype of THP-1 and J urkat cells and represents a mechanism by which αβ1 integrin-mediated cell adhesion is likely to be regulated by cells in the circulation.

15/7 Recognizes an Activation Epitope Associated with β1 Integrin—15/7 Recognized the β1 integrin chain (Fig. 2B) and its epitope could be induced on cells by three distinct types of β1 integrin activators: Mn2+, PMA, and the anti-β1 integrin activating antibodies, TS2/16 and B.A2 (Figs. 2 and 3). Expression of the epitope on THP-1 and J urkat cells in the presence of the stimulating reagents strongly correlated with αβ1 integrin activity, as measured by cell adhesion to VCAM-1 and to a peptide derived from the CS1 domain of fibronectin (Figs. 1 and 4).

15/7 Enhances Cell Adhesion by Stabilizing an Active Conformation of β1 Integrin—15/7 promoted THP-1 cell binding to β1 integrin ligands, even though the cells did not express a significant level of the 15/7 epitope (Fig. 5). However, in order to promote adhesion, it was important that 15/7 was present during the adhesion incubation (Fig. 8). These results suggested that expression of the 15/7 epitope was facilitated by the presence of ligand in the assay well, and that 15/7 stabilized the ligand interaction. Consistent with this idea was the observation that soluble peptide ligand and VCAM-1, itself, promoted expression of the 15/7 epitope in a dose-dependent fashion (Figs. 6 and 7). Once 15/7 had bound β1 integrin in the presence of peptide, the antibody interaction was stable when the peptide was removed, and the integrin remained locked in the ligand-occupied, or active, conformation to promote cell adhesion in a subsequent assay (Fig. 8). Thus, it cannot be argued that 15/7 is simply a low affinity antibody that must be present during an adhesion assay in order to promote ligand interaction. Instead, these results imply that β1 integrin ligands, such as VCAM-1, fibronectin, or laminin, facilitate expression of the 15/7 epitope by engaging low affinity, or transiently active receptors. During a cell adhesion assay with resting THP-1 cells, 15/7 promoted cell attachment by engaging β1 integrin in the presence of ligand, and by stabilizing what would have otherwise been a transient ligand interaction.

15/7 Is Different Than β1 Integrin “Activating” Antibodies—15/7 is distinct from other antibodies against β1 integrin that promote cell adhesion, such as TS2/16 and B.A2. These antibodies recognize β1 integrin regardless of the state of activation, and can induce a more active form. Thus, by FACS analysis, TS2/16 and B.A2 reacted with THP-1 and J urkat cells at levels comparable to control antibodies against β1 integrin (Fig. 2), and this high level of recognition was not influenced by the activating effects of Mn2+ (not shown). In addition, the 15/7 epitope has recently been mapped to the central region of β1 integrin at a site distinct from that for the activating antibodies. Since 15/7 reacted strongly with the isolated β1 integrin chain under the denaturing conditions of Western analysis.
(Fig. 2), it may recognize a linear epitope of \( \beta_1 \) integrin that is exposed or masked by conformational changes associated with the receptor heterodimer.

15/7 as a "Ligand-induced Binding Site" Antibody versus an Antibody Against an Activation Epitope of \( \beta_1 \) Integrin—Antibodies have been described that recognize ligand-occupied conformations of \( \alpha_IIb\beta_3 \) integrin, or ligand-induced binding sites (32, 42). The epitopes for these antibodies can be induced on platelets by peptide ligands derived from fibrinogen, and by fibrinogen itself, when the platelets are activated. 15/7 also functions as a ligand-induced binding site antibody since its epitope on \( \beta_1 \) integrin was induced by soluble VCAM-1 or by peptide ligands (Figs. 6 and 7). However, ligand was not required for expression of the 15/7 epitope since it could be induced by several distinct activators of cell adhesion in the absence of any known \( \beta_1 \) integrin ligands (Figs. 2 and 3); expression of the 15/7 epitope under these conditions correlated with adhesive activity (Fig. 4). Furthermore, 15/7 stabilized the conformation of \( \alpha_IIb\beta_3 \) integrin induced by Mn\(^{2+}\) (as an activating agent) or LDV peptide (as a ligand) to enhance cell adhesion when the reagents were removed (Fig. 8). These results indicate that 15/7 recognizes \( \beta_1 \) integrin when the receptor is either active or occupied by ligand, and suggests that these two receptor forms exhibit a similar conformation. Thus, 15/7 is both a ligand-induced binding site antibody for \( \beta_1 \) integrin, as well as an antibody against a \( \beta_1 \) integrin activation epitope.

The ligand occupied forms of other integrins may also exhibit an active conformation, since it has been shown that peptide ligand can induce the activity of purified or fixed \( \alpha IIb \beta 3 \) integrin (33). In addition, 15/7 has characteristics similar to antibody 24, which recognizes an activation epitope associated with the \( \beta_3 \) integrins (30). Following transient cell stimulation with PMA, antibody 24 engages LFA-1 in the presence of ligand (ICAM-1 on adjacent lymphocytes) and prevents the normal decline in receptor activity. However, since LFA-1 on lymphocytes cannot interact with ICAM-1 in the absence of cell stimulation (43), its activity is likely to be regulated in a manner different than that of \( \alpha_IIb\beta_3 \) integrin.

Subsets of \( \beta_1 \) Integrin on the Cell Surface—The model depicted in Fig. 11 summarizes studies with 15/7 which suggest that there are at least three subsets of \( \beta_1 \) integrin on the cell surface that can be distinguished by their state of activation: 1) active or high affinity; 2) transiently active or low affinity; and 3) inactive. The first population expressed the 15/7 epitope constitutively and is likely to be comprised of "high affinity" receptors described in other reports (17, 27). These receptors represented a small percentage of the total \( \beta_1 \) integrin on the surface of resting J urkat cells, and were virtually absent on THP-1 cells and on lymphocytes in the circulation.

The second population of receptors was detected by 15/7 in the presence of saturating concentrations of peptide ligand or Mn\(^{2+}\) and is likely to correspond to the population of "low affinity" receptors that have been described in other reports (17, 27). These receptors were required for cell adhesion to VCAM-1, as shown in Fig. 9. Resting THP-1 cells bound well to VCAM-1 when the ligand was plated at a sufficient density, and binding was not affected significantly by soluble VCAM-1. These results suggest that the affinity of the receptors that normally mediate THP-1 cell adhesion is too low to form a stable association with soluble VCAM-1. Presumably, such low affinity receptors can mediate cell adhesion to immobilized ligand through multivalent interactions. When the Mn\(^{2+}\)-responsive receptors were held active by 15/7, soluble VCAM-1 was able to completely inhibit even the resting levels of cell adhesion. These results indicate that Mn\(^{2+}\) affects the population of receptors that are normally available for ligand binding. These receptors do not constitutively express the 15/7 epitope, nor do they exhibit a stable high affinity conformation for interaction with ligand, and, therefore, are not normally occupied by soluble VCAM-1; however, these receptors can initiate or mediate cell adhesion when ligand is present at sufficient density. Recently, it has been shown that in vivo administration of recombinant soluble VCAM-1, at initial circulating levels of 100–200 ng/ml, will inhibit the onset of experimental diabetes, even though interaction of the construct with lymphocytes in the circulation could not be measured (44). Based on the results shown in Fig. 6, it is likely that the majority of low affinity \( \alpha_IIb\beta_3 \) integrin receptors were occupied by the VCAM-1 construct at this concentration, but that the interaction of the construct with the lymphocytes could not be measured by indirect FACS analysis (which involves multiple cell washings).

The third population of receptors on the cell surface appeared to be conformationally restrained and unavailable for ligand interactions regardless of ligand concentration or density. This population represented the majority of \( \beta_1 \) integrin on the surface of THP cells. In the presence of saturating concentrations of Mn\(^{2+}\), sVCAM-1, or peptide ligand, THP-1 cells expressed 3–10-fold lower levels of the 15/7 epitope than J urkat cells (Figs. 3, 6, and 7), even though THP-1 cells expressed higher levels of \( \beta_1 \) integrin than J urkat, and equivalent levels of \( \alpha_IIb\beta_3 \) integrin (Fig. 2). This population of receptors resisted expression of the 15/7 epitope even when directly engaged by the activating antibodies against \( \beta_1 \) integrin (Figs. 3 and 7). However, in lysates of THP-1 and J urkat cells, \( \alpha_IIb\beta_3 \) integrin exhibited equivalent levels of activity for binding immobilized VCAM-1 (not shown). Furthermore, by Western analysis, 15/7 exhibited equivalent levels of activity for binding immobilized VCAM-1 (not shown). Furthermore, by Western analysis, 15/7 reacted equally well with the isolated \( \beta_1 \) integrin subunit (Fig. 2), regardless of the type of cell from which the receptor was obtained (not shown). These results suggest that factors associated with the integrin heterodimer on the cell surface, or within the cytoplasm, prevent exposure of the 15/7 epitope and regulate receptor activity. Such negative regulatory factors are likely to play a pivotal role in controlling patterns of cell migration and localization by defining the capacity of cells to respond to ligand at a given density.

The regulation of \( \alpha IIb\beta 3 \) integrin appears to be different than that of \( \alpha_IIb\beta_3 \) integrin, in that subsets of \( \alpha IIb\beta 3 \) integrin were not apparent on the platelet cell surface; essentially all of the platelet receptors were responsive to RGD peptide or to PMA (45). The existence of \( \beta_1 \) integrin subsets is likely to reflect the specialized function of the many different cell types that express this receptor.

Activators of \( \beta_1 \) Integrin Can Affect Either the Apparent Affinity of \( \beta_1 \) Integrin or the Size of the Responsive Receptor Pool—Mn\(^{2+}\) and soluble peptide ligand appear to interact with the same subset of low affinity receptors to induce the 15/7 epitope since the effects of the reagents were not additive when peptide was present at a saturating level (Fig. 7). However, Mn\(^{2+}\) increased receptor sensitivity to LDV peptide by 10-fold on J urkat cells, and by 25-fold on THP-1 (see Table I), so that receptor saturation was reached at a lower concentration of peptide. These results suggest that Mn\(^{2+}\) facilitates cell adhesion by increasing the affinity of the available receptors, favoring an active conformation of \( \beta_1 \) integrin and expression of the 15/7 epitope, but does not increase the number of receptors that are available for cell adhesion.

The activating antibody against \( \beta_1 \) integrin, 8A2, affected the same receptor population that was responsive to Mn\(^{2+}\) since their inductive effects were not additive (Fig. 3); however, 8A2 also recruited additional receptors from the otherwise
Ligand-responsive β1 Integrin Receptor Pool

Ligand Specificity of α4β1 Integrin-mediated Cell Adhesion—

Different cells that express α4β1 integrin can differ greatly in their ability to bind VCAM-1 and FN CS1. In general, cell adhesion to FN CS1 requires higher levels of α4β1 integrin activity than adhesion to VCAM-1; however, the fact that some cells demonstrate absolute preference for VCAM-1 (lacking the ability to bind FN CS1) has lead to the idea that α4β1 integrin exhibits binding sites for VCAM-1 and for FN CS1 that can be regulated independently (14, 46). In contrast, there is evidence that VCAM-1 and FN CS1 occupy the same ligand binding site on α4β1 integrin, but that the affinity for CS1 is simply lower than that for VCAM-1 (47). In the current study, THP-1 cells bound strongly to VCAM-1 when the ligand was plated at a high density (Fig. 1), but could not bind to a peptide derived from FN CS1 even when the peptide was plated at a saturating level (Fig. 4). This difference could not be explained by differential regulation of independent ligand-binding sites, since the apparent affinity of α4β1 integrin on THP-1 cells for both VCAM-1 and CS1 peptide was much less than the apparent affinity of α4β1 integrin on THP-1 cells for both FN CS1 and VCAM-1. Despite this, the low affinity of α4β1 integrin on THP-1 cells for ligand, in general, appears to have precluded cell adhesion to the lower affinity CS1 peptide. Even in the presence of Mn2+ or PMA, when receptors on THP-1 and Jurkat cells exhibited similar affinity for soluble CS1 peptide (Table I), THP-1 cell adhesion to the immobilized peptide continued to be lower than that of Jurkat cells (Fig. 4). A likely explanation for the difference between these two cell types is that in the presence of Mn2+ or PMA, THP-1 cells continued to exhibit a lower number of receptors that were available for ligand interaction than Jurkat cells (Fig. 7). Stimulation of THP-1 cells with BA2, where both receptor affinity and the number of available receptors was increased, allowed THP-1 cells to bind to the CS1 peptide at a level approaching that of resting Jurkat cells (Fig. 4). Therefore, regulation of receptor number, as well as receptor affinity, is likely to contribute to differences in ligand binding specificity of α4β1 integrin expressed on different cells.

Conclusions—The most novel proposal to emerge from the studies with 15/7 is that α4β1 integrin-dependent cell adhesion can be regulated by the size and affinity of a ligand-responsive population of receptors; a population that is available for ligand interactions, but with low affinity or transient activity. Under resting conditions, the receptors that mediate cell adhesion change conformation in response to ligand, and do not require an exogenous stimulus to trigger ligand binding. These findings are of particular interest since it has recently been demonstrated that α4β1 integrin can support lymphocyte rolling and adhesive interactions on VCAM-1 in the presence of shear forces encountered within normal blood flow (see Introduction). Based on the findings of the current study with 15/7, activating signals or cytokines would not be required to initiate the interaction of cells with VCAM-1 at inflammatory sites. Instead, the ability of cells to roll on VCAM-1 may reflect their stable expression of a low affinity receptor population; the larger the receptor pool, the lower the density of VCAM-1 required to initiate cell binding. In this regard, it is interesting that memory cells in the circulation were found to express a greater number of receptors that were responsive to α4β1 integrin ligand than naive cells, and the receptors exhibited a higher apparent affinity. Chemokines or other activating signals would play an important role following initial cell contact with the vessel wall, and may act to increase α4β1 integrin affinity in a manner similar to that observed in the current study with PMA. The size of the ligand responsive pool, and relative affinity of the receptors within it, are likely to represent the major differences between cell types with different adhesive capacities under resting conditions. The regulation of β1 integrin on cells in the circulation appears to be distinct from that of other integrins, such as αIIbβ3 integrin on platelets, LFA-1 on lymphocytes, or Mac-1 on neutrophils, which require activation to initiate adhesive contact. J just as antibodies that recognize activation and ligand-dependent epitopes have been invaluable tools for understanding the regulation of β2 and β3 integrins, antibodies such as 15/7 will greatly help to understand the complex regulation of β1 integrin activity.

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