Affinity Modulation of Integrin α5β1: Regulation of the Functional Response by Soluble Fibronectin

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Abstract. We report that a β1 integrin (α5β1) can exist in different affinity states for its soluble ligand, fibronectin. The α5β1 expressed by the erythroleukemic cell line K562 binds soluble fibronectin with low affinity ($K_d > 1$ μM), but is induced to bind it with 20-fold higher affinity (K_d-54 nM) in the presence of the anti-β1 mAb 8A2. This activation seems to be due to direct antibody-induced change in the receptor that does not require intracellular signaling, and is a plausible basis for the 8A2-induced enhancement of β1-dependent adhesion to fibronectin and other immobilized ligands (Kovach, N. L., T. M. Carlos, E. Yee, and J. M. Harlan. 1992. *J. Cell Biol.* 116: 499–509). Fab fragments of 8A2 bind with higher affinity to α5β1 receptor that is occupied by the GRG-DSP peptide ligand suggesting that the antibody functions by stabilizing a high affinity (occupied) conformer of the receptor. A functional consequence of the affinity modulation is that soluble fibronectin (at physiological concentrations) occupies the high affinity receptors, and so becomes an effective inhibitor of adhesion to immobilized fibronectin. In contrast, the majority of low affinity receptors remain unoccupied and are still able to mediate cellular adhesion.

C ELL adhesion is controlled by interactions between adhesion receptors and their ligands. Changing the repertoire of adhesive receptors, by either modifying biosynthetic patterns (16, 39, 45, 47) or by receptor translocations (8, 35), are strategies to modify cell adhesion. The function of adhesion receptors can also be changed by structural alterations, by changes in mRNA splicing (5, 10, 30, 41, 54), proteolytic cleavage (31), or variations in glycosylation (17). Finally, cellular adhesion can be rapidly regulated through reversible modulation of receptor function, and this mechanism is a key feature of the integrin family of adhesion receptors (4, 15, 42, 51, 52).

The most widespread integrins are the β1 subfamily (VLA family) (27–29), and there is dynamic regulation of β1 integrin–mediated adhesion. T lymphocytes and T lymphoid cell lines, activated either with phorbol esters, by cross-linking CD3, or with activating combinations of anti-CD2 mAbs, exhibit increased β1-dependent adhesion to immobilized ligands (51, 52). This occurs without alterations in their repertoire of integrins. Cellular adhesion to immobilized ligand is a complex multi-step process, involving initial receptor–ligand interaction, followed by strengthening of adhesion through organization of the cytoskeleton, focal contact formation, and cell spreading (11, 55). Cells could enhance adhesion by the facilitation of any of these steps. For example, Danilov et al. (14) found that phorbol esters enhanced the adhesion of CHO cells to immobilized fibronec-

tin without any detectable change in the affinity of their fibronectin receptors for soluble ligand. Alternatively, the ligand binding affinity of certain integrins can be rapidly altered. For example, platelet receptor αIIbβ3 (or GPIIbIIIa) exists in at least two affinity states for its soluble ligand, fibrinogen (7, 33, 50), which appear to be a result of different conformations of the receptor (13, 40). Conversion from low to high affinity binding of fibrinogen can be induced by treating the platelets with agonists such as phorbol esters, ADP, and thrombin (43, 44), and by certain mAbs directed against the β3 chain (40). Interconversion between low and high affinity fibrinogen binding states also occurs with αMβ2 (4). Direct demonstration of affinity modulation of β1 integrins has not been reported. However, it is known that soluble fibronectin binds with moderate affinity to β1 integrins expressed by cell lines such as CHO cells ($K_d = 100$ nM) (14) and BHK cells ($K_d = 800$ nM) (1).

Several anti-β1 mABs which enhance β1 integrin–dependent cellular adhesion have been described (6, 32, 38, 53). In this paper we have used one of these antibodies (8A2, reference 32), to investigate affinity modulation of the fibronectin receptor, α5β1. We show that the α5β1 expressed by the erythroleukemic cell line K562 binds soluble fibronectin with low affinity which can be increased 20-fold by 8A2. The alteration in affinity occurs in the presence of inhibitors of intracellular signaling, and it appears that the antibody functions by directly inducing a high affinity conformation of the

receptor. Finally, the biologic consequences of affinity modulation of $\alpha5\beta1$ were found to depend on the ambient soluble fibronectin concentration.

Materials and Methods

Antibodies and Reagents

Production and initial characterization of the murine anti–human $\beta1$ mAb 8A2 (IgG1-κ isotype) has been described (32). The rat anti–human $\alpha5$ mAb BIIG2 and rat anti–human $\beta1$ mAb AIIB2 (23) were used as dilutions of ascites. The murine anti–human $\alpha4$ mAb HP2/1 (49) was used as dilutions of tissue culture supernatant. The murine anti–human $\beta1$ mAbs A1A5 and TS2/16 (25) were used as dilutions of ascites. The murine anti–human fibronectin mAb FnI.11 was produced in our laboratory as described (9), and used as dilutions of ascites. The murine anti–human HLA class I mAb W6/32 was obtained from the American Type Culture Collection (Rockville, MD) and used as dilutions of purified IgG. The peptide GRGDSP was obtained from Peninsula Laboratories (Belmont, CA) and the peptide GRGESP was synthesized in the Scripps Research Institute core facility (La Jolla, CA). Glucose and 2-deoxyglucose were purchased from Sigma (St. Louis, MO) and sodium azide from Fisher Scientific Co. (Pittsburgh, PA).

Cells

The erythroleukemic cell line K562 was obtained from the ATCC, and was maintained in RPMI medium (Biowhittaker, Walkersville, MD) supplemented with 10% FCS (Biowhittaker), 1% glutamine (Sigma), and 1% penicillin and 1% streptomycin (Sigma). U937, Jurkat (both from ATCC), and Molt-13 (from Dr. Dario Altieri, The Scripps Research Institute) cells were maintained in the same medium. THP-1 cells (from the ATCC) were maintained in this medium with the addition of 10 mM Hepes and 20 mM 2-mercaptoethanol. Human T lymphocytes were purified from the peripheral blood of normal donors by centrifugation on a Ficoll-Paque gradient (Pharmacia Fine Chemical, Piscataway, NJ), panning for monocytes on serum-coated dishes, and passaging over a nylon wool column.

Purification of 8A2 IgG and Fab Fragments

8A2 whole IgG was purified from ascites by binding to protein A immobilized on sepharose beads (Pharmacia LKB, Uppsala, Sweden). The antibody was incubated with the protein A-sepharose under high salt conditions (3.3 M NaCl and 0.5 M Na borate, pH 8.9), and eluted with 100 mM glycine, pH 3.0 into tubes containing 1.0 M Tris, pH 8.0. The peak fractions were pooled and dialyzed overnight against PBS. Fab fragments were produced by overnight incubation of the purified 8A2 IgG with papain cross-linked to beaded agarose (Pierce Chemical Co., Rockford, IL), according to the manufacturer's instructions. After removal of the immobilized papain by centrifugation, the supernatant was passed over a protein A-sepharose column, and the non-binding fraction analyzed for purity of the Fab fragments by SDS-PAGE under non-reducing conditions. Densitometry confirmed that <2% of the 8A2 Fab preparation remained in the form of intact IgG. The concentration of purified IgG and Fab fragments was determined by absorbance at 280 nm (1 OD = 0.8 mg/ml) (24).

Purification of Human Fibronectin

Fibronectin was isolated from fresh human citrated plasma by affinity chromatography on gelatin-sepharose (Pharmacia Fine Chemical), as we have previously described (42). The isolated fibronectin yielded a single band on SDS-PAGE under non-reducing conditions, and a closely spaced doublet of 215,000 and 230,000 D under reducing conditions, consistent with the reported properties of plasma fibronectin (12). The concentration of fibronectin was determined by absorbance at 280 nM ($A_{0.1\%}^{280nm}$ = 1.3) (36).

Radiolabeling of Fibronectin and 8A2 Fab Fragments

The fibronectin was labeled with ^{125}Iodine by a modified Chloramine-T procedure, as described (42). The 8A2 Fab fragments were radiolabeled by the same Chloramine-T method, the sample run over a PD10 Sephadex column (Pharmacia Fine Chemicals), and the peak activity fractions pooled. Greater than 90% of the radioactivity was precipitated by 10% TCA. Aliquots were stored at −70°C following the addition of BSA to 1% final concentration.

Binding Assays

Assays of the binding of ^{125}I-labeled fibronectin and 8A2 Fab fragments were based on assays of fibronectin binding to platelets that we have previously described (42). The standard buffer used in the assays was modified Tyrode's buffer (150 mM NaCl, 2.5 mM KCl, 12 mM NaHCO₃, 1 mg/ml BSA, 1 mg/ml glucose, and 2 mM MgCl₂), pH 7.4. Cells were washed twice in the appropriate buffer before being suspended in the same at the indicated density. The incubation time was typically 30 min at room temperature, as pilot studies showed that >90% of the maximal fibronectin binding occurred by this time. A typical binding assay, performed in a 1.5-ml microcentrifuge tube (Sarstedt, Germany), was in a 200-μl vol composed of 120 μl of cells (2 × 10⁶ per tube), 40 μl of radiolabeled protein (fibronectin or 8A2), and 40 μl of stimulus (e.g., cold 8A2) and/or inhibitor (e.g., EDTA, mAb, GRGDSP). After the incubation, 50-μl aliquots were layered in triplicate onto 300 μl of 20% sucrose in the same buffer in microfuge tubes (West Coast Scientific Inc., Hayward, CA), and centrifuged for 3 min at 12,000 rpm in a microfuge (Beckman Instruments, Fullerton, CA). The tips were amputated and counted, and the bound protein was calculated from the specific activity of the ligand (assuming a molecular weight of fibronectin as 440,000 and 8A2 Fab fragments as 50,000 D). For fibronectin binding, the residual bound radioactivity in the presence of 5 mM EDTA was subtracted as non-specific binding (i.e., non-integrin-dependent binding). The bound fibronectin did not undergo detectable proteolytic degradation or transglutaminase-mediated cross-linking during these experiments, as the bound fibronectin subunits showed identical mobility to input subunits on reduced SDS gels. Reversibility of the fibronectin binding was established in control experiments where EDTA was added to the mixture of cells and radiolabeled fibronectin 30 min after the commencement of the incubation. Greater than 90% of the radiolabel was eluted from the cells after 90 min of incubation with the EDTA. For 8A2 Fab fragments, the non-specific binding was determined in the presence of a 50× excess of cold 8A2 IgG. The data was fitted to equilibrium binding models using the LIGAND program (37).

Assays of Cell Adhesion to Immobilized Fibronectin

The assay of K562 adhesion to immobilized fibronectin were based on that described by Prater et al. (46). Fibronectin was diluted in PBS, pH 7.4, and 50-μl aliquots incubated in the wells of a 96-well Immulon II plate (Dynatech Laboratories Inc., Chantilly, VA) for 2 h at 37°C. After washing with PBS, the wells were blocked with 1% BSA in PBS for 1 h at 37°C. The buffer used in the adhesion assay was the same as in the binding assays. Cells were washed twice in this buffer, resuspended at 1 × 10⁶ cells/ml, and 100-μl aliquots added to the emptied wells. Where required, appropriate concentrations of stimulus or inhibitor (e.g., antibody EDTA) were mixed with the cells when they were added to the wells, except in assays involving soluble fibronectin. In this case, the cells were preincubated with antibody (8A2 or W6/32) and soluble fibronectin for 30 min rocking at room temperature, before addition to the wells. After a 30-min incubation at room temperature, the non-adherent cells were washed off with three rounds of gentle pipetting. The residual adherent cells were checked by visual inspection, and quantified with a colorimetric reaction using endogenous cellular acid phosphatase activity by adding 100 μl of the following substrate/lysis solution to each well: 1% Triton X-100 (Fisher Scientific Co.), 6 mg/ml p-nitrophenylphosphate (Sigma), in 50 mM sodium acetate buffer, pH 5.0. After a 1-h incubation at 37°C, the reaction was terminated by the addition of 50 μl of 1 M NaOH, and read in an ELISA plate reader (Molecular Devices Corp., Menlo Park, CA) with a 410 nm filter. Background values, determined in wells coated with 1% BSA alone, were subtracted from each point. Adherence was expressed as a percentage of the number of cells originally added to each well, and determined from a standard curve generated using known numbers of cells.

Results

$\alpha5\beta1$ on Nonadherent Cells Binds Little Soluble Fibronectin

As noted in the introduction, at least two adherent cell lines (1, 14) express $\alpha5\beta1$ that binds soluble fibronectin with moderate affinity. To investigate the affinity state of $\alpha5\beta1$ on cells that grow in suspension, we assessed fibronectin binding to the K562 erythroleukemic cell line. These cells were
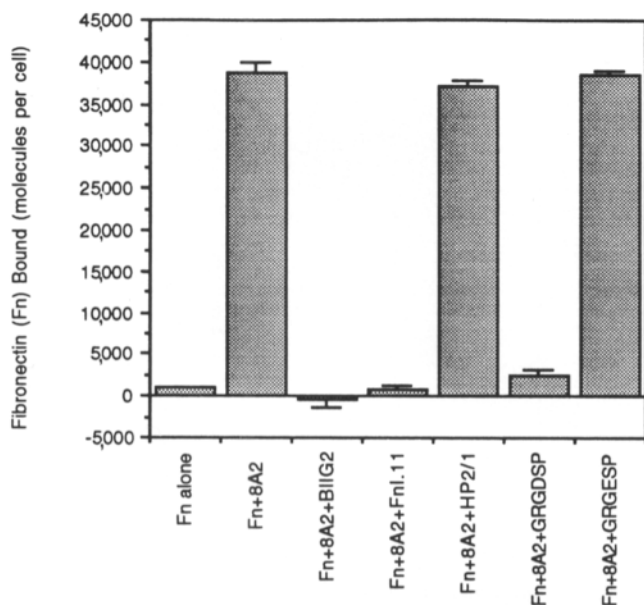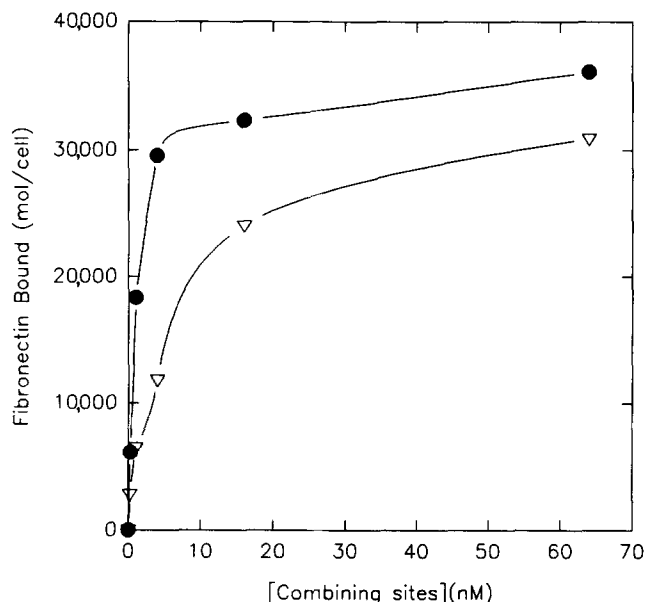
Figure 1. Binding of soluble fibronectin to K562 cells. Soluble ^{125}I-labeled fibronectin (100 nM) was added to 2 × 10^{6} K562 cells in a final volume of 200 μl. After incubation at 22°C for 30 min, bound fibronectin was assayed as described in Materials and Methods. The added reagents were: 8A2 (anti-β1; reference 32) (20 nM purified IgG), BIIG2 (anti-α5; reference 23) (1:200 dilution of ascites), FnI-11 (anti-cell binding domain of fibronectin; reference 9) (1:50 dilution of ascites), HP2/1 (anti-α4; reference 49) (1:10 dilution of culture supernatant), and peptides GRGDSP and GRG-ESP (250 μM). Results are the mean ± SD of three determinations.

chosen because they express only α5β1 of the β1 integrins, and do not express β3 integrins without induction (26). In contrast to BHK or CHO cells (1, 14), very little binding of soluble fibronectin to the K562 cells was observed (Fig. 1). Nevertheless, the α5β1 on these cells had the potential to bind substantial soluble fibronectin in the presence of the monoclonal antibody 8A2. The 8A2-induced binding was completely inhibited by a blocking antibody against the α5 chain (Fig. 1), confirming that α5β1 was responsible for all the observed binding. Moreover, it was mediated by the "classical" cell binding site in fibronectin, as it was blocked by a monoclonal anti-fibronectin antibody that recognizes this site (9), and by an RGD- but not an RGE-containing peptide (Fig. 1). Consistent with integrin-mediated binding, it

*Table I. 8A2 Induces the Binding of Soluble Fibronectin to Purified Peripheral Blood T Lymphocytes*

[8A2 IgG] nM	Fibronectin bound (molecules per cell)
0.0	1,515 ± 748
0.2	2,040 ± 628
2.0	4,505 ± 960
20	5,515 ± 1,534

Peripheral blood T lymphocytes were isolated from heparinized venous blood of volunteer donors by centrifugation on a Ficoll-Paque gradient, panning on serum-coated dishes to remove monocytes, and passage over a nylon wool column. ^{125}I-labeled soluble fibronectin (200 nM) was incubated with 2 × 10^{6} T lymphocytes in a final volume of 200 μl in the presence of increasing concentrations of 8A2 whole IgG. After incubation at 22°C for 30 min, bound fibronectin was assayed as described in Materials and Methods. Results are expressed as mean ± SD of three determinations.
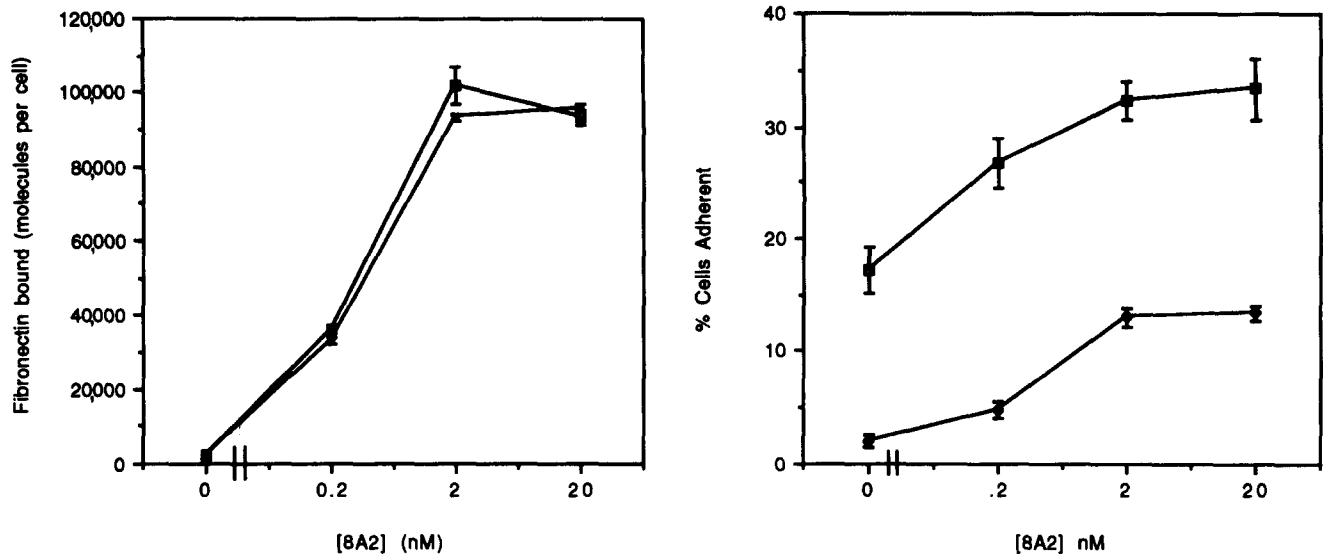

Figure 2. The binding of soluble fibronectin to K562 cells is induced by both 8A2 whole IgG and Fab fragments. Purified 8A2 IgG and monovalent Fab fragments were produced as described in Materials and Methods. Increasing concentrations of each were added to 2 × 10^{6} K562 cells in a final volume of 200 μl. After incubation at 22°C for 30 min, bound fibronectin was assayed as described in Materials and Methods.

failed to occur in buffer depleted of divalent cations or in the presence of EDTA (data not shown). The lack of high affinity fibronectin binding to α5β1 appears to be a general property of non-adherent hematopoietic cell lines, as similar results
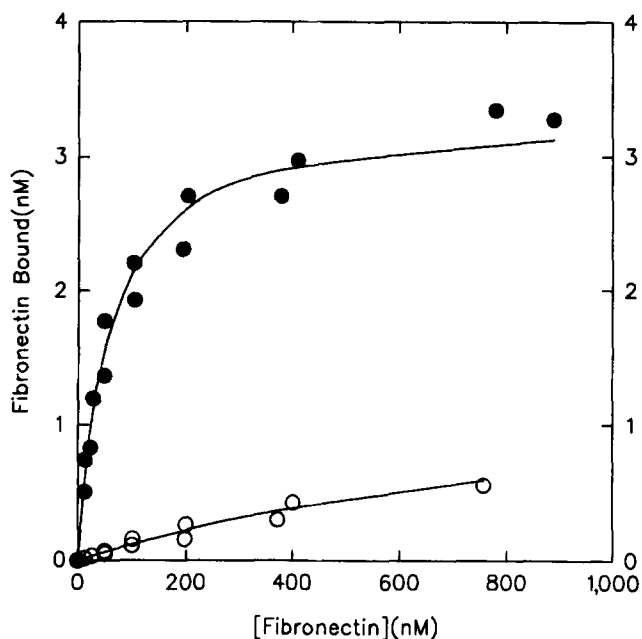
Figure 3. Specific binding of increasing concentrations of soluble fibronectin to K562 cells, either in the presence (●—●) or absence (○—○) of 20 nM of 8A2 whole IgG. Soluble ^{125}I-labeled fibronectin of known concentration was added to 2 × 10^{6} K562 cells in a final volume of 200 μl. After incubation at 22°C for 30 min, bound fibronectin was assayed as described in Materials and Methods. Nonspecific binding was estimated from the amount of fibronectin bound in the presence of 5 mM EDTA.

Figure 4. The metabolic inhibitors sodium azide and 2-deoxyglucose inhibit the adhesion of K562 cells to immobilized fibronectin, but have no effect on the 8A2-enhanced binding of soluble fibronectin to K562 cells nor on the 8A2-enhanced adhesion of K562 cells to immobilized fibronectin. (*a*) Binding of ^{125}I-labeled soluble fibronectin to K562 cells was determined in the presence of increasing concentrations of 8A2 whole IgG, as described in Materials and Methods. Before the binding assay, the cells were incubated for 30 min at 37°C in either the standard modified Tyrode's buffer, or in buffer with the glucose replaced by sodium azide (0.1%) and 2-deoxyglucose (5 mM), and the binding assay performed in the same buffer. Results are the mean ± SD of three determinations. (*b*) The adhesion of K562 cells to immobilized fibronectin was determined as described in Materials and Methods. Fibronectin (0.5 μg/well) was coated onto plastic micro-titer wells for 2 h at 37°C. Cells were preincubated in either buffer as above, and the 30 min adhesion step performed in triplicate wells at 22°C in the presence of increasing concentrations of 8A2 whole IgG. Percent adherence was determined and results expressed as mean ± SD of three determinations. (-□-) Standard buffers; (-●-) metabolic inhibitor.

were obtained with the monocytic cell lines THP-1 and U937, and the lymphoid cell lines Jurkat and Molt-13 (data not shown). In addition, peripheral blood T lymphocytes also manifested little basal fibronectin binding, and an increased in the presence of 8A2 (Table I).

The binding of fibronectin to K562 cells in response to varying concentrations of 8A2 has been quantified (Fig. 2). Fibronectin binding approached saturation at above 4 nM of 8A2 IgG, and the EC50 was ~1.0 nM. Specificity of this effect was documented by the failure of another anti-β1 mAb (AIIB2, reference 23) or an irrelevant antibody (W6/32 – anti-HLA class I) to induce this response (data not shown). Moreover, purified Fab fragments of 8A2 were also able to induce fibronectin binding (Fig. 2), albeit with lower efficiency than intact IgG. The contaminating whole IgG in the Fab preparation could account for <10% of this activity. Thus, the 8A2 effect was not dependent on bivalency nor on the Fc portion of the antibody molecule.

In additional experiments, the mAbs A1A5 and TS2/16, which also stimulate cellular adhesion to immobilized extracellular matrix proteins (6, 53), were able to induce the binding of soluble fibronectin to K562 cells (results not shown).

Affinity Modulation of α5β1

To characterize the mechanism of the increase in fibronectin binding induced by 8A2, detailed equilibrium binding isotherms were analyzed. In the absence of 8A2 there was little binding of fibronectin, although at higher fibronectin concentrations some specific binding was observed (Fig. 3). This data could be fitted to a single site binding model with

an estimated affinity, K_a, = 8.6 (±3.4) × 10^5 M^{-1} (K_d = 1.2 μM), and receptor concentration of 1.5 (±0.5) nM (9.0 × 10^4 receptors/cell). In the presence of 20 nM of 8A2 IgG, fibronectin bound with an estimated K_a of 1.8 (±0.3) × 10^7 M^{-1} (K_d = 54 nM), and receptor concentration of 3.3 (±0.3) × 10^7 M^{-1} (K_d = 54 nM), and receptor concentration of 3.3 (±0.25) nM (19.9 × 10^4 receptors/cell) (Fig. 3). The 20-fold difference in receptor affinity observed in the presence of 8A2 was highly significant by constrained parameter (37) curve fitting (F = 63.4, df = 21, P < 0.0001). In sharp contrast, the twofold difference in estimated receptor number was not significant (F = 1.7, df = 21, P = 0.22). Thus, the 8A2 antibody changes the binding affinity of α5β1 for fibronectin, and β1 integrins, like β2 and β3 integrins, can undergo affinity modulation.

Mechanism of Affinity Modulation by 8A2

Active cellular responses have been implicated in the capacity of anti-β1 monoclonal antibodies to promote cell adhesion (6, 32, 53), suggesting that these antibodies might work by activating intracellular signaling systems. However, in sharp contrast to previous results, the 8A2-induced increase in fibronectin binding was not affected by the metabolic inhibitors sodium azide and 2-deoxyglucose (Fig. 4 *a*). The effect of these drugs on the K562 cells was documented in concurrent measurements of cellular adhesion to immobilized fibronectin (Fig. 4 *b*). The metabolic inhibitors profoundly suppressed basal adhesion to fibronectin. Nevertheless, 8A2 promoted similar increases in adhesion in the presence of absence of the inhibitors. Thus, the combination of the inhibitors suppressed α5β1-dependent cellular adhe-
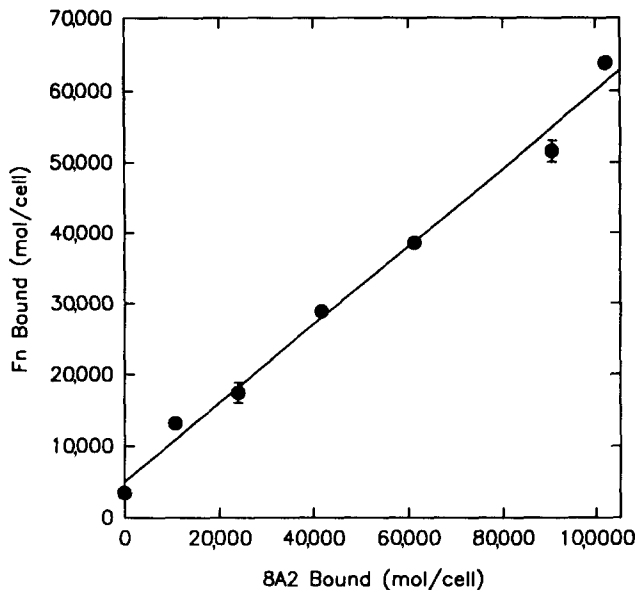
Figure 5. The number of fibronectin molecules bound per cell directly correlates with the number of 8A2 molecules bound. Parallel binding assays were performed as described in Materials and Methods. A fixed concentration of radiolabeled fibronectin (50 nM) was incubated with 2×10^6 K562 cells for 30 min at 22°C in the presence of increasing concentrations of unlabeled 8A2 Fab fragments. The number of fibronectin molecules bound per cell was determined at each concentration of 8A2 Fab. In parallel, the number of ^{125}I-labeled 8A2 Fab fragments bound per cell in the presence of the same concentration (50 nM) of unlabeled fibronectin was determined for the same concentrations of 8A2 Fab.

sion to fibronectin without affecting the affinity of the fibronectin–integrin interaction. In addition, treating the cells with 0.02 μg/ml of cytochalasin D did not reduce 8A2-induced fibronectin binding even though it effectively blocked basal cell adhesion to immobilized fibronectin (data not shown).

We also examined the relationship between the binding of 8A2 and fibronectin to K562 cells. To do this we simultaneously measured the binding to K562 cells of 50 nM of radiolabeled fibronectin and varying doses of radiolabeled 8A2 Fab fragments. As the concentration of 8A2 Fab increased, the binding of 8A2 Fab fragments (cf. Fig. 6 b) and fibronectin (cf. Fig. 2) approached saturation. Replotting of this data as 8A2 bound versus fibronectin bound showed a remarkable correlation between the number of 8A2 molecules bound and the number of high affinity $\alpha 5\beta 1$ fibronectin receptors, i.e., the number of fibronectin molecules bound ($r = 0.995$) (Fig. 5). This suggests that only those $\alpha 5\beta 1$ molecules that have physically associated with 8A2 bind fibronectin with high affinity.

Taken together, the lack of energy requirement and direct nature of the effect of 8A2 suggests that it binds $\alpha 5\beta 1$ and directly promotes an increase in ligand binding affinity through a change in receptor conformation. To determine whether 8A2 also preferentially recognizes an occupied conformer of $\alpha 5\beta 1$, the binding of 8A2 Fab fragments to K562 cells was quantitated in the presence or absence of the GRGDSP peptide ligand. The addition of increasing concentrations of GRGDSP resulted in a progressive increase in the binding of 10 nM 8A2 Fabs (Fig. 6 a). The EC50 for this

effect was 7.0 μM, which is similar to the IC50 we determined for the inhibition of 8A2-induced fibronectin binding to K562 cells (5.5 μM, data not shown). To define the basis for this effect, the binding of 8A2 Fab fragments to K562 cells in the presence or absence of 200 μM GRGDSP was analyzed (Fig. 6 b). In the presence of GRGDSP there was an approximate twofold increase in the affinity of 8A2 binding ($K_a = 5.5$ [$\pm$ 0.7] $\times 10^7$ M^{-1} to $K_a = 10.5$ [± 2.0] $\times 10^7$ M^{-1}), which was statistically significant (F = 6.8, df = 28, P = 0.015). In contrast, the receptor concentration (3.1 to 3.0 nM) was not significantly different in the presence of the peptide (F = 0.2, df = 28, P = 0.65). Thus, ligand binding to the $\alpha 5\beta 1$ increases the affinity of 8A2 binding.

Binding Affinity Governs the Inhibition of Cellular Adhesion by Soluble Fibronectin

Although soluble fibronectin and fibronectin fragments inhibit the adhesion of some cells to immobilized fibronectin (2, 56), other workers have failed to observe this dualistic role for fibronectin (22). We examined the possibility that affinity modulation of $\alpha 5\beta 1$ alters the capacity of soluble fibronectin to block cell adhesion. K562 cells were incubated with either an irrelevant antibody (W6/32) or 100 nM of 8A2 IgG in the presence or absence of 675 nM of soluble fibronectin, and permitted to adhere to immobilized fibronectin. Soluble fibronectin modestly ($\sim$50%) inhibited the low basal adhesion of K562 cells (Fig. 7). As expected, 8A2 nearly tripled adhesion to immobilized fibronectin. Strikingly, the addition of physiological plasma levels of soluble fibronectin (450–900 nM; 1, 36), in the presence of 8A2, completely suppressed specific adhesion to fibronectin. In the presence of soluble fibronectin, the net effect of the 8A2 antibody was profound inhibition of cell adhesion. Thus, the functional consequences of affinity modulation of $\alpha 5\beta 1$ are influenced by the ambient concentration of soluble fibronectin.

Discussion

The most important findings in this work are: (*a*) a $\beta 1$ integrin ($\alpha 5\beta 1$) can exist in different affinity states; (*b*) the anti-$\beta 1$ mAb 8A2 stimulates cell adhesion by converting the receptor from a low to a high affinity state; (*c*) this activation seems to be due to antibody-induced change in the receptor without intracellular signaling; and (*d*) the functional consequences of affinity modulation of the $\alpha 5\beta 1$ integrin depend on the ambient soluble fibronectin concentration.

The $\alpha 5\beta 1$ integrin can manifest differing affinities for soluble fibronectin. The K562 cells (as well as the other non-adherent cell lines that we tested) bind soluble fibronectin with low affinity ($K_d > 1$ μM), but can be induced to bind it with 20-fold higher affinity ($K_d = 54$ nM). It is noteworthy that the fibronectin receptors on the adherent CHO and BHK cell lines have a measured affinity for soluble fibronectin that lies between these two values (1, 14), implying that multiple affinity states exist. We have also found that soluble fibronectin binds to CHO cells with a similar affinity to that previously reported ($K_d = 95$ nM vs. 100 nM reported reference 14) (results not shown). This suggests that at plasma concentrations of fibronectin, high affinity receptors on adherent cells (e.g., fibroblasts) will be fully occupied. In contrast,
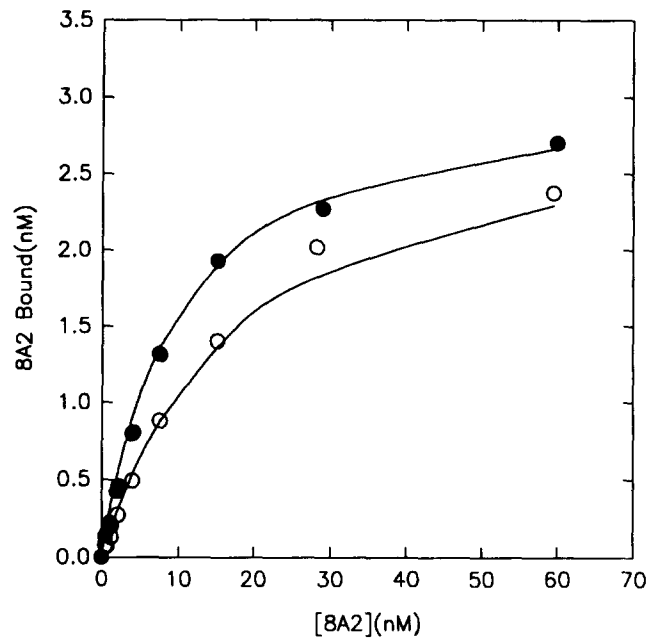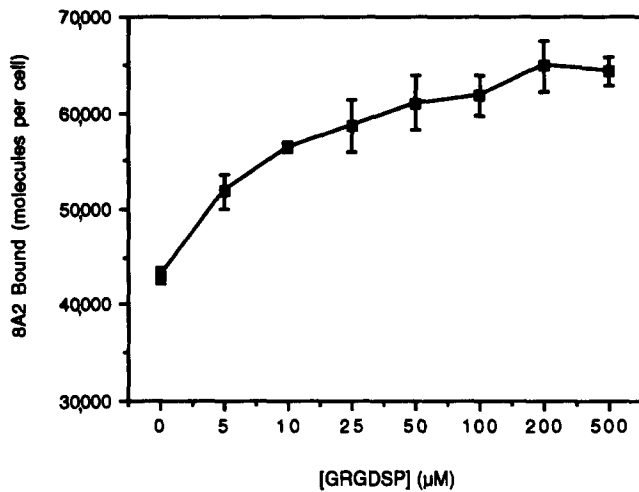
Figure 6. 8A2 Fab fragments bind with higher affinity to K562 cells in the presence of the GRGDSP peptide ligand. (a) ^{125}I-labeled 8A2 Fab fragments (10 nM) were incubated with 2 × 10^{6} K562 cells in a final volume of 200 μl in the presence of increasing concentrations of GRGDSP (0 to 500 μM). After incubation at 22°C for 30 min, bound 8A2 was assayed as described in Materials and Methods. Results are the mean ± SD of three determinations. (b) Known concentrations ^{125}I-labeled 8A2 Fab fragments were incubated with 2 × 10^{6} K562 cells in a final volume of 200 μl in the absence or presence of a fixed concentration of GRGDSP peptide (200 μM). After incubation at 22°C for 30 min, bound 8A2 was assayed as described in Materials and Methods. Nonspecific binding was determined in the presence of a 50× excess of unlabeled 8A2, and results are expressed as the estimated specific binding. (●) GRGDSP; (○) no addition.

low affinity $\alpha5\beta1$ on non-adherent cells (e.g., circulating T lymphocytes) would be expected to have much lower fractional occupancies.

The anti-$\beta1$ mAb 8A2 induces the K562 cells to bind solu-
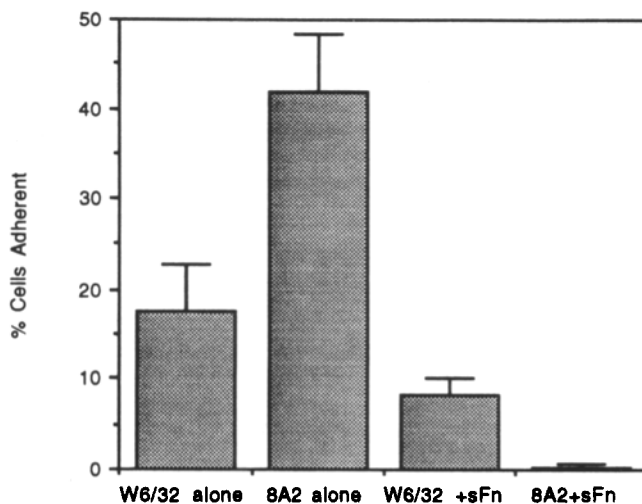
Figure 7. Soluble fibronectin (sFn) more effectively inhibits K562 cell adhesion to immobilized fibronectin in the presence of 8A2. K562 cells were preincubated in buffer for 30 min at 22°C with 100 nM of the irrelevant monoclonal antibody W6/32 or of 8A2, and with or without 675 nM of soluble fibronectin. Cellular adhesion to fibronectin (0.5 μg/well) immobilized on microtiter wells was performed in triplicate as described in Materials and Methods. Percent adherence was determined and results expressed as mean ± SD of three determinations.

ble fibronectin with higher affinity, which is a plausible basis for antibody enhancement of cellular adhesion to immobilized fibronectin. It is likely that the capacity of 8A2 to stimulate adhesion via other $\beta1$ integrins (32) is by the same mechanism. The 8A2-induced fibronectin binding has the appropriate characteristics for an integrin–ligand interaction, in that it is inhibited by the appropriate blocking antibodies as well as GRGDSP, and is dependent on the presence of divalent cations.

The 8A2-induced increase in receptor affinity is due to a direct effect on the receptor that is apparently independent of intracellular signaling. We found that the increased binding of soluble fibronectin in the presence of 8A2 was unaffected by treating the cells with the metabolic inhibitors sodium azide and 2-deoxyglucose, even though the same treatment effectively inhibited the adhesion of these cells to immobilized fibronectin. In addition, the metabolically "poisoned" cells still had enhanced adhesion in the presence of increasing concentrations of 8A2. This suggests that directly increasing receptor affinity for its ligand will by itself produce a measurable increase in adhesion, but that maximal adhesion requires the participation of further events that are dependent on the generation of intracellular ATP, e.g., cytoskeletal organization, focal contact formation, and cell spreading (11, 55). The tight correlation between fibronectin and 8A2 binding to the cells also suggests that the high affinity binding state of each receptor is directly dependent on an attached 8A2 molecule. Since the 8A2 Fab fragments bind with higher affinity to receptor that is occupied by the GRGDSP peptide ligand, the antibody may stabilize a high affinity (occupied) conformer of the receptor. Several activating anti-$\beta3$ mAbs also bind with higher affinity to the

occupied form of $\alpha IIb\beta3$, and have been termed anti-LIBS (ligand-induced binding sites) (19–21, 40). Thus it may be that many activating anti-integrin antibodies are also anti-LIBS.

The functional consequences of affinity modulation of the $\alpha5\beta1$ receptors depends on the ambient soluble fibronectin concentration. At physiological plasma concentrations of soluble fibronectin, a minority of the low affinity fibronectin receptors expressed by a cell will be occupied. In contrast, at the same concentration of fibronectin virtually all of the high affinity receptors will be occupied. For example, at the soluble fibronectin concentration of 675 nM that is used in the experiments summarized in Fig. 7, the calculated fractional occupancy of the low affinity receptors is 36%, whereas the fractional occupancy of the high affinity receptors is calculated to be 93%. Therefore, in the presence of saturating concentrations of 8A2, soluble fibronectin becomes a potent inhibitor of cellular adhesion to immobilized fibronectin by binding to the high affinity receptors. This "dualistic role" for fibronectin has been observed in cells which express moderate affinity fibronectin receptors (56). Hence, circulating leukocytes expressing low affinity receptors will retain their capacity to adherent to immobilized fibronectin that is laid down at sites of injury and during wound healing. In contrast, adherent cells express high affinity fibronectin receptors which could serve to bind soluble fibronectin from solution. This may be central to the involvement of $\alpha5\beta1$ in the assembly of an extracellular fibronectin matrix (3, 18, 34, 48).

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