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# Structural Interlock between Ligand-Binding Site and Stalk-Like Region of β1 Integrin Revealed by a Monoclonal Antibody Recognizing Conformation-Dependent Epitope[1]

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**Integrin activation and sebsequent ligand binding to it are regulated by intracellular mechanisms called inside-out signaling, which are not fully understood and are accompanied by dynamic structural changes of the integrin molecule itself. A monoclonal antibody recognizing a conformation-dependent epitope on human β1 integrin was produced and characterized in detail. This antibody, AG89, reacted with human integrin β1 chain regardless of the α subunit. AG89 can recognize resting state β1 integrin on the cells, but the reactivity is increased ~2-fold upon integrin activation by activating anti-β1 antibodies and ~3-fold by $Mn^{2+}$. Furthermore, occupation of the ligand-binding pocket by a soluble ligand (RGD peptide for αvβ1 and CS-1 peptide for α4β1) resulted in maximum binding of AG89, indicating that the epitope for AG89 is exposed during the conformational changes of β1 integrin upon activation/ligation. Epitope mapping by using interspecies chimeric β1 revealed that the epitope for AG89 lies within residues 426-587, which corresponds to the cysteine-rich repeat structure located in the middle of the β1 chain. The fact that binding of AG89 itself could activate the resting β1 integrin indicates that exposure of the AG89 epitope in the membrane-proximal stalk-like domain and "opening" of the ligand-binding pocket at the outermost domain are physically linked. We propose that the integrin "signaling" is mediated by this direct physical transduction of conformational information along the integrin molecule.**

**Key words: cell adhesion, inside-out signaling, integrin, ligand binding, monoclonal antibody.**

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits, and mediate cell adhesion to extracellular matrix proteins as well as cell-cell interactions (*1-3*). To date, more than fourteen α subunits and eight β subunits have been identified, and the combination of α and β subunits determines the ligand specificity of an individual integrin. Integrin-mediated cell adhesion plays crucial roles in regulating the morphology, proliferation, migration, and differentiation of cells. It is widely accepted that availability of integrins as adhesion receptors is dynamically regulated (*4-9*). For example, integrins on floating cells such as leukocytes are usually inactive; they need to be "activated" by various stimuli prior to displaying binding potential for their extracellular ligands (*10, 11*). Conversion of integrin from the resting to the activated state is accompanied by conformational change of the integrin molecule (*12-14*). Furthermore, ligand recognition by activated integrin appears to cause additional conformational changes resulting in the expression of epitopes known as ligand-induced binding sites (LIBS) (*15, 16*). Monoclonal antibodies (mAbs) have been the most valuable tools to investigate the molecular mechanisms of integrin functions. There are a number of both anti-α and β subunit mAbs capable of blocking the ligand binding of integrin. It is also known that some mAbs can activate the resting integrin and enhance its ligand-binding activity. There is another group of mAbs called "conformation-dependent" mAbs, which preferentially recognize activated and/or ligand-occupied conformation of integrin (*15, 17-19*). In the case of the β3 integrin family, dynamic conformational changes upon activation and subsequent ligand occupancy have been precisely investigated by using a number of anti-LIBS mAbs (*14-20*). In the case of other members of the integrin family, however, there is much less information about the structural changes controlling their affinity state, though several conformation-dependent mAbs against β1 (*21, 22*) as well as β2 subunit (*23*) have been reported. We report herein production and characterization of a mAb that recognizes a unique conformation-dependent epitope on the human integrin β1 subunit.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence activated cell sorting; LIBS, ligand-induced binding site; mAb, monoclonal antibody; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline.

Characterization of the relation between binding of this novel anti-LIBS mAb and integrin activating activity revealed that there is a conformational interlock between the ligand-binding site and a membrane-proximal stalk-like region.

## EXPERIMENTAL PROCEDURES

*Cells and Cell Culture*—Human melanoma cell line G-361, human normal diploid fibroblast cell line TIG-1, human lymphoma cell line MOLT-3, human monocytic cell line THP-1, and human erythroleukemic cell line K562 were provided by the Japanese Cancer Research Resources Bank (Tokyo) and cultured in either Eagle's minimum essential medium (MEM) containing 10% fetal calf serum or RPMI 1640 medium containing 10% fetal calf serum and non-essential amino acids. CHO cell clones stably expressing human β1 or α4 integrin subunits were established as described previously (*24*) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and non-essential amino acids.

*Monoclonal Antibodies*—Mice were immunized with G-361 human melanoma cells ($1\times10^7$ cells/500 μl PBS/mouse) four times every 10 days and the spleen was isolated 3 days after the last immunization. Spleen cells were fused with the mouse myeloma P3U1 essentially as described before (*25*). HAT selection and the subsequent cloning of the fused cells were carried out by using S-Clone cloning medium (Sanko Junyaku, Tokyo) with no feeder cells added. Antibodies were screened for cell surface reactivity with G-361 in the presence of $Mn^{2+}$ ion and a clone with high reactivity (AG89) was obtained. AG89 (IgG1, κ) was grown as ascites and purified by ammonium sulfate precipitation and anion exchange chromatography. MAb 4B4 recognizing human β1 integrin was a gift from Dr. C. Morimoto, Dana-Farber Cancer Institute, Boston, MA. MAbs A1A5 and TS2/16 (both anti-human β1) were obtained from Dr. M.E. Hemler (Dana-Farber Cancer Institute). Mouse mAb 8A2 (anti-β1) was from Dr. N. Kovach (University of Washington, Seattle, WA). Mouse mAb LM609 (anti-αvβ3) was purchased from Chemicon International (Temecula, CA).

*Immunoprecipitation and Western Analysis*—Reactivity of the resultant clone, AG89, against integrin β1 chain was confirmed as follows. TIG-1 human normal diploid fibroblasts were lysed in a buffer containing 1% Brij 96, 10 mM Tris-HCl, 150 mM NaCl, 5 mM $MnCl_2$, 5 μg/ml leupeptin, 5 μg/ml pepstatin A (lysis buffer) for 15 min on ice and the insoluble materials were removed by centrifugation. The lysates were subjected to immunoprecipitation by using anti-human β1 mAb (4B4) or anti-human αvβ3 mAb (LM609) which had been absorbed on anti-mouse IgG-Sepharose (American Qualex, La Mirada, CA). After extensive washing with the lysis buffer, the resultant immunoprecipitates were run on a 7.5% polyacrylamide gel under non-reducing conditions, and then transferred to Immobilon-P membrane (Millipore). The blot was probed with peroxidase-conjugated AG89 which had been prepared according to the method described previously (*26*), and detected by chemiluminescence reaction using the ECL system (Amersham). The supernatant from the anti-β1 immunoprecipitate, which represents the β1-depleted fraction, and the untreated cell extract were also analyzed by Western blotting with AG89.

*Fluorescence Activated Cell Sorting (FACS) Analysis*—Cells were detached from culture dishes and treated with primary antibody at an appropriate concentration (1:500 diluted ascites for A1A5 and 10 μg/ml for AG89 purified IgG) in serum-free medium for 30 min on ice. After having been washed once with serum-free medium, the cells were incubated with 1:50 dilution of FITC conjugated anti-mouse IgG for 30 min on ice, washed once with serum-free medium, and analyzed on a FACScan flow cytometer (Beckton Dickinson and Co., Mountain View, CA). Binding of AG89 to cells was also measured directly by using FITC-conjugated AG89. Briefly, purified AG89 IgG (0.3 mg) was incubated with 2 mg of FITC isomer I on Celite (Sigma) in 300 μl of 0.1 M $NaHCO_3$, pH 9.3, for 1 h in the dark, and free FITC was removed by gel filtration. FITC-AG89 was incubated with cells in the presence of various reagents for 30 min on ice, washed once with serum-free medium, and subjected to FACS analysis. Data were collected for 10,000 events, and values were expressed as the mean fluorescence intensity of the gated cell population.

*Mapping of the Epitope for mAb AG89 Using Interspecies Chimeric β1 Integrin*—cDNAs for human/mouse interspecies chimeras of β1 were prepared and used to transfect CHO cells as described (*27*). CHO cells stably expressing β1 chimeras were then cloned to obtain cells expressing a high level of β1 chain by single cell sorting with anti-β1 mAb A1A5. These cloned cells expressing various chimeric β1 integrins were analyzed for their reactivity with FITC-AG89 using flow cytometry.

*Cell Adhesion Assay*—Cell adhesion assay was carried out essentially as described previously (*28*). In brief, wells of 96-well microtiter plates were coated with bovine plasma fibronectin (Itoham Foods, Nishinomiya) in PBS and incubated at 4°C overnight. Nonspecific binding sites were blocked by incubating the plates with 1% bovine serum albumin for 30 min at room temperature. K562 cells ($10^5$ cells/well) in 100 μl of DMEM were added to the wells and incubated for 1 h at 37°C in the presence or absence of 5 μg/ml mAbs. After the removal of the unbound cells by rinsing the wells three times with PBS, the bound cells were quantified by incubating them with 100 μl of phosphatase assay buffer (50 mM sodium acetate, 1% Triton X-100, 6 mg/ml *p*-nitrophenyl phosphate, pH 5.2) for 1 h followed by addition of 50 μl of 1 N NaOH and measurement of the absorbance at 405 nm.

*Binding of FITC-Labeled Fibronectin to Cells*—Fibronectin was labeled by the same method used in the preparation of FITC-AG89. β1-CHO cells were harvested with 3.5 mM EDTA in PBS and washed with PBS. Cells were first incubated with mAbs TS2/16 or AG89 in DMEM at various concentrations for 30 min on ice, washed once with PBS, incubated with FITC-fibronectin (at a final concentration of 25 μg/ml) in DMEM for 30 min on ice, and then subjected to FACS analysis.

## RESULTS

We have reported previously that a unique non-matrix protein called propolypeptide of von Willebrand factor (pp-vWF) promotes melanoma cell adhesion in a β1 integrin-dependent manner (*29*). In the course of identifying the α subunit of this adhesion receptor, we obtained a

series of mAbs that react with the cell surface of a human melanoma cell line G-361. A clone designated AG89, which did not inhibit cell adhesion to pp-vWF, was established as a control antibody. When the cell surface reactivity of this mAb was checked by FACS analysis, expression of AG89 antigen was not restricted to G-361; AG89 reacted with hematopoietic cell lines such as K562 and MOLT-3 (Fig. 1). Furthermore, the AG89 antigen was also present on almost all the human cells tested, including normal fibroblasts, blood platelets, endothelial cells, and various tumor cell lines (data not shown). It is clear that the antigen for AG89 is widely distributed among various human cell types, and it seems to be a common cell surface protein. As CHO cells are completely negative for AG89 binding, it seems that AG89 cannot recognize the hamster protein. When CHO cells transfected with human $\beta1$ integrin were tested, however, bright staining with AG89 was observed (Fig. 1). Other transfectants, such as $\alpha4$-CHO, were again completely negative. These staining patterns paralleled that with anti-$\beta1$ integrin mAb A1A5, though the fluorescence intensity that resulted from the staining with AG89 was always somewhat lower. These results strongly suggest that the cell surface protein recognized by AG89 is integrin $\beta1$ chain. In order to confirm this, Western blotting experiments were performed. AG89 was conjugated with peroxidase and used to detect the corresponding antigen in cell extract of human fibroblast TIG-1. As shown in Fig. 2A, AG89 specifically reacted with a band of 115 kDa, band which is very similar to the relative molecular weight of $\beta1$ integrin under non-reducing conditions. Furthermore, this band was lost upon treatment of the extract with the known anti-$\beta1$ mAb 4B4 (lane 2). Recognition of the $\beta1$ chain by AG89 was further confirmed by immunoprecipitation-Western experiments. $\beta1$ or $\beta3$ integrin complexes were immunoprecipitated from the cell extract with the respective mAb, followed by detection with AG89. As is clearly depicted in Fig. 2B, AG89 again reacted with ~115 kDa $\beta1$ chain from the immunoprecipitate of 4B4, but not from that of LM609 (anti-$\alpha v\beta3$ integrin). It can be concluded at this point that the antigen recognized by AG89 is human integrin $\beta1$ chain.

In the preliminary observation, reactivity of AG89 with G-361 cells in serum-free medium (Fig. 1) was somewhat lower than that observed in the presence of $Mn^{2+}$ ion. As it is known that $Mn^{2+}$ ion can "activate" $\beta1$ integrin by binding to the metal ion-dependent adhesion site (MIDAS) (*30*), we anticipated that epitope expression of AG89 would be augmented upon $\beta1$ integrin activation. As is clearly shown in Fig. 3, this was the case; binding of FITC-AG89 to $\beta1$-CHO increased ~3-fold in the presence of 5 mM $Mn^{2+}$. Moreover, activating anti-$\beta1$ mAbs, TS2/16 and 8A2, also increased the AG89 reactivity, suggesting that AG89 recognizes an activation-dependent epitope on $\beta1$ integrin. Though the activating mAbs were used at saturating concentrations, AG89 epitope expression of these mAbs was only increased by ~2-fold and the combination of $Mn^{2+}$ and activating mAb resulted in a synergistic increase in

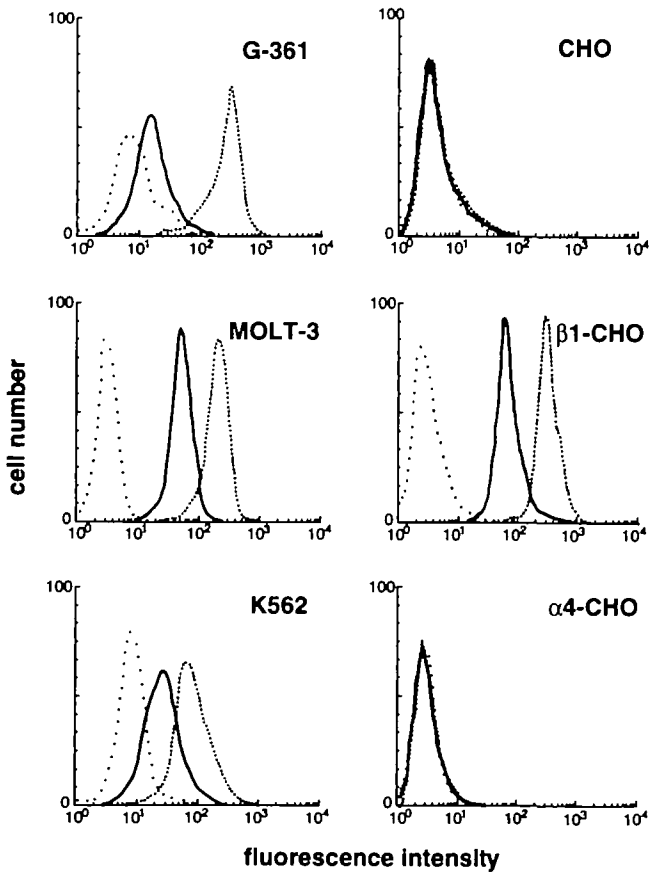


Fig. 1. **Expression of the antigen for AG89 on various cell lines.** Cells were first incubated with mAb AG89 or A1A5 or control mouse IgG for 30 min on ice. They were washed once with serum-free medium, then incubated with FITC-labeled anti-mouse IgG and analyzed by flow cytometry. Solid line, AG89; tight dotted line, A1A5; sparse dotted line, control IgG.

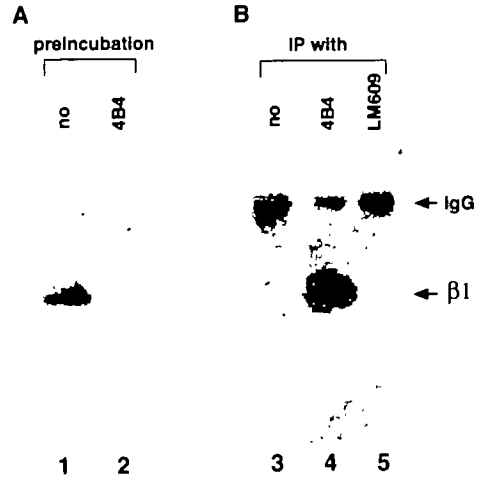


Fig. 2. **AG89 recognizes human integrin $\beta1$ subunit.** A: Western analysis of whole cell lysate with AG89. Human normal fibroblast TIG-1 cells were lysed by 1% Brij 96 and incubated with control mouse IgG (lane 1) or anti-$\beta1$ mAb 4B4 (lane 2). The immunocomplex was removed by using anti-mouse IgG-Sepharose, and the cell lysates were electrophoresed on a 7.5% polyacrylamide gel under nonreducing conditions, transferred onto Immobilon-P membrane, and blotted with peroxidase-conjugated AG89. B: Immunoprecipitation-Western analysis of integrin by AG89. TIG-1 cell lysate was immunoprecipitated with control IgG (lane 3), anti-$\beta1$ mAb 4B4 (lane 4), or anti-$\alpha v\beta3$ mAb LM609 (lane 5) and the immunoprecipitates were subjected to Western blotting by using AG89 as in A. The positions of IgG and human $\beta1$ integrin are indicated on the right.

AG89 binding. It seems likely that the conformational changes of β1 integrin induced by $Mn^{2+}$ and activating mAb are different, and both activations are necessary to induce the most favorable conformation of β1 integrin for recognition by AG89. It is also clear that the binding of AG89 to activated as well as resting β1 integrins is specific, since addition of an excess amount of unlabeled AG89 completely reversed the FITC-AG89 binding.

There are two groups of anti-β3 mAbs known to bind preferentially to activation-dependent conformation of β3 integrin. One group recognizes the ligand binding pocket itself and competes with ligand (*20*), and the other recognizes the so-called "ligand-induced binding site (LIBS)" and binds more avidly upon ligand occupation (*12, 17*). By analogy with the situation for β3, AG89 reactivity to β1 integrin may be affected by ligand binding. In order to clarify this point, binding of FITC-AG89 was evaluated in the presence of soluble ligands. Although integrins on suspended cells usually cannot bind soluble ligands unless they are activated (*7*), synthetic peptides containing the RGD sequence can bind to resting αIIbβ3 integrin when added at high concentration. We used αvβ1-CHO (CHO cells transfected with both human αv and β1 subunits) for this purpose, because α5β1 (a major integrin complex expressed on β1-CHO) is less effective in recognizing the RGD sequence in fibronectin without the aid of a synergistic site (*31*). As shown in Fig. 4, ligation of αvβ1 integrin by RGD peptide resulted in a more than 10-fold increase in AG89 epitope expression. This was much higher than that induced by activation with TS2/16, and the increase was dependent on the RGD peptide concentration, suggesting that the ligand-occupied conformation of β1 integrin is the most favorable for recognition by AG89. Specificity of the amino acid sequence was revealed by the lack of effect of RGE control peptide. This ligand-induced epitope expression was not limited to the RGD-dependent β1 integrin, because a lymphocyte-like cell line MOLT-3, which almost exclusively expresses α4β1 as β1 class integrin, showed increased reactivity with AG89 upon ligation with the α4β1 ligand CS-1 peptide (Fig. 4). In this case, the extent of epitope expression by the ligand was also higher (about 15-fold) than that by activating mAb. RGD peptide did not cause a significant increase in epitope expression because this cell line lacks RGD-dependent integrins. In the case of human monocytic cell line THP-1, which also expresses α4β1, the induction of the AG89 epitope by CS-1 peptide was somewhat lower (about 1.6-fold). However, addition of $Ca^{2+}$ together with CS-1 greatly increased AG89 epitope expression to about 500%, suggesting a difference in β1 integrin basal state on MOLT-3 and THP-1, in that α4β1 on MOLT-3 can readily recognize the soluble ligand, while that on THP-1 requires $Ca^{2+}$ to bind the soluble peptide with sufficient affinity. Moreover, induction of AG89 epitope by CS-1 peptide was reversed in the presence of EDTA, but the level of AG89 binding was almost the same as that to resting cells. Therefore, it is likely that the

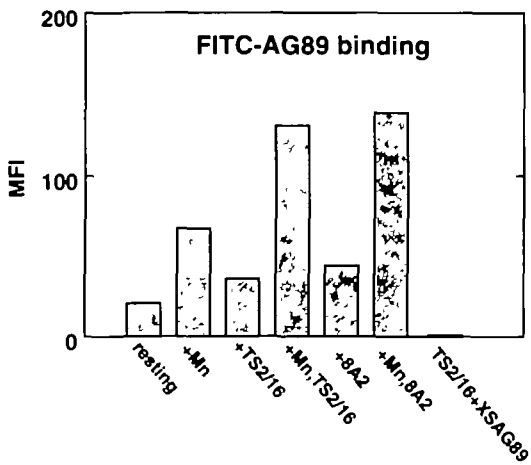


Fig. 3. **Augmentation of AG89 reactivity upon activation of β1 integrin.** β1-CHO cells were exposed to FITC-AG89 (10 μg/ml) in the presence of the following concentrations of the indicated reagents for 30 min on ice: $Mn^{2+}$, 5 mM; TS2/16 and 8A2, 1:500 dilution of ascites. One sample was incubated with excess unlabeled AG89 (314 μg/ml) in the presence of TS2/16 (XS AG89). After incubation, the cells were subjected to FACS analysis without washing and the binding was expressed as mean fluorescence increase (MFI), which represents mean fluorescence intensity of the sample minus that of cells stained with FITC-labeled mouse control IgG.

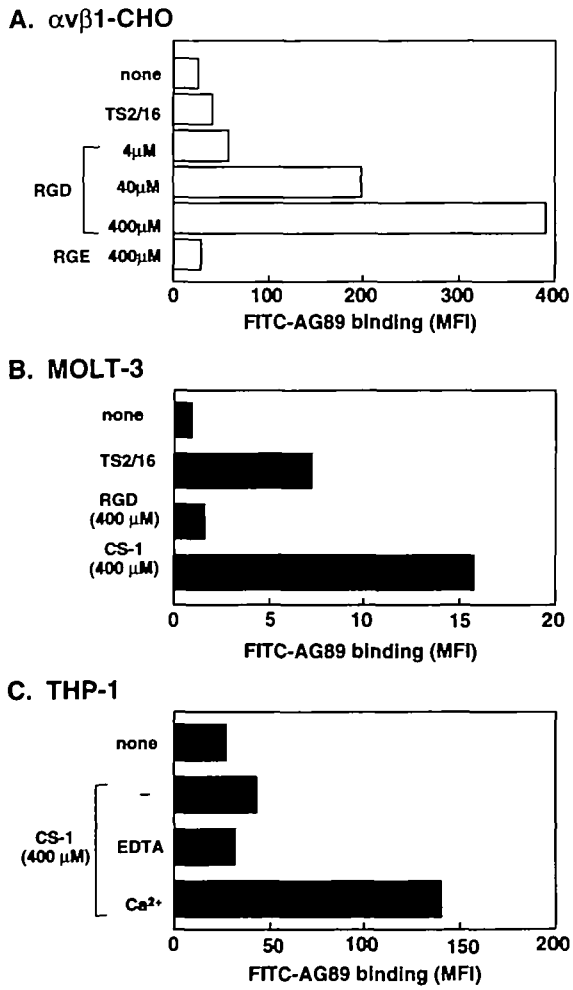


Fig. 4. **Induction of the AG89 epitope by soluble ligands.** αvβ1-CHO (A), MOLT-3 (B), and THP-1 (C) cells were exposed to FITC-AG89 (10 μg/ml) in the presence of TS2/16 (1:500 dilution of ascites) or indicated concentrations of the soluble peptides. In the case of THP-1 cells, the effect of CS-1 was also investigated in the presence of EDTA (5 mM) or $Ca^{2+}$ (10 mM). Bindings are expressed as described in the legend to Fig. 3.

binding of AG89 to the resting cells represents the partial reactivity of AG89 with inactive $\beta 1$ integrin, but not the existence of a small receptor pool which is constitutively active. Thus, we conclude that AG89 is an anti-LIBS antibody that preferentially recognizes a conformation of activated and ligand-occupied $\beta 1$ integrin.

To date, two mAbs have been reported to recognize conformation-dependent epitopes on $\beta 1$ integrin; 15/7 (*22*) and 9EG7 (*21*). The epitope for the former was mapped to residues 354-425 in $\beta 1$ integrin structure and that for the latter, to residues 495-602. In order to see whether AG89 recognizes a similar structure, epitope mapping was performed by using various human/mouse interspecies chimeric $\beta 1$ integrins. Figure 5 shows the structures of the human/mouse interspecies chimeras used in the following experiments. CHO cell clones expressing these chimeras to almost the same degree were subjected to FACS analysis using AG89. As shown in Table I, AG89 reacted with wt and h587/m $\beta 1$, but not with h425/m or h354/m $\beta 1$. Endogenous hamster $\beta 1$ on CHO cells showed almost no reactivity to AG89. It is concluded that the epitope for AG89 lies within residues 426-587 in $\beta 1$, which corresponds to a domain composed of four cysteine-rich repeat structures. This overlaps with the putative location of the epitope for mAb 9EG7, but is clearly distinct from that for 15/7.

MAb 9EG7 was originally reported to block lymphocyte adhesion to endothelial cells (*32*), but recently it was reported to recognize an epitope induced by soluble ligand and $Mn^{2+}$ ion and, furthermore, it could stimulate cell adhesion under certain conditions (*21*). In addition, Faull *et al.* (*33*) have recently established an anti-$\beta 1$ mAb with stimulating activity whose epitope lies in the region 426-587 (the same as that for AG89), though the epitope is not induced by activation or ligation of integrin. We then decided to look into the effect of AG89 on the $\beta 1$ integrin affinity state by using two different methods. Human erythroleukemic cell line K562 expresses the fibronectin receptor $\alpha 5\beta 1$ as its only $\beta 1$ integrin (and does not express $\beta 2$ or $\beta 3$ integrin), while most of the adherent cells, such as TIG-1 and CHO, express other fibronectin receptor integrins ($\alpha v\beta 3$, $\alpha v\beta 5$, *etc.*). So the effect of activatig anti-$\beta 1$ mAb on the adhesion to fibronectin can be unambiguously determined using this cell line. As shown in Fig. 6, AG89 significantly increased the adhesion of K562 cells to bovine fibronectin in a way very similar to that of the known activating mAb TS2/16 (Fig. 6). The enhancing effect was especially marked at lower concentrations of fibronectin. As it is known that low-affinity-state integrin can mediate cell adhesion and spreading only when the density of the coated ligand is high enough (*22*), it seems that both TS2/16 and AG89 promoted cell adhesion to low concentrations of fibronectin by increasing the affinity of $\alpha 5\beta 1$ integrin on K562 cells. The augmentation of receptor avidity by AG89 was also investigated by another approach utilizing soluble fibronectin binding assay. On the floating cells such as K562, as well as the cells detached from culture dishes, the $\alpha 5\beta 1$ integrin usually binds the soluble ligand (fibronectin) at very low affinity (*7*). As shown in Fig. 7, TS2/16 induced FITC-fibronectin binding to $\beta 1$-CHO in a suspension and the maximum binding was achieved at 1 $\mu$g/ml TS2/16. Though AG89 also induced fibronectin binding in a dose-dependent manner, the concentration of AG89 required for the induction was much higher than that of TS2/16 and fibronectin binding was not saturated even in the presence of 100 $\mu$g/ml AG89. Nevertheless, this activating effect of

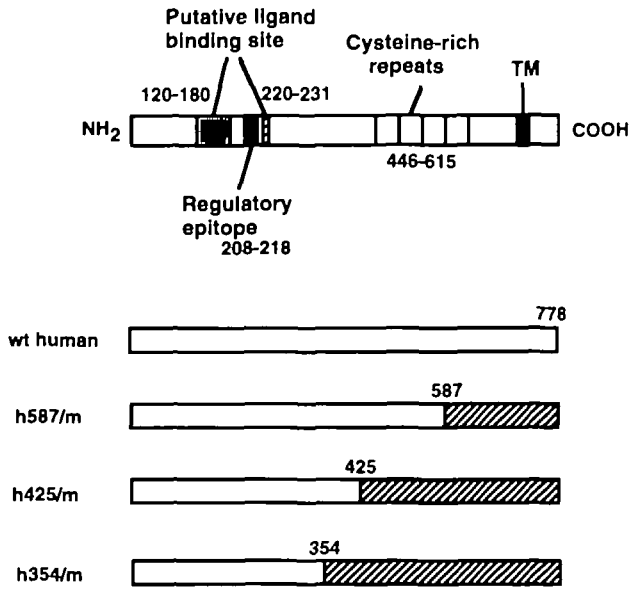


Fig. 5. **Schematic representations of various interspecies chimeric $\beta 1$ integrins used for epitope mapping.** The constructs were prepared as described previously (*27*). The upper part of this figure shows the structural features of the $\beta 1$ chain. The lower part shows the structures of chimeras, where the open box represents human sequences and the hatched box represents mouse sequences.

TABLE I. **Reactivity of AG89 to various human/mouse chimeric integrin $\beta 1$ chains.** CHO cells stably expressing wild-type or chimeric $\beta 1$ chains (see Fig. 5) were stained either with AG89 or control mouse IgG and analyzed by flow cytometry. Numbers represent % positive cells.

| Antibody | CHO | Human wt | h587/m | h425/m | h354/m |
|---|---|---|---|---|---|
| AG89 | 1.4 | 95.1 | 89.6 | 2.68 | 4.42 |
| mIgG | 2.45 | 5.92 | 3.67 | 2.71 | 3.45 |

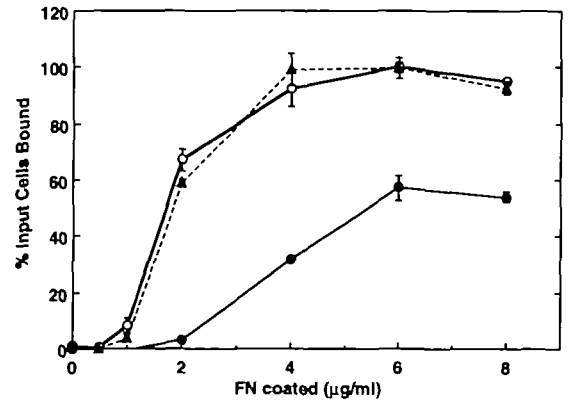


Fig. 6. **AG89 activates $\beta 1$ integrin.** K562 adhesion to plasma fibronectin coated at various concentrations was evaluated in the presence of 5 $\mu$g/ml control mouse IgG (closed circles), AG89 (open circles), or TS2/16 (closed triangles). Adhering cells were quantitated by phosphatase assay as described under "EXPERIMENTAL PROCEDURES" and expressed as percent of the input cells. Data represent mean ± SE of triplicate samples.

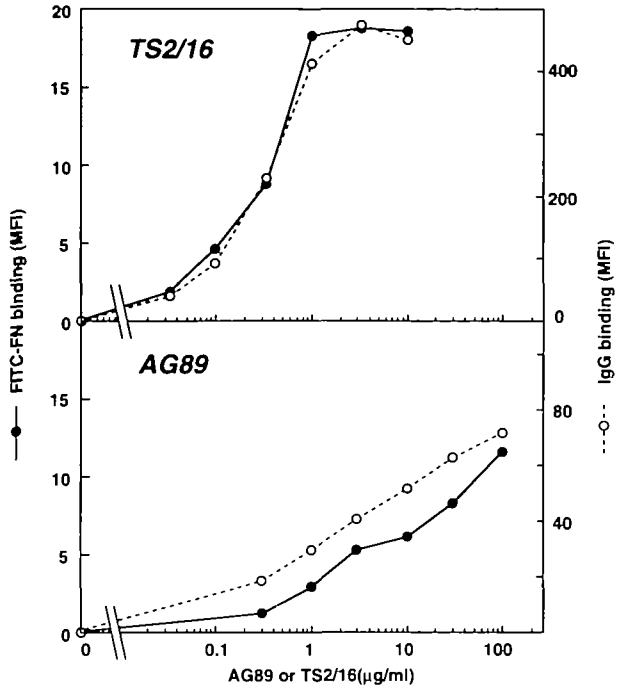


Fig. 7. **Induction of soluble FITC-fibronectin binding to β1-CHO cells by AG89 and TS2/16.** β1-CHO cells were first incubated with the indicated concentration of TS2/16 (upper panel) or AG89 (lower panel) and then exposed either to FITC-fibronectin (closed circles) or FITC-anti-mouse IgG (open circles). Each binding was expressed as the mean fluorescence increase (MFI), which represents mean fluorescence intensity of the sample minus that of basal binding obtained in the absence of antibodies.

AG89 was specific, because control mouse IgG did not induce fibronectin binding at all (data not shown). When the binding of each mAb was evaluated by using FITC-labeled secondary antibody, very similar dose response curves were obtained (Fig. 7, open circles). It is obvious that the requirement of a high concentration of AG89 to induce fibronectin binding reflects the low binding affinity of AG89 to the resting β1 integrin. The reason for the inability of AG89 to attain maximum binding even at 100 μg/ml, which is more than 300-fold higher than the concentration at which significant binding is observed (0.3 μg/ml), is not clear. The difference between TS2/16 and AG89 in the ability to induce soluble fibronectin binding, but not in the ability to support cell adhesion to immobilized fibronectin, may indicate that they induce and/or stabilize slightly different conformations of β1 integrin to activate the receptor complex.

## DISCUSSION

In a physiological condition, integrin activation is achieved by a process called "inside-out signaling" whereby cells convert intracellular signaling events into changes in the extracellular conformation of integrins (*2*). For example, leukocyte integrins are activated during the response to inflammatory mediators and gain adhesive activity, resulting in successful extravasation at the inflammatory site (*34-36*). It is also known that activation of platelet integrin (αIIbβ3) upon platelet stimulation by thrombotic agents, which allows fibrinogen binding to it, is essential to the hemostatic plug formation at the site of vascular injury (*37, 38*). Therefore, elucidation of the mechanisms of dynamic change in the integrin affinity state is central to the understanding of the adhesive events of cells in physiological as well as pathological states. Direct evidence that integrin activation is accompanied by conformational changes has been obtained by using fluorescence resonance energy transfer (*13*), but the conformational changes are more easily detected by mAbs. The use of many conformation-dependent mAbs has indicated that integrins on the cells take different conformations depending on the circumstances and, even on the resting cells, exist as subpopulations in different activation states (*7, 22*). However, it is not clear that this information about conformational modulation upon integrin activation is applicable to all classes of integrins, because most of these observations were made using conformation-dependent mAbs against αIIbβ3 integrin. As AG89 preferentially recognizes the activated and ligated form of the β1 integrin, it enables us to detect and quantitate the high-affinity β1 on the cells under particular conditions. Monitoring the β1 integrin affinity state by using AG89 would be very useful in studying candidate intracellular signaling molecules that mediate inside-out-type integrin activation.

Several conformation dependent anti-β1 mAbs have been reported. Two of these, mAb 9EG7 and 15/7, have rather similar properties to AG89 in that they recognize the ligand-induced binding site and can activate integrin. However, they differ greatly in some respects. 9EG7 epitope expression is induced by $Mn^{2+}$ but is greatly reduced by $Ca^{2+}$ (*21*), while both divalent cations induced the AG89 epitope (Figs. 3 and 4C). 15/7, whose epitope lies in a different region (residues 354-425) from that of AG89 (*39*), failed to react with resting β1 integrin on cells such as THP-1, but bright staining of resting THP-1 is observed with AG89 (Fig. 4C). It is possible that the structures recognized by these mAbs are closely related but different in detail, resulting in a difference in the specificity. It will therefore be interesting to compare the binding properties of these mAbs very precisely to investigate the molecular mechanisms of conformational changes in the β1 integrin molecule.

It is now accepted that β1 integrin contains a domain similar to the "I domain" which is present in certain α subunits of integrin, as well as other adhesive proteins (*40, 41*) and this I domain-like structure represents a putative ligand-binding site in β1 chain (*42*). Mapping of the epitope for AG89 has revealed that it binds to residues 426-587 in β1, a region comprising cysteine-rich repeat structure that is located far from the putative ligand-binding domain. The fact that binding of AG89 is affected by activation and ligand occupation of β1 integrin suggests that β1 integrin undergoes drastic conformational change upon activation/ligation, not only in the ligand-binding pocket itself, but also in the distant membrane proximal region. Reciprocally, binding of AG89 itself could activate the resting β1 integrin, suggesting that stabilization of the altered conformation in the cysteine-rich repeat region by AG89 will "open" the distant ligand-binding pocket. Thus, the signal is "transferred" as conformational information along the integrin molecule in both directions. It is possible that this structural information is also transferred all the way to the

cytoplasmic tail of the $\beta 1$ chain, thus providing a mechanism for inside-out as well as outside-in signal transduction. Elucidation of the physiological machinery organizing the molecular dynamics of integrin conformation will greatly improve our understanding of the complex regulation of adhesion events of cells.

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