Monoclonal Antibody 9EG7 Defines a Novel β1 Integrin Epitope Induced by Soluble Ligand and Manganese, but Inhibited by Calcium*

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The monoclonal antibody 9EG7 has been previously found to recognize an epitope induced by manganese on the integrin β1 chain (Lenter, M., Uhlig, H., Hamann, A., Jeno, P., Imhof, B., and Vestweber, D. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 9051–9055). Here we show that treatment of β1 integrins with manganese or soluble integrin ligands (e.g. fibronectin and RGD peptide) induced the 9EG7 epitope. This epitope was also induced upon EGTA treatment to remove calcium, and the addition of calcium inhibited 9EG7 epitope induction by manganese or by ligand. Further emphasizing the importance of the 9EG7 epitope, the 9EG7 antibody itself stimulated adhesion mediated by multiple β1 integrins, and conversely, ligands for αbβ2, αbβ3, αbβ1, and αbβ2 all stimulated 9EG7 expression. Together these results support a model whereby (i) calcium inhibits β1 integrin function because it prevents the appearance of a conformation favorable to ligand binding and (ii) manganese enhances β1 integrin function because it induces the same favorable conformation that is induced by adding ligand, or removing calcium. Notably, other β1-stimulating agents (magnesium and mAb TS2/16) did not induce 9EG7 expression unless ligand was also present. Thus, although 9EG7 may reliably detect the ligand-bound conformation of β1 integrins, its expression does not always correlate with integrin “activation.” Finally, mouse/chicken β1, chimeric molecules were used to map the 9EG7 epitope to β1 residues 495–602 within the cysteine-rich region, and antibody cross-blocking studies showed that the 9EG7 epitope is distinct from all previously defined human β1 epitopes.

Adhesive events mediated by transmembrane αβ heterodimers in the integrin family are dynamically regulated (1, 2). Dramatic changes in integrin-mediated adhesive functions can be observed upon cell differentiation (3, 4), during hemostatic (5) and immunological (6) responses, and upon triggering with various cellular agonists (2, 7). Studies with the platelet integrin αIIbβ3 have indicated that both integrin activation (8–10) and ligand binding (11, 12) are associated with conformational changes, and that an “activated” integrin sometimes resembles a “ligand-bound” integrin (13).

The study of variable integrin activation states has been facilitated by the use of monoclonal antibodies that selectively recognize distinct integrin conformations. Notably, the expression of several integrin epitopes is regulated by cell triggering with various agonists, divalent cation gain or loss, ligand binding, or combinations of these events (9, 11, 12, 14–19). Also, several antibodies can stimulate integrin adhesive functions, presumably by stabilizing an active conformation (4, 12, 15, 20–26). Interestingly, some antibodies not only recognize epitopes induced by ligand, but also stimulate integrin function themselves, whereas other antibodies apparently have only one or the other of these properties.

The mechanism for ligand binding and activation of β1 integrins has not been well studied, partly because few antibodies that specifically recognize activated or ligand-bound forms have been available. One possible “activation-specific” antibody (called 15/7) has been found to selectively recognize β1 on activated T cell subsets in vivo (27). Also, two recently described antibodies (9EG7 and SG/7) define β1 neoepitopes induced by divalent cations. The former recognizes an epitope up-regulated in response to Mn2⁺ treatment (28), whereas the latter defines a β1 epitope induced by either Mn2⁺ or Ca2⁺, but not Mg2⁺ (29). However, the association of these epitopes with integrin functions has not been extensively studied. Also, there are two antibodies that recognize ligand-induced β1 epitopes (30, 31). In other studies of β1 integrins, the inhibitory effects of Ca2⁺ (32–36), and the stimulatory effects of manganese (34, 35, 37–39) have often been noted, but few mechanistic insights have emerged.

Because little is known regarding β1 integrin conformational changes, we have utilized the 9EG7 mAb4 to study events accompanying β1 integrin activation and ligand binding. We define “activation” as an increase in the potential of an integrin to bind ligand, and/or to mediate the more complex function of cell adhesion. We wish to clearly distinguish “activation” as a distinct phase that precedes “ligand binding.”

Here we have found that the 9EG7 antibody defines a β1 conformation of fundamental importance because (i) the epitope is negatively regulated by calcium, (ii) it is induced by manganese, (iii) it is induced by the binding of all β1 integrin ligands tested, and (iv) the 9EG7 epitope only stimulates all β1 adhesive functions tested. Finally, we mapped the 9EG7 epitope to a site within β1, distinct from that recognized by all other known anti-human β1 antibodies, and not previously shown to be regulated by divalent cations and ligand binding.

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1 The abbreviations used are: mAb, monoclonal antibody; BCECF-AM, 2',6'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; CLIBS, calcium- and ligand-influenced binding site; hBSA, heat-denatured BSA; LIBS, ligand-induced binding site; PMA, phorbol 12-myristate 13-acetate; TBS, Tris-buffered saline; aa, amino acid(s); BSA, bovine serum albumin.
MATERIALS AND METHODS
Antibodies and ECM Proteins—The Mn$^{2+}$-dependent anti-$\beta_1$, mAb 9EG7 (IgG2a, rat anti-mouse with human cross-reactivity), has been described previously (28). Other anti-$\beta_1$, mAbs were mouse anti-human, TS2/16 (40), 15/7 (27), K20 (41), LM534 and LM442 (42); rat anti-human, mAb 13 (43); and mouse anti-chicken, CSAT (44) and W1B10 (45). Also used were mouse mAb anti-human $\alpha_\text{v},$ A5-PUJ 2; rabbit polyclonal anti-$\beta_2$, cytoplasmic domain (46) and rabbit anti- $\beta_3$, cytoplasmic domain (47). Human fibronectin was from Life Technologies, Inc., the peptides GRGDSP and GRGESP were from Telios Co. (La Jolla, CA), and phorbol 12-myristate 13-acetate (PMA) was from Sigma.

Cell Adhesion Assay—K562 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cell attachment to fibronectin was carried out as described previously (48). Briefly, fibronectin (5 g/ml) was coated onto 96-well microtiter plates (Flow Laboratories), and incubated overnight at 4°C. Then 0.1% heat-denatured BSA (hBSA) was added (45 min) to block nonspecific adhesion. Cells were labeled by incubation with the fluorescent dye BCECF-AM (Molecular Probes, OR) for 30 min and then washed sequentially with divalent cation-free PBS, 1 mM EDTA in PBS, and TBS (Tris-buffered saline: 24 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl), before resuspending in TBS containing 0.1% hBSA and 2 mM glucose (assay buffer). Cells (5 x 10$^5$/well) were then added to each well in triplicate in the presence of various concentrations of divalent cations, mAbs TS2/16, or PMA. After incubation for 25 min at 37°C, the plate was washed three times with TBS to remove unbound cells. Cells remaining attached to the plate were analyzed using a fluorescence analyzer (CytoFluor 2300, Millipore Co.).

For flow cytometry, cells were washed once with TBS and preincubated on ice for 10 min with 5% BSA in TBS containing 0.02% sodium azide (washing buffer) and then were incubated, with divalent cations or other stimulants. Aliquots of 3 x 10$^5$ cells were then incubated for 45 min on ice with primary antibodies (ascites at a final dilution of 1:250 or purified mAbs at a final concentration of 1 $\mu$g/ml). Cells were washed three times with washing buffer and incubated for 30 min on ice with fluorescein isothiocyanate-conjugated goat anti-mouse (Life Technologies, Inc.) or anti-rat IgG (Sigma). Cells were washed three times and analyzed using a FACScan machine (Becton Dickinson, Oxnard, CA).

Immunoprecipitation—The mouse/chicken $\beta_1$ integrin chimeras (MC3 and MC5) were expressed in NIH-3T3 cells as described previously (50). These cells were labeled with ImmunoPure NHS-LC-biotin (Pierce) and then lysed with extraction buffer (1% Nonidet P-40 in PBS containing 2 mM MgCl$_2$, 1 mM phenylmethylsulfonyl fluoride, 2 $\mu$g/ml aprotinin, and 2 $\mu$g/ml leupeptin). After immunodepletion to remove proteins binding nonspecifically to protein A-Sepharose beads, mouse anti-chicken $\beta_1$ antibodies were added and immune complexes were directly collected on protein A beads that had been preadsorbed with rabbit anti-mouse sera. Rabbit anti-$\beta_2$, immune complexes were then incubated with TBS containing 0.1% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated with Extravidin-HRP and finally detected with the Renaissance chemiluminescent kit (DuPont). For precipitation experiments, biotinylated $\beta_1$ integrin MC3 and MC5 complexes were precipitated using the appropriate anti-chicken mAb, and then eluted at 70°C for 10 min in 1% Nonidet P-40 detergent. The eluates were then precipitated with mAb 9EG7 that had been preadsorbed onto protein A beads and analyzed on SDS-polyacrylamide gel electrophoresis as above.

RESULTS
Stimulation of Cell Adhesion by the 9EG7 mAb—The observation that mAb 9EG7 detects a Mn$^{2+}$-dependent $\beta_1$ integrin conformation (28) prompted us to evaluate whether this conformation is functionally relevant for cell adhesion. For these experiments we used the erythroleukemia cell line K562, expressing the $\alpha_\text{v}^\beta_2$ fibronectin receptor but no other $\beta_1$ integrins. In the absence of any added divalent cations, K562 cells did not adhere to immobilized fibronectin, and the addition of the 9EG7 mAb was unable to induce adhesion (Fig. 1). However, in the presence of intermediate levels of Mn$^{2+}$ (0.01–0.05 mM), mAb 9EG7 stimulated strong dose-dependent adhesion (Fig. 1). The effect of 9EG7 was more evident as Mn$^{2+}$ was increased from 0.01 to 0.05 mM. However at higher Mn$^{2+}$ levels (>0.1 mM), Mn$^{2+}$ itself already had a maximal stimulatory effect, such that the effect of 9EG7 became less evident. In all cases, K562 cell adhesion to fibronectin was completely inhibited by the anti-$\alpha_\text{v}$ mAb A5-PUJ 2 (data not shown), consistent with mediation by $\alpha_\text{v}^\beta_1$. In another experiment, $\alpha_\text{v}$-transfected K562 cells showed $\alpha_\text{v}$-dependent adhesion to kalinin-containing matrix that was also stimulated by mAb 9EG7 (data not shown). These results suggest that the 9EG7 mAb may recognize and stabilize a functionally relevant integrin conformation. In this respect, 9EG7 resembles several other anti-$\beta_1$ antibodies that stimulate integrin adhesive function (4, 23, 24).

9EG7 Epitope Induction by Mn$^{2+}$, but Not by Other Integrin-Stimulating Agents—We next compared the ability of various integrin-activating stimuli to induce the epitope. Treatment with Mn$^{2+}$ yielded a dose-dependent increase in 9EG7 expression from nearly 0% (relative to total $\beta_1$), up to more than 30%, with maximal expression at 5.0 mM Mn$^{2+}$ and half-maximal at 1 mM (Fig. 2A). Representative flow cytometry profiles are shown in Fig. 3 (upper panels). Notably, upon Mn$^{2+}$ stimulation, the amount of 9EG7 bound/cell was increased, relative to total $\beta_1$ on K562 cells, with no evidence for cellular subpopulations. In a separate experiment, stimulation with Mn$^{2+}$ also caused increased cell adhesion to fibronectin, with maximal adhesion at $>$5.0 mM and half-maximal at 0.02–0.03 mM Mn$^{2+}$ (Fig. 2A). Again, adhesion was completely inhibited by mAb A5-PUJ 2 (data not shown). In contrast to Mn$^{2+}$, Mg$^{2+}$ did not induce the 9EG7 epitope on K562 cells, even though Mg$^{2+}$ did induce $\alpha_\text{v}$-$\beta_1$-dependent cell adhesion to immobilized fibronectin, with maximal adhesion at $>$4 mM and half-maximal at 1–2 mM Mg$^{2+}$ (Fig. 2B). Neither expression of the 9EG7 epitope nor adhesion to fibronectin was induced by Ca$^{2+}$ over a wide range of concentrations (0.01–25 mM; data not shown).

Similar to Mg$^{2+}$, the stimulatory antibody mAb TS2/16 (Fig. 2C) and the phorbol ester PMA (Fig. 2D) induced little or no 9EG7 expression, but did promote cell adhesion to fibronectin in a dose-dependent manner. Because integrin-mediated adhesion requires divalent cations, 1 mM MgCl$_2$ and 1 mM CaCl$_2$ were added during the adhesion assays and were also present during the analysis of 9EG7 epitope expression (Fig. 2, C and D). If MgCl$_2$ and CaCl$_2$ were omitted, TS2/16 and PMA still failed to induce 9EG7 epitope expression (data not shown).

Induction of the 9EG7 Epitope by Soluble Ligand—Next we tested the effects of soluble ligands on 9EG7 epitope expression. As seen in representative flow cytometry profiles (Fig. 3,
lower panels) and in Fig. 4B, the ligand-mimetic peptide GRGDSP, but not the control peptide GRGESP, caused increased 9EG7 expression relative to total \(\beta_1\). Also, the 9EG7 epitope was consistently induced by intact fibronectin at concentrations greater than 100 nM (Fig. 4A), and also by soluble collagen, kalinin, VCAM-1, and CS1 peptide on K562 transfec-tants expressing \(\alpha_2\beta_1\), \(\alpha_3\beta_1\), and \(\alpha_4\beta_1\), respectively (data not shown). To facilitate ligand binding, these experiments were carried out in the presence of 1 mM MgCl\(_2\) and 1 mM CaCl\(_2\). Also, it was essential to utilize serum-free conditions, to avoid complications due to serum fibronectin. We conclude that both ligand occupation and Mn\(^{2+}\) induce a similar \(\beta_1\) integrin con-formation, recognized by 9EG7.

Induction of the 9EG7 Epitope by Soluble Fibronectin Is Potentiated by Mg\(^{2+}\) and mAb TS2/16, but Not by PMA—For those agents (Mg\(^{2+}\), TS2/16, PMA) able to stimulate integrin adhesive function but not 9EG7 expression (Fig. 2), further experiments were carried out, testing their ability to synergize with a suboptimal dose of soluble fibronectin. As indicated in Table I, fibronectin (100 nM), Mg\(^{2+}\) (5 mM), or TS2/16 (3 mg/ml) added by themselves induced minimal 9EG7 expression. However, in the presence of 100 nM fibronectin, both MgCl\(_2\) (5 mM) and TS2/16 (3 mg/ml) each stimulated a marked increase in the 9EG7 epitope above the basal level. Also, the stimulatory effect of Mn\(^{2+}\), obvious even in the absence of fibronectin, was more pronounced when fibronectin was present. In sharp contrast, a relatively high dose of PMA did not induce 9EG7 expression, even in the presence of fibronectin.

Inverse Correlation between the Presence of Calcium and 9EG7 Expression—During preliminary experiments aimed at testing divalent cation effects on 9EG7 expression, we found that preincubation with EDTA itself caused an increase in the 9EG7 epitope. As shown in Fig. 5, incubation of K562 cells with EDTA yielded a dose-dependent increase in 9EG7 expression. At EDTA concentrations higher than 0.5 mM, the percentage of total \(\beta_1\) expressing the 9EG7 epitope was 25–30%, a level comparable to the maximal level induced by Mn\(^{2+}\). Preincubation with EGTA yielded essentially the same result as EDTA (Fig. 5), indicating that removal of Ca\(^{2+}\) rather than Mg\(^{2+}\) was most critical for causing 9EG7 epitope expression. Notably, the effect of EDTA was temperature-dependent (inset), since the high level of 9EG7 induction seen at 37°C was decreased at 20°C and negligible at 4°C. Also, the effect of EGTA on 9EG7 epitope expression was fully reversible. Incubation of K562 cells with 2.5 mM EGTA (for 30 min at 37°C) caused elevated 9EG7 expression (from 5.2% up to 32.6%). Continued incubation for another 30 min in 2.5 mM EGTA, or after addition of buffer that slightly diluted the EGTA (to 2.3 mM), did not markedly alter 9EG7 expression.
had only a very minor inhibitory effect on Mn$^{2+}$ whereas even more pronounced (data not shown). In contrast, Mg$^{2+}$ (at 1 mM) exerted a slight to moderate stimulatory effect above that seen with 25 mM MgCl$_2$ and 1 mM CaCl$_2$) with either soluble human fibronectin for 30 min at 37 °C (panel A) or GRGDSP and GRGESP peptides for 5 min at room temperature (panel B). The percent of 9EG7 relative to total $\beta_1$ expression was determined by flow cytometry.

**FIG. 4. Effect of fibronectin and RGD peptide on 9EG7 expression.** K562 cells were incubated (in the presence of 1 mM MgCl$_2$ and 1 mM CaCl$_2$) with either soluble human fibronectin for 30 min at 37 °C (panel A) or GRGDSP and GRGESP peptides for 5 min at room temperature (panel B). The percent of 9EG7 relative to total $\beta_1$ expression was determined by flow cytometry.

**TABLE I**

**Effect of soluble fibronectin on 9EG7 epitope expression: potentiation by various other agents**

In both experiments (A and B), K562 cells were incubated for 30 min at 37 °C with the indicated stimuli in the presence or absence of soluble fibronectin. Cells were then stained with mAb 9EG7 and mAb 13, and the percentage of 9EG7 expression relative to total $\beta_1$ was determined.

| Stimulus          | Soluble fibronectin |
|-------------------|---------------------|
|                   | None | 100 nM | 250 nM |
| Exp. A            |       |        |        |
| None              | 6    | 9      | 16     |
| MgCl$_2$ (5 mM)   | 5    | 18     | 31     |
| mAb TS2/16 (3 μg/mL) | 7  | 44     | 57     |
| Exp. B            |       |        |        |
| None              | 4    | 16     | 21     |
| PMA (50 mM)       | 7    | 14     | 18     |
| MnCl$_2$ (0.1 mM) | 5    | 28     | 34     |
| MnCl$_2$ (1.0 mM) | 18   | 42     | 48     |

In contrast, subsequent addition of Ca$^{2+}$ (to 12.5 mM, for 30 min at 37 °C) lowered 9EG7 expression back to a basal level (4.1% relative to total $\beta_1$, Fig. 6A). Remarkably, simply removing EGTA by washing cells and resuspending them in PBS for an additional 30 min also resulted in a loss of much of the 9EG7 epitope (down to 9.5%). Because this occurred even when the PBS was pretreated with Chelex 100 resin, we suspect that the calcium responsible for this effect is derived from the cell, rather than buffer contamination. This result is consistent with very tight binding of Ca$^{2+}$ to $\beta_1$ integrins (a $\beta_1$ epitope) on K562 cells, as also suggested from Fig. 5.

Notably, 9EG7 expression was also elevated to a roughly similar extent upon preincubation with either 5 mM MnCl$_2$ or 25 μM GRGDSP peptide (Fig. 6, B and C). Again, this expression was nearly completely reversed upon the addition of 12.5 mM Ca$^{2+}$. When 2.5 mM EGTA was added subsequent to treatment with either 5 mM MnCl$_2$ or 25 μM GRGDSP peptide, no further increase in the 9EG7 epitope was observed. These results suggest that Ca$^{2+}$ had already been depleted due to incubation with 5 mM MnCl$_2$ or 25 μM GRGDSP peptide.

Detailed Ca$^{2+}$ titrations confirmed that the addition of Ca$^{2+}$ (at >0.1 mM) could reverse both the stimulatory effects of 5 mM Mn$^{2+}$ (Fig. 7A) and 25 μM GRGDSP ligand (Fig. 7B). At lower doses of Mn$^{2+}$ and GRGDSP, the percent inhibition by Ca$^{2+}$ was even more pronounced (data not shown). In contrast, Mg$^{2+}$ had only a very minor inhibitory effect on Mn$^{2+}$ stimulation, and in fact, Mg$^{2+}$ exerted a slight to moderate stimulatory effect above that seen with 25 μM GRGDSP alone.

Analysis of the 9EG7 Epitope—The majority of anti-$\beta_1$ antibodies, including both function enhancers (e.g. TS2/16) and blockers (e.g. mAb 13), have been mapped to a common epitope that includes residues 207–218 (42). Thus as expected, preincubation with mAb 13 did block TS2/16 binding, and similarly it blocked the binding of another interesting antibody, mAb 15/7 (27), that had not previously been mapped. Also as expected, mAb 13 did not block binding of three other antibodies (LM534, LM442, K20), that define two distinct epitopes near the $\beta_1$ cysteine-rich region (Table II). In comparison, mAb 9EG7 did not block the common epitope seen by TS2/16, mAb 13, 15/7, SG/7, and many other anti-$\beta_1$ antibodies not listed here, and also did not block the less common epitopes defined by K20, LM534, or LM442 binding.

Because 9EG7 did not overlap strongly with any previously defined epitope, precise localization required a chimeric $\beta_1$ mapping approach. For this, previously described mouse/chicken chimeras (50) were utilized, since 9EG7 recognizes mouse $\beta_1$ (28) but fails to recognize chicken $\beta_1$ (data not shown). As shown in a reprecipitation experiment (Fig. 8), the mouse/chicken MC3 chimera was isolated from NIH-3T3 cells by the CSAT mAb (lane 1), and then reprecipitated using the 9EG7 mAb (lane 6), but not by a negative control antibody (lane 5). In contrast, 9EG7 did not recognize the MC5 chimera (lane 5).

**FIG. 5. Effect of EDTA and EGTA on 9EG7 epitope expression.** K562 cells were washed in PBS and then resuspended in TBS (+5% BSA and 0.02% NaN$_3$) containing either EDTA or EGTA. After 30 min at 37 °C cells were analyzed by flow cytometry to determine the percent of 9EG7 relative to total $\beta_1$ expression. In parallel experiments cells were incubated with 2.5 mM EDTA for 30 min at the indicated temperatures (inset).
FIG. 6. 9EG7 epitope expression induced by Mn2+ and soluble ligand is reversed by Ca2+. K562 cells (in a 100-μl volume) were preincubated for 30 min at 37°C with either 2.5 mM EGTA (A), 5 mM Mn2+ (B), or 25 μM GRGDSP peptide (C). Then, additional 10-μl volumes of control buffer, CaCl2 (to 12.5 mM), or EGTA (to 2.5 mM) were added, and the cells were incubated for another 30 min before being assayed for 9EG7 expression by flow cytometry. In one experiment (part A), cells were washed and resuspended for 30 min in PBS that had been treated with Bio-Rad Chelex 100 resin (buffer + wash).

DISCUSSION

Here we have characterized a novel β1 integrin epitope that is highly relevant to ligand binding and adhesive functions. The 9EG7 epitope was induced by all β1 integrin ligands tested (GRGDSP peptide, fibronectin, soluble collagen, kalinin, VCAM-1, and CS1 peptide) and the antibody itself could stimulate the adhesive function of all β1 integrins, most likely by stabilizing a conformation favorable to ligand binding. In contrast to the previous report (28), we saw no evidence for blocking of β1 integrin-mediated adhesion.

Calcium and Ligand Effects—Our results suggest that constitutive expression of the 9EG7 epitope on β1 integrins is prevented by very tightly bound calcium. First, neither EDTA or EGTA was effective in promoting 9EG7 expression unless K562 cell temperature was elevated to 37°C. Second, elevated 9EG7 epitope expression was readily lost upon removal of EDTA or EGTA, presumably due to trace amounts of calcium derived from the cell itself. Assuming that calcium released from the cell could reach as high as 1–10 μM, the presence of 300 μM EGTA (enough to induce half-maximal 9EG7 expression) would chelate all but 1–10 nm free calcium (51). Thus, the apparent Ki for calcium inhibition of 9EG7 binding (in the absence of Mn2+ or ligand) could be as low as 1–10 nM.

Notably, binding of ligand to β1 integrin also triggered the appearance of the 9EG7 epitope. The addition of excess Ca2+ could reverse this effect, but under these conditions, levels in the nM range were required. We assume that ligand binding is inhibited by this excess Ca2+ because (i) this is far in excess of the amount needed to prevent 9EG7 binding in the absence of ligand, and (ii) binding of 9EG7 itself is not appreciably increased in affinity (data not shown). In this regard, there is recent biophysical evidence for a mechanism whereby bound ligand can displace β1 integrin cations (52) from a site (aa 118–131) that is very well conserved in β1 (aa 129–142), and also required for ligand binding to β1 integrins (53).

The inhibitory effect of Ca2+ on several β1 integrin functions (32–36, 54) and on some β1 integrin functions (55) has been well established. Now we have the fundamental new insight that Ca2+ but not Mg2+ may act largely by obstructing the appearance of a conformation (defined by 9EG7) that is favorable for ligand binding. In this regard, it is likely that the enhanced cell migration associated with a lower Ca2+/Mg2+ ratio in wound fluid (56) probably involves a more favorable β1 ligand-binding conformation such as described here.

Mn2+ Stimulation Effects—The induction of the 9EG7 epitope by Mn2+ confirms results noted earlier (28). Just as for soluble ligand, the effects of Mn2+ were almost completely reversed by the addition of Ca2+. Also, the addition of EGTA following induction by Mn2+ or soluble ligand caused no further 9EG7 induction. Experiments analyzing Ca2+ inhibition effects on Mn2+-induced 9EG7 expression yielded results consistent with non-competitive inhibition (data not shown). Thus, the interaction between Mn and Ca may be complex, with separate sites involved, as suggested for a β3 integrin (55). In this regard, Mn2+ but not Ca2+ supported α5β1 integrin clustering in a ligand-independent fashion.2 3

It has been well established that Mn2+ is a strong stimulator of β1 integrin function (34, 35, 37–39). Now we gain a new insight into this activity since Mn2+ not only supports ligand binding, but also, by itself, stabilizes an epitope favorable to ligand binding, and causes a diminished inhibitory effect of Ca2+. In contrast, Mg2+ is able to support ligand binding, but does not appear to have these other activities. It has been suggested elsewhere (55) that Mn2+ could reach 1–12 μM in many tissues, and up to 50 μM upon bone resorption. These levels approach the point at which Mn2+ begins to stimulate α5β1-dependent cell adhesion and 9EG7 expression (i.e., see Fig. 2A).

Is 9EG7 a Detector of Activated Integrins?—While the 9EG7 antibody is a reliable detector of ligand binding, and itself can stimulate cell adhesion, the 9EG7 epitope does not closely correlate with integrin activation. To avoid confusion regarding the term activation, we have defined it here as the increased potential of an integrin to bind ligand or mediate adhesion. By this definition, 9EG7 does not define an activation epitope as previously suggested (28). First, agents such as EDTA and EGTA inhibit ligand binding, but nonetheless stimulated increased 9EG7 expression. Second, agents such as Mg2+ and TS2/16 did not directly induce 9EG7 expression themselves (in the absence of ligand), but could facilitate 9EG7 expression indirectly, by enhancing ligand binding. This divergence between the effects of Mn2+ and soluble ligand, compared to

2 C. Pujades, J. Teixido, and M. E. Hemler, submitted for publication.
3 C. Pujades, S. K. Craef, R. Alon, A. Masumoto, L. Burkly, T. A. Springer, L. B. Chen, R. R. Lobb, M. Hemler, submitted for publication.
Fig. 7. Effects of calcium and magnesium on manganese-induced and ligand-induced 9EG7 expression. K562 cells were washed twice with PBS then stimulated for 30 min at 37 °C with either 5 mM MnCl₂ (A) or 25 μM GRGDSP peptide (B) and then incubated for 25 min at 37 °C with CaCl₂ or MgCl₂ at the indicated concentrations. In the absence of added CaCl₂ or MgCl₂, expression of the 9EG7 epitope was determined by flow cytometry to be 39.7 and 37.6 mean fluorescence intensity units (in A and B, respectively).

Table II

| Rat mAb preincubated with K562 cells* | Mouse mAbs: | Control | mAb 13 | mAb 9EG7 |
|--------------------------------------|-------------|---------|--------|---------|
| Mouse mAbs:                          |             |         |        |         |
| TS2/16                               | 100         | 1       | 89     |
| 1S7                                  | 100         | 5       | 98     |
| K20                                  | 100         | 84      | 70     |
| LM534                                | 100         | 86      | 83     |
| LM442                                | 100         | 88      | 83     |

*K562 cells were pretreated for 10 min at 20 °C in the presence of 5 mM Mn²⁺ with either control buffer, or mAb 9EG7 or mAb 13 (both rat antibodies) at 10 μg/ml. Cells were then incubated with saturating concentrations of the indicated mouse anti-β₁ mAbs for 45 min on ice, and subsequently stained with FITC-conjugated anti-mouse second antibody that did not cross-react with the rat mAb 13 and 9EG7 primary antibodies.

Fig. 8. Epitope mapping for mAb 9EG7. A, β₁ integrin chimeras were precipitated from NIH-3T3 cells using anti-chicken β₁ antibodies CSAT (lane 1) and W1B10 (lane 3), respectively. As a control, mouse β₁ was precipitated from the same lysates (lanes 2 and 4). B, precipitation of material precipitated by CSAT (lanes 5–7) or by W1B10 (lanes 8 and 9) was carried out using either negative control mAb P3 (lane 5), mAb 9EG7 (lanes 6 and 8) or an antiserum to the cytoplasmic domain of β₁ (lanes 7 and 9). Note that the diminished intensity of the β₁ bands seen by the anti-β₁ tail serum is likely due to competition by a pool of immature β subunit that is not surface labeled. C, a schematic diagram of the MC3 and MC5 β₁ chimeras is shown.

Mg²⁺ and TS2/16, had not previously been recognized.

Rather than defining an activation epitope, the 9EG7 epitope can be better characterized as a “ligand-induced binding site” or “LIBS,” such as have been described for β₁ integrins (11, 57).

However, even the term LIBS is only partially correct, since in the absence of ligand, both the addition of Mn²⁺ and removal of Ca²⁺ also induce 9EG7 expression (see below).

Although the phorbol ester PMA stimulated adhesive activity, it did not directly stimulate 9EG7 expression, nor did it synergize with soluble fibronectin to give increased 9EG7 expression. We are not sure why PMA was previously found to cause increased 9EG7 expression (28), except that possibly peripheral blood T cells could differ from K562 cells in this regard. Nonetheless, our results are consistent with previous findings that PMA could stimulate adhesion without increasing β₁ integrin ligand binding affinity (59, 60). Rather than altering affinity, PMA could alter receptor clustering as previously reported (61), thus leading to enhanced avidity for ligand. Consistent with this, reduced clustering of α₂β₁ integrin did not change 9EG7 expression, but caused markedly diminished cell adhesion (3). Likewise, α chain cytoplasmic tail deletion did not change Mn²⁺-inducible 9EG7 expression, despite causing diminished adhesive function (62).

All of these results reinforce the idea that there are at least three ways to increase the functional potential of β₁ integrins. First, agents such as Mn²⁺ increase ligand binding potential as well as 9EG7 expression; second, agents such as Mg²⁺ and TS2/16 increase ligand binding potential without directly inducing 9EG7 expression; and third, agents such as PMA can stimulate cell adhesion without either stimulating ligand binding or inducing 9EG7 epitope expression.

In preliminary data, we have found that increasing doses of Mn²⁺ or GRGDSP peptide uniformly increased the number of 9EG7 binding sites (relative to total β₁), without appreciably altering the apparent Kᵦ (10 nm) for 9EG7 binding to β₁ (data not shown). This result is consistent with each individual receptor existing in either a ‘‘+’’ or ‘‘−’’ state with regard to the 9EG7 epitope. Notably, we have never observed 9EG7 expression to reach more than 40–50% relative to the total β₁ expression, when measured either by flow cytometry or by immunoprecipitation from solution (data not shown). In this regard, several other antibodies that report ligand-induced or activated integrin conformations also fail to bind to more than 50% of the integrins (9, 17, 55). As yet, no adequate explanation for this widely observed phenomenon has been offered. Possibly, it may be an intrinsic property of integrins to exist in multiple conformations, with it being very difficult to shift equilibrium completely toward the conformation resembling the ligand-bound state.

Location of the 9EG7 Epitope—Most anti-human β₁ inhibitory and stimulatory antibodies (42), the Mn²⁺-inducible anti-β₁ antibody 15/7 (27), the Mn²⁺ and Ca²⁺ -inducible mAb 5G/7 (29), and the ligand-inducible 8A2 (31) and 12G10 (30) antibodies all map to a narrow region including β₁ residues 207–218 (30, 42, 63). The 9EG7 mAb, mapping to the 495–602
region, thus becomes perhaps the first known cation-regulatable and/or ligand-inducible epitope to map elsewhere in $\beta_1$. Furthermore, although 9E7 mapped to an area in the cysteine-rich domain partly overlapping the region containing the K20 and LM442/LM534 sites, 9E7 did not cross-block those antibodies, further indicating that it recognizes a novel human $\beta_1$ epitope. Comparison of mouse (64), human (65), and chicken (66) $\beta_1$ sequences within the 495–602 region reveals only 6 positions (all between 577–599) where chicken $\beta_1$, which is not recognized by 9E7, shows non-conservative differences from both the mouse and human sequences, which are recognized by 9E7. However, 9E7 binding to that subregion remains to be formally proven by further mutagenesis experiments. Also, it remains to be determined whether the anti-chicken antibodies TASC and G, mapping to the 495–602 region (50), recognize the chicken equivalent of the 9E7 epitope. Binding of neither the TASC nor G antibodies has yet been reported to be regulated by divalent cations or ligand, although the TASC mAb does induce $\beta_1$ adhesive function (4).

Notably, several anti-$\beta_1$ LIBS antibodies have been mapped to $\beta_1$ regions 602–690 (19, 67), or 422–490 (19), both of which are mostly non-overlapping with the 495–602 region in $\beta_1$. Thus, between the $\beta_1$ and $\beta_2$ subunits, there now have been described a total of five different LIBS sites. Notably, these five sites on the $\beta_1$ or $\beta_2$ subunits are also sensitive to divalent cations as indicated for $\beta_1$ aa 1–6 (19), $\beta_2$ aa 422–490 (12, 19), $\beta_2$ aa 602–690 (67, 68), $\beta_2$ aa 207–218 (29, 42, 63), and $\beta_1$ aa 495–602 (described here). Underlining the fact that these cation- and ligand-regulated sites are apparently inseparable, we refer to them here as cation- and ligand-induced binding sites, or CLIBS. These sites, labeled as $\beta_1$ aa Cl1–$\beta_3$ Cl2 and $\beta_2$ Cl1 and $\beta_1$ Cl2, are shown schematically in Fig. 9.

Together these results emphasize that conformational changes due to ligand and divalent cation binding events are not limited to a small area within the primary structure. Rather, our results help to demonstrate that profound conformational changes occur throughout the integrin molecule upon ligand binding and/or cation manipulation. This point is additionally reinforced by several anti-integrin $\alpha$ chain epitopes that are also induced by ligand, and regulated by divalent cations (eg. Refs. 11, 18, 69, 70).

In conclusion, the 9E7 antibody defines a novel epitope near the cysteine-rich region of the $\beta_1$ integrin, now known for the first time to be conformationally involved in both ligand and cation regulation. As such, 9E7 has been exceptionally useful. For example, it has allowed us to demonstrate that a fundamental role for Ca$^{2+}$ may be to counterbalance those agents (including ligand) and Mn$^{2+}$ that stimulate 9E7 expression. In addition, it has allowed us to elucidate three categories of function-activating stimuli. Finally, the appearance of the 9E7 epitope not only provides a useful tool for studying the regulation of $\beta_1$ integrin function, but also it implies that ligand binding may expose this epitope, which could potentially play a role in interactions with other integrins or other proteins, and ultimately in outside-in signaling.

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