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β1 Integrin Cytoplasmic Domain Regulates the Constitutive Conformation Detected by MAb 15/7, But Not the Ligand-Induced Conformation

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Abstract The anti-integrin β1 MAb 15/7 sometimes may be a reporter of integrin activation or ligand occupancy. However, certain β1 tail deletions eliminate ligand binding despite inducing maximal constitutive 15/7 expression [Puzon-Mclaughlin et al. (1996): J Biol Chem 271:16580–16585]. Here we describe β1 tail mutations (e.g., double point mutations [D759L/F763L, F766L/E769L], or replacement of the β1 tail by the β5 tail) that prevent rather than induce constitutive appearance of the 15/7 epitope. Despite variable losses of *constitutive* 15/7 epitope, these mutants all retained a similar *inducible* 15/7 epitope component as seen upon incubation with GRGDSP peptide ligand. In addition, *constitutive* 15/7 expression did not correlate with integrin localization into focal adhesions. In conclusion, we show for the first time for a fully functional integrin that specific mutations within the β1 tail can down-regulate the constitutive appearance of an extracellular conformation defined by MAb 15/7. Because this regulation occurs away from the ligand binding site and does not correlate with responsiveness to integrin ligand, cell adhesion, or localization into focal adhesions, a novel type of conformational regulation is suggested. J. Cell. Biochem. 71:63–73, 1998.

Key words: integrin; activation epitopes; ligand binding; focal adhesions; cytoplasmic domains

Cell surface receptors in the integrin family mediate cell adhesion that regulates growth, differentiation, and apoptosis. Upon binding to ligands, integrins form complexes with an array of actin-associated cytoskeletal proteins, and signaling molecules [Hynes, 1992; Schwartz et al., 1995; Clark and Brugge, 1995; Miyamoto et al., 1995b; Burridge and Chrzanowska-Wodnicka, 1996]. Complexes forming at sites of tight cell adhesion to the underlying extracellular matrix are called focal adhesions (FA) [Burridge and Chrzanowska-Wodnicka, 1996]. Not only do integrins initiate signaling events subsequent to ligand binding (outside-in signaling), but also the initial ligand binding and adhesive functions of integrins can themselves be regulated by cytoplasmic/cytoskeletal interactions (inside-out signaling) [Schwartz et al., 1995; Faull and Ginsberg, 1995]. Importantly, integrin cytoplasmic domains play key regulatory roles, both for "inside-out" regulation of ligand binding and adhesion functions, and for "outside-in" in regulation of focal adhesion formation and other post-ligand binding functions [Hemler et al., 1994; LaFlamme et al., 1992; Sastry and Horwitz, 1993; Agrez et al., 1994; O'Toole et al., 1995; Crowe et al., 1994; Hughes et al., 1995; Van Nhieu et al., 1996]. Within the integrin β1 tail, cyto-1, cyto-2, and cyto-3 regions are particularly important for focal adhesion formation [Reszka et al., 1992] (Fig. 1).

It is widely assumed that activation of β1 integrins is accompanied by a significant conformational change, that can be measured using MAb recognizing β1 integrin activation epitopes. However, we are not yet aware of an anti-β1 MAb that is truly "activation-specific" [Bazzoni and Hemler, 1998]. Appearance of an epitope defined by anti-β1 MAb 15/7 correlates with increased β1 integrin affinity for ligand, after manganese stimulation [Yednock et al., 1995]; and also with increased cell adhesion resulting from cell differentiation [Bohnsack et al., 1995] or phorbol ester stimulation [Yednock et al., 1995]. Thus, MAb 15/7 binding has been frequently suggested to indicate integrin activation [Koyama et al., 1996; Nieto et al., 1996;

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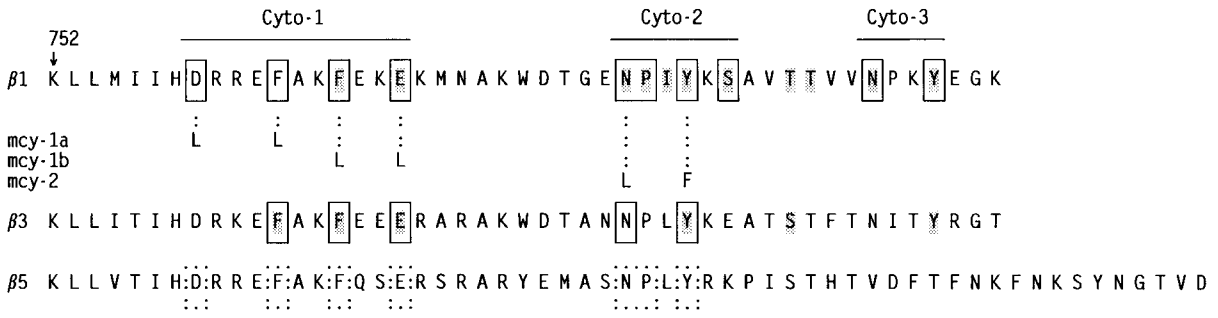

Fig. 1. Wild-type and mutant β chain cytoplasmic domains. Boxed β1 [Reszka et al., 1992] and β3 [Ylänne et al., 1995] residues important for localization into focal adhesions were defined previously. Shaded residues correspond to those needed for ligand binding, as determined using the PAC-1 antibody [O'Toole et al., 1995]. Double point mutations in β1, as indicated: mcyto-1a, D759L/F763L; mcyto-1b, F766L/E769L; and mcyto-2, N780L/Y783F. Note that most of the key residues in the cyto-1 and cyto-2 regions are conserved in the β5 tail.

Neelamegham et al., 1996; Seki et al., 1996; Werfel et al., 1996; Yednock et al., 1995; Arroyo et al., 1995; Bohnsack et al., 1995; Kovach et al., 1995; Picker et al., 1993]. However, much of the expression of the 15/7 epitope and of other β1 "activation" epitopes is perhaps more likely due to ligand occupancy [Yednock et al., 1995; Gomez et al., 1997; Bazzoni et al., 1995; Bazzoni and Hemler, 1998] than to integrin activation. Furthermore, for some αβ1 combinations (e.g., α3β1), the 15/7 epitope is not induced when the integrin is activated [Bazzoni et al., 1998]. Conversely, the 15/7 epitope can be strongly induced when β1 integrins are *inactivated* by either denaturation [Yednock et al., 1995], or by β1 tail deletion [Puzon-Mclaughlin et al., 1996].

In contrast to a previous report [Puzon-Mclaughlin et al., 1996], in the present paper we have analyzed β1 integrin cytoplasmic domain regulation of constitutive MAb 15/7 expression under conditions in which integrins are not inactivated. We chose to study β1 cytoplasmic tail point mutations, as these should not be unduly disruptive. We also studied an integrin β1/5 chimera, previously shown to be fully functional in cell adhesion assays [Pasqualini and Hemler, 1994]. Indeed, ligand-inducible 15/7 levels were maintained for all mutants, consistent with retention of activity, while constitutive 15/7 expression was lost partially or nearly completely for some of the β1 tail mutants. Constitutive 15/7 expression did not correlate with focal adhesion formation, cell adhesion, or ligand-inducible 15/7 (an indicator of ligand binding). Thus, alterations in constitutively displayed 15/7 epitope may be indicative of a novel type of conformational regulation by the β1 tail.

METHODS

Antibodies

Monoclonal antibodies used were: mouse anti-hamster β1 7E2 [Brown and Juliano, 1988]; mouse anti-human β1, A-1A5 [Hemler et al., 1983]; mouse anti-human β1, 15/7 [Picker et al., 1993]; and negative control antibody, P3 [Lemke et al., 1978]. Anti-phosphotyrosine rabbit antiserum was from Transduction Laboratories (Lexington, KY).

Generation of β1 Mutants

Double point mutations were generated by overlapping polymerase chain reaction (PCR) [Higuchi et al., 1988]. Paired inner oligonucleotide primers were CTCAGAAGGGAGTTAGCTAAATTTGAAAAG (sense) and GCTAACTCCCTTCTGAGATGAATTATC (antisense) for the D759L/F763L mutation, TTAGAAAAGCTGAAAATGAATGCCAAATGG (sense) and CATTTTCAGCTTTTCTAATTTAGCAAACTC (antisense) for the F766L/E769L mutation, and GAACTTCCTATTTTTAAGAGTGCCGTAAC (sense) and CTTAAAAATAGGAAGTTCACCCGTGTCC (antisense) for the N780L/Y783F mutation. The outer oligonucleotide primers for all mutants were CATTCCAATTGTAGCTGGTGTGGTTGC (5′ end primer) and GTCAGTCCCTGGCATGAATTACAAC (3′ end primer). Amplified fragments (containing the β1 integrin *Hin*dIII site just downstream of the trans-

membrane domain) were ligated into the TA cloning vector (Invitrogen, San Diego, CA) and excised with *Hin*dIII and *Xba*I (the latter present in the TA cloning vector polylinker). Wild-type β1 integrin insert was cloned into the pECE vector as described [Giancotti and Ruoslahti, 1990] and digested with *Eco*RI/*Hin*dIII or *Eco*RI/*Xba*I. To replace the wild-type cytoplasmic domain with the mutated region, the 2.3-kb fragment from the pECE *Eco*RI/*Hin*dIII digest (containing wild-type β1 integrin extracellular and transmembrane domains) and the 2.9-kb fragment from the pECE *Eco*RI/*Xba*I digest (containing vector alone) were joined to the mutated cytoplasmic tail (from the TA cloning vector *Hin*dIII/*Xba*I digest) in a three-way ligation. The β1/5 integrin chimera (consisting of the β1 extracellular and transmembrane domains and the β5 cytoplasmic tail) was produced as previously described [Pasqualini and Hemler, 1994]. Sequences of PCR products and ligated regions of all constructs were verified by DNA sequencing.

For transfection, Chinese hamster ovary (CHO) cells negative for the dihydrofolate reductase gene ($dhfr^-$) were harvested in 2 mM EDTA/PBS, washed, and then suspended in serum-free MEM-α+ medium. The cells were combined with 200 μg salmon sperm DNA (carrier DNA) and two plasmids: 1 μg p901 ($dhfr^+$) vector [Kaufman and Sharp, 1982], and 30 μg of either wild-type β1-pECE, mutant β1-pECE, or β1/5-pECE. Mock-transfected CHO cells were combined with 1 μg p901 vector alone. Electroporation was carried out (at 960 μF and 280 V) using a gene pulser (Bio-Rad Laboratories, Cambridge, MA). Cells were then diluted in MEM-α+ medium, centrifuged, and resuspended in MEM-α+ complete medium, with 10% fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml). Three days after electroporation, the cells were switched to MEM-α^- complete medium. After 2–3 weeks of selection, the transfectants were stained with the human β1-specific MAb A-1A5 and then positive cells were isolated using a flow cytometer (Epics; Coulter Corp., Hialeah, FL). For each transfectant, at least 1–4 × 10^5 cells were selected each time from a series of sorts. Furthermore, independent sorts yielded cells with similar properties, thus assuring that results were not due to atypical clonal variation.

Immunofluorescence

Coverslips were coated overnight with fibronectin at 40 μg/ml in 0.1 M $NaHCO_3$. Then cells (50,000 per 12 × 12 mm^2 surface area; in MEM-α^- medium with 1% FCS) were allowed to adhere and spread for 2 h at 37°C in 10% CO_2. Then, coverslips were fixed in freshly prepared 1.5% paraformaldehyde/PBS (pH 7.4) for 15 min at room temperature (RT), permeabilized with 0.5% TX-100 in PBS for 2 min, blocked in 20% goat serum/PBS (blocking solution) for 1 h, and and then stained with various antibodies (each diluted to 1–2 μg/ml in blocking solution). Antibodies were visualized using rhodamine-labeled goat anti-mouse or anti-rabbit antibody (1:200 dilution; Biosource International, Camarillo, CA) and then coverslips were dipped in water, air dried for 5–10 min and mounted in FluorSave (Calbiochem, La Jolla, CA).

Flow Cytometry

Cells were harvested in 2 mM EDTA/PBS, washed once with Tris-buffered saline (TBS), consisting of 24 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and resuspended at 4°C in TBS with 5% bovine serum albumin (BSA) (Fraction V; Sigma, St. Louis, MO) and 0.02% sodium azide. Where indicated, aliquots of 3 × 10^5 cells in 96-well microtiter plates were preincubated with GRGDSP or GRGESP peptide (4–500 μM final dilution; Life Technologies, Rockville, MD), and/or freshly prepared $MnCl_2$ (2 mM final dilution) for 15 min at 37°C. Cells were then mixed 1:1 with primary antibodies (at 1–2 μg/ml) previously diluted in TBS with 5% BSA and 0.02% sodium azide, and incubated for 45 min at 4°C. After washing two times with ice-cold TBS containing 1% BSA and 0.02% sodium azide (wash buffer), cells were incubated with FITC-labeled goat anti-mouse antibody (Life Technologies) for 45 min at 4°C. The cells were washed two more times at 4°C, and fluorescence was analyzed using a FACScan (Becton Dickinson, Oxnard, CA).

Total 15/7 expression in human β1-transfected CHO cells included a small contribution due to cross-reactivity of MAb 15/7 with hamster β1. Thus, 15/7 expression specific for human β1 (15/7% of Hu-β1) was calculated using the following formula:

$$15/7\ \text{total} = [15/7\%\ \text{of Hu-}\beta1][\text{Hu-}\beta1] + [15/7\%\ \text{of ha-}\beta1][\text{ha-}\beta1]$$

Mean fluorescence intensity (MFI) values were obtained for 15/7 total (using MAb 15/7), Hu-β1 (using MAb A-1A5), and ha-β1 (using MAb 7E2). To obtain the percentage of hamster β1 expressing 15/7 (15/7% of ha-β1), we determined 15/7 levels as a percentage of total ha-β1 (measured using MAb 7E2) in untransfected CHO cells.

RESULTS

Generation and Expression of β1 Integrin Cytoplasmic Tail Mutants

Using overlapping PCR mutagenesis, we introduced two different double missense mutations (D759L/F763L, F766L/E769L) into the cyto-1 domain, and one double mutation (N780L/Y783F) into the cyto-2 domain of the human β1 integrin. These mutants are called mcyto-1a, mcyto-1b, and mcyto-2, respectively (Fig. 1). We also studied a chimeric molecule (β1/5) containing extracellular and transmembrane domains of human β1 and the cytoplasmic tail of human β5 (KLLVTIHD...) that was previously generated [Pasqualini and Hemler, 1994].

The three double point mutants, β1/5 and wild-type human β1 were stably transfected into CHO cells, and cells expressing transfected molecules at the same level were isolated by fluorescent-activated cell sorting (FACS). As shown in Figure 2, all human β1 (Hu-β1), including mcyto-1a, mcyto-1b, mcyto-2, β1/5 and wild-type β1 (solid lines), were expressed at levels similar to each other and comparable to or slightly higher than the endogenous hamster β1 (ha-β1) integrin (dotted lines). Anti-human β1 (A-1A5) and anti-hamster β1 (7E2) antibodies showed no cross-species reactivity. Previously it was shown that β5 is the predominant β subunit associated with hamster β1, human β1, and mutant human β1 (β1/5) molecules in CHO cells [Pasqualini and Hemler, 1994].

Localization of β1 Integrin to Focal Adhesions

To confirm that focal adhesion formation was diminished for our mutant β1 integrins, focal adhesions (FA) were assessed by immunofluorescent staining of transfectants plated on fibronectin for 2 h at 37°C. As shown by staining with anti-human β1 antibody (Fig. 3) wild-type human β1 integrin was found in FA-like structures in nearly every transfected cell (Fig. 3a). However β1 cyto-region mutants showed diminished focal adhesion localization (Fig. 3b–d). More than 50% of mcyto-1a-transfected cells did not show mcyto-1a in focal adhesions, while

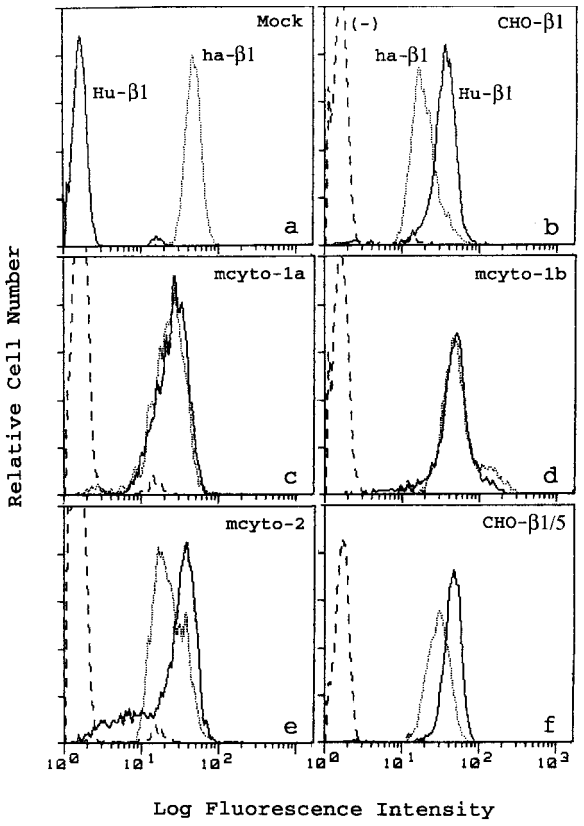

Fig. 2. Flow cytometry of β1-CHO transfectants. Cells were stained for human β1 (Hu-β1; MAb A-1A5; heavy lines) and hamster β1 (ha-β1; MAb 7E2; close dotted lines), while background staining was obtained using MAb P3 (dashed lines).

the mcyto-1b and mcyto-2 β1 were each distributed diffusely on the cell surface and showed no detectable focal adhesions. Likewise, the β1/5 chimera (included as a negative control) did not mobilize into focal adhesions (Fig. 3e), as previously reported [Pasqualini and Hemler, 1994]. In positive control experiments, endogenous hamster β1 integrin was concentrated in focal adhesion structures in nearly every cell visualized for every transfectant (Fig. 3f–j). Likewise, an anti-phosphotyrosine antibody showed abundant staining of focal adhesions in each of the transfectants (Fig. 3k–o).

Constitutive Binding of the 15/7 Antibody

The anti-human β1 antibody, MAb 15/7, detects human β1 conformational changes that may accompany integrin activation, inactivation, ligand occupancy, or denaturation (see Introduction). Here we employed MAb 15/7 to assess possible changes in the extracellular conformation of β1 as a consequence of nondisruptive cytoplasmic tail mutations. Flow cytometry profiles showed that 15/7 bound wild-type

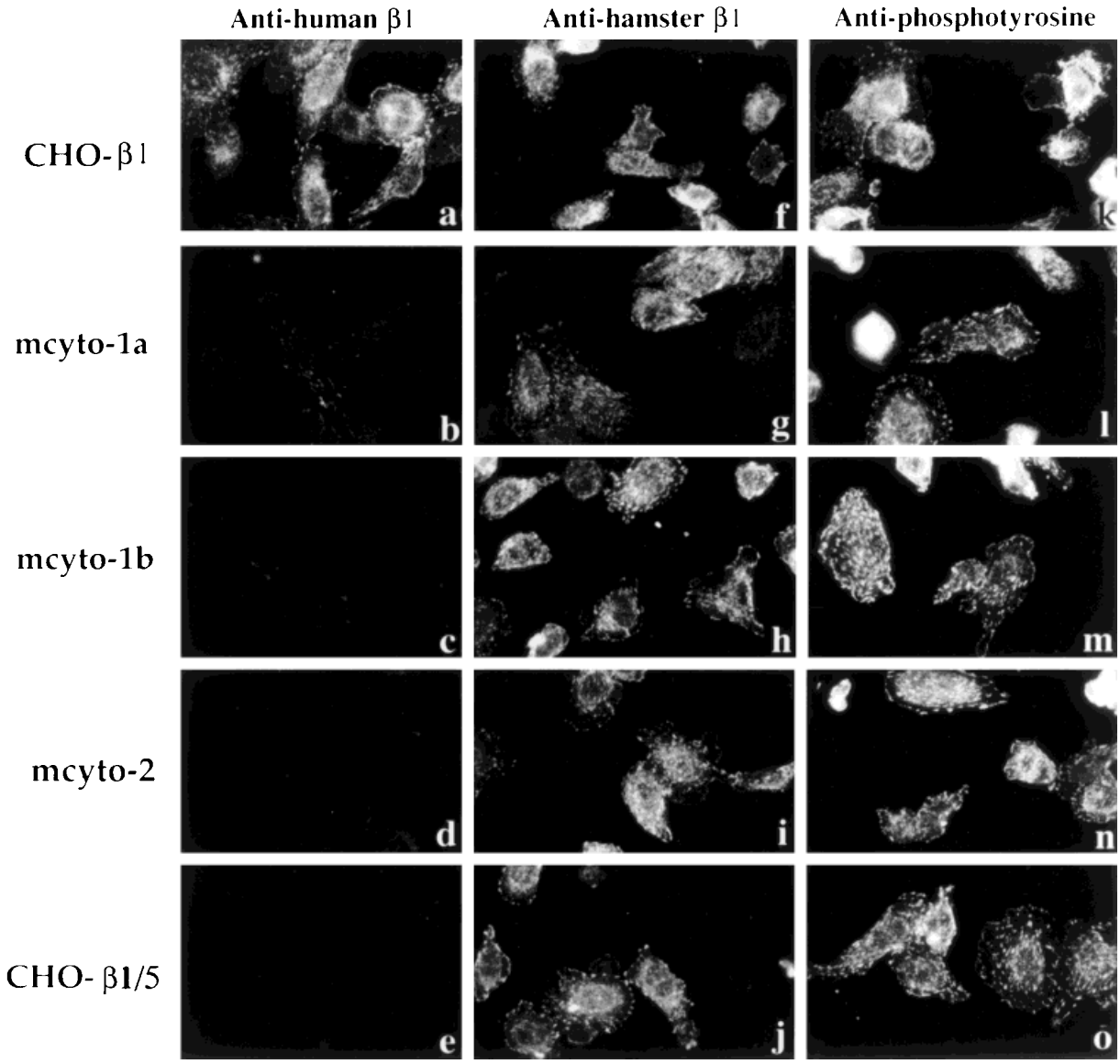

Fig. 3. Localization of β1 into focal adhesions. CHO cells were plated on fibronectin and then stained and analyzed as described under Experimental Procedures. Staining was with monoclonal antibodies to human β1 (A-1A5), hamster β1 (7E2) or with rabbit anti-phosphotyrosine antiserum.

human β1 and the mcyto-2 β1 at reasonably high levels (Fig. 4b,e). Because the cells had been treated with EDTA, and washed multiple times to remove traces of bound ligand, this 15/7 binding was constitutive, rather than ligand-induced. In contrast to wild-type and mcyto-2 β1, the mcyto-1a β1 showed biphasic 15/7 binding (Fig. 4c), whereas the mcyto-1b mutant and β1/5 chimera showed very low 15/7 levels (Fig. 4d,f). Notably, the 15/7 binding profiles in the latter two cases were largely superimposable with the background staining pattern of hamster β1, as seen in untransfected cells (marked M). Negative control staining by MAb P3 is shown for wild-type β1 cells (Fig. 4a) and was essentially identical for all transfectants.

As described under Experimental Procedures, we then calculated constitutive 15/7 staining of human β1, relative to total human β1. As indicated in Figure 5, MAb 15/7 bound comparably to wild-type β1 (16%) and to the mcyto-2 mutant (14%). However, 15/7 binding was substantially reduced for mcyto-1a (7%), mcyto-1b (1.3%), and β1/5 (2.6%). These results were highly reproducible. Pairwise Student's

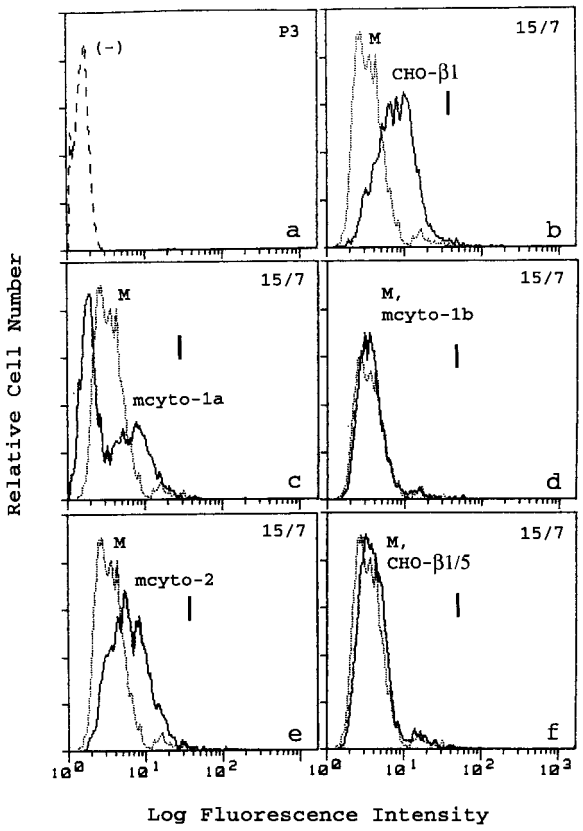

Fig. 4. Variable constitutive binding of MAb 15/7. After washing in 2 mM EDTA to remove all traces of ligand, cells were stained as in Fig. 2 and analyzed by flow cytometry. **a:** Wild-type β1 cells stained with negative control MAb P3. **b–f:** MAb 15/7 staining of five different Chinese hamster ovary (CHO) transfectants (solid lines), each compared with staining of mock-transfected CHO cells (M). Vertical dashes, peak staining positions for total human β1 in the transfectants. For unknown reasons, mcyto-1a showed consistent heterogeneity in 15/7 staining, despite homogeneity in staining for total human β1 (e.g., see Fig. 2c).

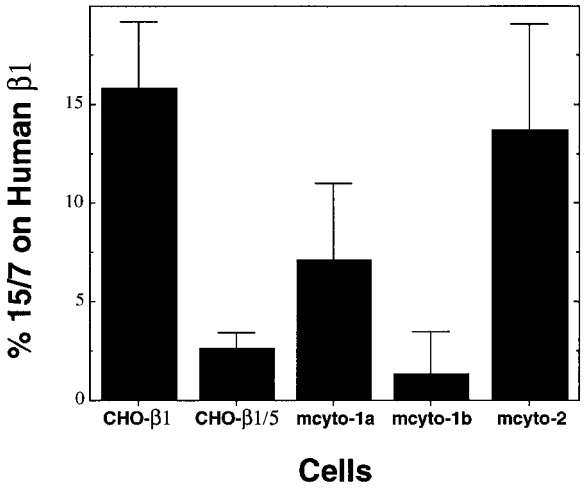

Fig. 5. Constitutive 15/7 binding to human wild-type and mutant β1. The percentage 15/7 on human β1 was calculated as described under Experimental Procedures. Background 15/7 binding to hamster β1 never exceeded more than 10–15% of the total 15/7 constitutively present in wild-type Chinese hamster ovary (CHO)-β1 cells (i.e., 15/7 on wild-type β1 was at least 7- to 10-fold greater than background 15/7). All analyses of constitutive 15/7 epitope expression were carried out in Tris-buffered saline (TBS) without any added divalent cations.

t-tests showed that these values (means of 5–9 experiments) distributed into three groups of 15/7 binding that were distinct from each other ($P < 0.05$): high (wild-type β1, mcyto-2), low (mcyto-1b, β1/5 chimera), and intermediate due to biphasic binding (mcyto-1a).

Ligand-Induced Binding of 15/7

Because of the background hamster β1, we were unable to obtain an accurate direct measurement of ligand binding for the various β1 mutants. However, induction of the 15/7 epitope, or the related HUTS-21 epitope, itself is an established indirect indicator of ligand binding [Seki et al., 1996; Yednock et al., 1995; Gomez et al., 1997]. Thus to assess ligand binding indirectly, we analyzed induction of the 15/7 epitope in response to addition of soluble ligand for α5β1. Incubation with ≤1 mM RGD peptide (GRGDSP; in TBS with no added divalent cations) did not induce 15/7 epitope expression above that already seen in Figs. 4 and 5 (not shown). Likewise, addition of 1–50 mM $MnCl_2$ by itself did not alter 15/7 levels (not shown). However in the presence of 1 mM $MnCl_2$, the addition of RGD peptide did induce 15/7 binding. In two representative transfectants (CHO-β1, mcyto-1b; Fig. 6Ab,c) the addition of 500 μM RGD peptide triggered a highly reproducible elevation in 15/7 levels (of 6–7 MFI units) that was substantially greater than the small background increase in 15/7 (1.5 MFI units) seen on hamster β1 (Fig. 6Aa).

From data such as shown in Figure 6A, the percentage 15/7 induced on human β1 relative to total human β1 was calculated, and RGD peptide titration curves were constructed (Fig. 6B). On β1 and β1/5, 15/7 binding was similarly induced by RGD peptide (by 14%), even though pre-existing constitutive 15/7 expression (20 vs 2.0%, indicated in parentheses) was markedly different (Fig. 6Ba). Also, 15/7 was induced to a similar extent (13–14%) on mcyto-2 and mcyto-1b (Fig. 6Bb), even though, again, they differed

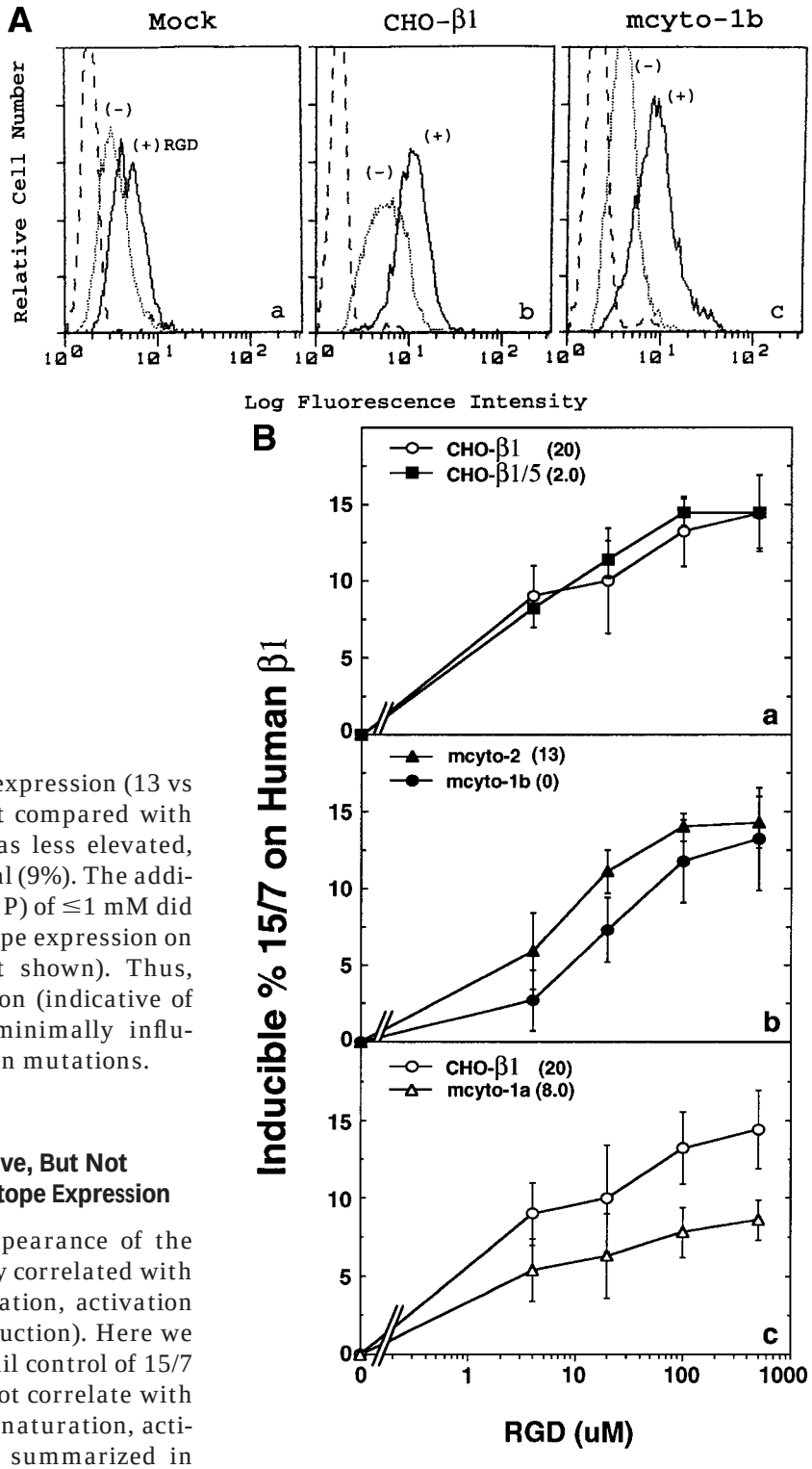

Fig. 6. Induction of 15/7 binding by RGD peptide. **A:** Staining for MAb 15/7 was analyzed on cells in suspension treated with 2 mM $MnCl_2$ alone (−) or with 2 mM $MnCl_2$ plus 500 μM GRGDSP (+). Dashed lines, background staining by negative control MAb P3. **B:** In the presence of 2 mM $MnCl_2$, titration with GRGDSP was carried out. The background 15/7 binding to hamster β1, induced by GRGDSP, was typically only 10–20% of the total 15/7 binding. This was subtracted to yield percentage 15/7 on human β1, as indicated under Experimental Procedures. Also in this experiment, contributions of constitutive 15/7 binding (listed in parentheses) are subtracted, such that the curves shown represent only ligand-inducible 15/7 binding. The β1, β1/5, and mcyto-2 curves each represent the mean of three experiments; the mcyto-1a and mcyto-1b curves are from two experiments. For all experiments, human β1 expression was in the range of 25–50 MFI units. The wild-type β1 curve **(a)** is shown again **(c)** to permit comparison with mcyto-1a.

markedly in constitutive 15/7 expression (13 vs 0%). For the mcyto-1a mutant compared with β1 (Fig. 6Bc), 15/7 binding was less elevated, but was nonetheless substantial (9%). The addition of control peptide (GRGESP) of ≤1 mM did not induce increased 15/7 epitope expression on any of the transfectants (not shown). Thus, ligand-inducible 15/7 expression (indicative of RGD peptide binding) was minimally influenced by β1 cytoplasmic domain mutations.

DISCUSSION

β1 Tails Control Constitutive, But Not Ligand-Induced MAb 15/7 Epitope Expression

In previous studies, the appearance of the 15/7 epitope has been variously correlated with integrin denaturation, inactivation, activation and ligand binding (see Introduction). Here we demonstrate β1 cytoplasmic tail control of 15/7 epitope expression that does not correlate with either integrin inactivation, denaturation, activation, or ligand binding. As summarized in Table I, the 15/7 epitope was displayed on wild-type β1, and on the mcyto-2 mutant, but was lost completely or partially on the other mutants. This *constitutive* 15/7 epitope expression is quite distinct from *ligand-induced* 15/7 epitope expression, which was fully retained in all of the mutants, except for mcyto-1a, where it was only slightly diminished. As seen elsewhere, ligand-induced appearance of the 15/7 or overlapping HUTS-21 epitope is a good indirect indicator of ligand occupancy upon addi-

TABLE I. Summary of β1 Tail Mutation Results

	Constitutive 15/7	Ligand-induced 15/7	Focal adhesions
β1 wild type	+	+	+
β1 mcyto-1a	+/−	+/−	−/+
β1 mcyto-1b	−	+	−
β1 mcyto-2	+	+	−
β1/5 chimera	−	+	−

tion of soluble ligand [Yednock et al., 1995; Seki et al., 1996; Gomez et al., 1997]. Maximal induction of the 15/7 epitope by soluble RGD peptide reached 15%, while total 15/7 (constitutive plus induced) reached as high as 30–35% of human β1. These results are comparable to the ~15% RGD-stimulated increase in 15/7 on α5β1, and 30% maximal 15/7 expression on β1 seen elsewhere [Seki et al., 1996]. The finding that all of our mutants retained a comparable capability for 15/7 induction by ligand suggests that our mutant structures are not unduly disrupted, and that ligand interaction capability is fully retained. Furthermore, the β1/5 mutation that here eliminates constitutive 15/7 expression was shown previously to have no effect on α5β1-dependent cell adhesion [Pasqualini and Hemler, 1994]. Thus again, ligand–interaction capability was not diminished, despite loss of constitutive 15/7 epitope expression.

In previous studies, specific β1 tail mutations were studied in the context of a β3 extracellular domain, and were shown to alter β3 integrin affinity for ligand [O'Toole et al., 1995]. However as far as we are aware, there has only been one previous study showing β1 tail mutation effects on the extracellular conformation of the β1 integrin [Puzon-Mclaughlin et al., 1996]. In that study, β1 tail deletions caused maximal appearance of the 15/7 epitope, but at the same time the integrin was inactivated as evidenced by nearly complete loss of ligand binding capability [Puzon-Mclaughlin et al., 1996]. As shown here with β1 point mutants and a chimeric β1/5 mutant, we have obtained markedly different results. For our mutants the 15/7 epitope was either retained or lost, but never enhanced. Meanwhile, capability for interaction with ligand was retained for all mutants. Thus our results may represent the first demonstration that relatively subtle changes in the β1 cytoplasmic tail can alter the extracellular conformation of a fully functional β1 integrin.

Focal Adhesions and the 15/7 Epitope

Previous studies have shown that β1 tail deletions of the type that abolish focal adhesion localization [Hayashi et al., 1990; Marcantonio et al., 1990], can stimulate 15/7 expression [Puzon-Mclaughlin et al., 1996]. By contrast, we now describe β1 tail mutations that abolish focal adhesion localization while the 15/7 epitope is either down-regulated or unchanged (Table I). The mcyto-2 mutant lost FA localization but retained constitutive 15/7 expression. Thus, cytoskeletal interactions at the cyto-2 site, that may be required for localization into focal adhesions, are not needed to maintain the β1 conformation displaying the 15/7 epitope.

Expression of the 15/7 epitope and FA formation were both lost in parallel for mcyto-1b (completely) and mcyto-1a (partially). Thus, cytoskeletal interactions in the cyto-1 region, such as α-actinin binding [Otey et al., 1993], could possibly affect both parameters. The β1/5 mutant also lost both FA localization (as previously seen [Pasqualini and Hemler, 1994])and the 15/7 epitope. Because the β1/5 mutant contains essentially unaltered cyto-1 and cyto-2 sites (Fig. 1), we speculate that it may fail to localize to focal adhesions because it lacks a cyto-3 site, or because of its added length. These distinct structural features of the β5 tail also could account for the loss of constitutive 15/7 epitope expression, but this remains to be determined.

Interpretation of 15/7 Expression

Expression of the 15/7 epitope on β1 integrins was initially suggested to correlate with integrin "activation" [Koyama et al., 1996; Nieto et al., 1996; Neelamegham et al., 1996; Seki et al., 1996; Werfel et al., 1996; Yednock et al., 1995; Arroyo et al., 1995; Bohnsack et al., 1995; Kovach et al., 1995; Picker et al., 1993]. However, more recent studies have shown that the 15/7 epitope not only sometimes fails to accompany integrin activation, but in fact can sometimes be induced upon integrin inactivation [Puzon-Mclaughlin et al., 1996], or denaturation during Western blotting [Yednock et al., 1995]. In addition, as seen here and elsewhere [Seki et al., 1996; Yednock et al., 1995] the 15/7 epitope may be induced as a consequence of ligand binding, and thus is reminiscent of several other anti-integrin MAb epitopes that are

induced by ligand [Bazzoni and Hemler, 1998]. The common theme linking this seemingly disparate regulation of the 15/7 epitope is integrin α-β chain unfolding [Bazzoni and Hemler, 1998]. For example, ligand binding was previously shown to induce partial integrin unfolding [Parise et al., 1987]. Likewise, the β1 tail deletions that caused 100% of β1 to express 15/7, and complete loss of ligand binding function, may also have caused integrin unfolding [Puzon-Mclaughlin et al., 1996]. Also in this regard, the 15/7 epitope is induced (by Mn^{2+}) at high levels on β1 integrins that readily undergo α-β dissociation, but hardly at all on α-β combinations that are very stable [Bazzoni et al., 1998]. Thus we suggest that the β1 tail mutants studied here also regulate integrin folding in the vicinity of the 15/7 epitope.

How can regulation of the 15/7 epitope, through β1 tail mutagenesis, be relevant if it does not correlate with ligand binding, cell adhesion, or focal adhesion functions? We think that these conformational changes detected by MAb 15/7 are likely to be physiologically meaningful precisely because (1) they occur in the context of a functionally active integrin, and (2) the mutations that cause loss of the 15/7 epitope are relatively subtle (at least compared with β1 deletions). Notably, the 15/7 epitope has been mapped to β1 residues 354–425, which is a region distinct from the ligand binding site [Puzon-Mclaughlin et al., 1996]. We speculate that possibly the region bearing the 15/7 epitope could participate in the lateral interactions of β1 integrins with each other [Miyamoto et al., 1995a; Yauch et al., 1997] and/or with other cell surface proteins [Berditchevski et al., 1997; Berditchevski et al., 1996]. In this regard, phorbol esters may induce integrin clustering [Detmers et al., 1987] and lateral diffusion [Kucik et al., 1996] as well as the 15/7 epitope [Yednock et al., 1995].

It is possible that our results may be at least partially influenced by aberrant dimerization of human β1 with hamster β5 subunit in CHO cells. However, this may not be a major concern. The amino acid sequence of the human β1A tail is 100% identical to the β1A tails of frog, chicken, and mouse [DeSimone and Hynes, 1988; Tominaga, 1988]. Furthermore, β1 from mouse shows 92.2% overall identity to human β1 [Tominaga, 1988]. Thus, the hamster β1 sequence (not yet available) is likely to be also very similar to human β1.

In conclusion, our results provide perhaps the first evidence that the integrin β1 tail can regulate the extracellular conformation of a functionally active β1 integrin. This occurs at a site defined by MAb 15/7 away from the ligand binding site. Because regulation of constitutive MAb 15/7 epitope appearance does not correlate with focal adhesion formation, cell adhesion, or ligand binding activity, our results provide an insight into a novel type of β1 conformational regulation.

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