Journal of Cell Science 112, 217-229 (1999)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS4637

Amino acid motifs required for isolated β cytoplasmic domains to regulate 'in trans' β1 integrin conformation and function in cell attachment

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Accepted 8 November; published on WWW 21 December 1998

SUMMARY

The role of β cytoplasmic domains in regulating β1 integrin conformation and function in cell attachment is not fully understood. In this study, we tested the ability of transiently expressed β cytoplasmic domains connected to an extracellular reporter domain to regulate 'in trans' the conformation of endogenous β1 integrins, and compared these effects on cell attachment. We found that chimeric receptors containing either the β1, β3 or β5 cytoplasmic domains inhibited the expression of the conformationally dependent 9EG7 and 12G10 epitopes on endogenous β1 integrins. In contrast, chimeric receptors containing the β4 or α5 cytoplasmic domain, or a control receptor lacking a cytoplasmic domain, had no effect. This inhibition occurred in a dose-dependent manner that required high levels of expression of the chimeric receptor. These results suggest that β1 integrin conformation can be regulated by conserved cytosolic interactions involving β cytoplasmic domains. This is further supported by our findings that mutations within amino acid motifs conserved among these β cytoplasmic domains, specifically the NXXY, NPXY and TST-like motifs, reduced the ability of these chimeric receptors to regulate β1 integrin conformation. Interestingly, the chimeric receptors inhibited cell attachment in a similar dose-dependent manner and required intact NXXY, NPXY, and TST-like motifs. The β1 chimera also inhibited the binding of soluble fibronectin to endogenous β1 integrins. Thus, the concomitant inhibition in the expression of conformation-dependent integrin epitopes, cell attachment and ligand binding by the chimeras, suggests that the expression of the 9EG7 and 12G10 epitopes correlates with integrin function. However, Mn^{2+}, which is an extracellular activator of integrin function, increased 9EG7 expression to basal levels in the presence of the β1 chimera, but did not rescue cell attachment to the same extent. Thus, although the β1 integrin conformation recognized by mAb 9EG7 may be required for cell attachment, it is not sufficient, suggesting that the β chimeras may be inhibiting both ligand binding and post-ligand binding events required for cell attachment. In addition, the inhibitory effects of the chimeric receptors on cell attachment were not reversed by the addition of the pharmacological agents that inhibit intracellular signals previously shown to inhibit integrin function. This finding, together with the requirement for high levels of the chimeric receptors and the fact that mutations in the same conserved motifs in heterodimeric β1 integrins have been reported to regulate β1 integrin conformation and function in cell attachment, suggest that β cytoplasmic domains regulate these processes by interacting with cytosolic factors and that the regulatory effect of the chimeras may be due to their ability to titrate proteins from endogenous integrins.

Key words: β cytoplasmic domain, Cell attachment, Integrin conformation

INTRODUCTION

The integrin family of heterodimeric transmembrane receptors regulates many cellular processes, including cell survival, proliferation and differentiation (Clark and Brugge, 1995; Yamada and Miyamoto, 1995; Burridge and Chrzanowska-Wodnicka, 1996). Integrins function in these processes by linking the extracellular matrix (ECM) to the cell's signal transduction and cytoskeletal networks. The interaction of integrins with their ECM ligands activates small GTP-binding proteins, phosphatidylinositol 3-kinase, tyrosine kinases, such as focal adhesion kinase (FAK), and serine/threonine kinases, including mitogen-activated protein kinases (Clark and Brugge, 1995; Yamada and Miyamoto, 1995; Burridge and Chrzanowska-Wodnicka, 1996; King et al., 1997). However, central to integrin function is their ability to mediate cell attachment. This requires integrins to be in a conformation that allows them to bind their ECM ligand, as well as to interact with the cytoskeleton to stabilize the attachment event. Experiments from several laboratories have demonstrated that integrin β subunit cytoplasmic domains are required for the adhesion process, including cell attachment,

cell spreading and the formation of focal adhesions (Sastry and Horwitz, 1993).

The largest family of integrins is the β1 integrins, which mediate cell adhesion to a variety of extracellular matrix ligands (Hynes, 1992). The identification of monoclonal antibodies (mAb) that bind conformation-dependent epitopes on the extracellular domain of the β1 subunit (Humphries, 1996), suggests that the conformation of β1 integrins may be dynamically regulated in a similar way to the platelet receptor, αIIbβ3 (Hughes and Plaff, 1998). Recent studies utilizing exogenously expressed heterodimeric integrins have demonstrated that the expression of several of these epitopes (15/7, 9EG7 and 12G10) depends on the amino acid sequence of the β cytoplasmic domain (Puzon-McLaughlin et al., 1996; Belkin et al., 1997; Sakai et al., 1998; Wennerberg et al., 1998). In many instances, the expression of these epitopes correlates with the ability of particular β1 integrins to mediate cell attachment (Belkin et al., 1997; Bazzoni et al., 1998; Sakai et al., 1998; Wennerberg et al., 1998). The mechanism by which β cytoplasmic domains regulate β1 integrin conformation and function in cell attachment is not fully understood. Particular amino acid sequences of β cytoplasmic domains or mutant cytoplasmic domains may alter the structure of heterodimeric integrins and thereby influence their ability to interact with extracellular ligands or other cellular factors, both cytosolic and transmembrane.

To isolate the role of cytosolic interactions with β cytoplasmic domains in integrin-mediated processes, β cytoplasmic domains have been expressed as chimeric receptors connected to heterologous extracellular domains, such as the interleukin-2 (IL-2) receptor tac subunit (LaFlamme et al., 1992), N-cadherin (Geiger et al., 1992), or CD4 (Lukashev et al., 1994). Using this approach, β cytoplasmic domains have been shown to interact with cytosolic components to direct focal adhesion localization (LaFlamme et al., 1992; Geiger et al., 1992), to induce FAK phosphorylation (Akiyama et al., 1994; Lukashev et al., 1994), and to inhibit cell attachment (Lukashev et al., 1994; Smilenov et al., 1994), cell spreading, cell migration, matrix assembly (LaFlamme et al., 1994), α5β1-mediated phagocytosis (Blystone et al., 1995) and αIIbβ3 high-affinity ligand binding (Chen et al., 1994). It has been suggested that chimeric receptors may inhibit integrin function by titrating cytoplasmic factors required for endogenous integrin function (LaFlamme et al., 1994), or by activating signaling pathways that have inhibitory effects on integrin function (Blystone et al., 1994, 1995).

In this study, we demonstrate that high levels of IL-2 receptor chimeras containing the β1, β3 or β5 cytoplasmic domain inhibit the expression of the conformation-dependent epitopes 9EG7 (Lenter et al., 1993) and 12G10 (Mould et al., 1995) on endogenous β1 integrins, suggesting that β1 integrin conformation can be regulated by interactions between the β cytoplasmic domain and cytosolic factors. In addition, mutagenesis studies indicate that the conserved NPXY, NXXY and TST-like motifs are involved in regulating this trans-inhibition of β1 integrin conformation. These chimeras also inhibit β1 integrin-mediated cell attachment in a similar dose-dependent manner and the conserved NPXY, NXXY and TST-like motifs are strictly required for this inhibition. Furthermore, the ability of endogenous β1 integrins to bind soluble fibronectin is also inhibited by the chimera containing the β1 cytoplasmic domain. Although extracellular activators of integrin function, such as Mn^{2+}, can rescue 9EG7 expression to basal levels, they cannot similarly rescue cell attachment, suggesting that the chimeras are inhibiting both ligand binding and post-ligand binding events required for cell attachment. We further demonstrate that the chimeric receptors do not inhibit cell attachment by activating inhibitory signaling pathways, as was previously shown for the inhibition of α5β1-mediated phagocytosis (Blystone et al., 1994, 1995), or by constitutively activating the Ras/Map kinase pathway previously shown to inhibit the binding of mAb PAC-1 to αIIbβ3 (Hughes et al., 1997).

MATERIALS AND METHODS

Cell culture and DNA transfection

The human osteosarcoma cell line, MG-63, was grown in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal bovine serum, 1 mM L-glutamine, 50 i.u./ml penicillin and 50 μg/ml streptomycin. Normal human fibroblasts (Vec Technologies, NY) were grown in the same medium as MG-63 cells with 10% fetal bovine serum. Sub-confluent monolayers of cells were transiently transfected by electroporation as previously described (LaFlamme et al., 1992). Cells were harvested 15-48 hours after transfection using trypsin/EDTA. The trypsin was inactivated with soybean trypsin inhibitor (Sigma, MO). Cells were then incubated in serum-free DMEM for 15 minutes at 37°C and analyzed for expression of various β1 epitopes and their ability to attach to fibronectin, as described below.

Chimeric receptors

The generation of chimeric receptors containing the wild-type β and α5 cytoplasmic domains and mutated β3 cytoplasmic domains has been previously described (LaFlamme et al., 1992, 1994; Tahiliani et al., 1997). The construction of the chimera containing the β4 intracellular domain (amino acids 854-1752) is described elsewhere (Homan et al., 1998). To construct the β5-756-758(*/A) mutant, two PCR products were generated using the plasmid encoding the β5 chimera as template DNA and the following primers: primer #1, 5′-CCATGGAGACGTCCA, and primer #2, 5′-GAAGTCCAC-AGTGTGCGCGGCGGCAGGCTTTCTGTATAATGG, for product 1 and primer #3, 5′-CCATTATACAGAAAGCCTGCCGCCGCGCAC-ACTGTGGACTTC, and primer #4, 5′-TTACCTTAGAGCTTTA-AATC, for product 2. These products were then used in a final reaction using primer #1 and primer #4 to generate a fragment encoding the mutant cytoplasmic domain, which was digested with *Xho*I and *Hin*dIII and then inserted into the appropriate vector immediately downstream of the transmembrane domain of the IL-2 receptor. Similarly, to construct the β5-752(Y/A) mutant, two PCR products were generated using primer #1 and primer #5, 5′-GGAGATAGGCTTTCTGGCTAATGGATTTGAAGC, for product 1 and primer #6, 5′-GCTTCAAATCCATTAGCCAGAAAGCCTATC-TCC, and primer #4 for product 2. These products were then used in a reaction with primers #3 and #4 to generate a fragment that was digested and cloned as described above for β5-756-758(*/A).

Flow cytometry

Cells (5×10^5) were suspended in 50 μl of cold phosphate-buffered saline (PBS) containing 0.01% sodium azide. Specific mAbs or isotype controls were then added at approximately 5-10 μg/ml. After a 30 minute incubation at 4°C in the dark, the cells were washed twice with cold PBS containing 0.01% sodium azide and then fixed with 1% formaldehyde in PBS. The samples were analyzed with a FACScan flow cytometer (Becton Dickinson, CA). Non-specific

antibody binding was determined using PE-conjugated or FITC-conjugated mouse IgG (Becton Dickinson). For indirect flow cytometry, non-specific antibody binding was determined using the fluorescence of the secondary antibody only.

Expression of specific β1 subunit epitopes on transiently transfected cells

Flow cytometry was used to determine the surface expression of several β1 subunit-specific epitopes on cells transiently expressing the chimeras. mAb 13 (Akiyama et al., 1989), which was generously provided by Dr Kenneth M. Yamada (National Institutes of Health), and the 9EG7 mAb (Lenter et al., 1993) (Pharmingen, CA) were conjugated with fluorescein using a labeling kit (Boehringer Mannheim Biochemica, IN). Fluorescein-conjugated mAb K20 (Amiot et al., 1986) was obtained commercially (Immunotech, MA). The mAb 9EG7, 13 or K20 and a phycoerythrin (PE)-conjugated mAb specific for the human IL-2 receptor (Becton Dickinson) were simultaneously added to cells expressing chimeric receptors so that the expression of the 9EG7, 13 or K20 epitope could be analyzed with respect to chimeric receptor expression. In order to determine the effects of the chimeras on the 12G10 (Mould et al., 1995) or TS2/16 (Hemler et al., 1984) epitopes, mAb 7G7B6 (hybridoma supernatant), which recognizes the human IL-2 receptor, was added to cells simultaneously with the 12G10 or TS2/16 mAb. PE-conjugated rat anti-mouse IgG1 (Becton Dickinson) and FITC-conjugated goat anti-mouse IgG2a (Pharmingen) were then used to detect the 12G10 or TS2/16 and 7G7B6 mAb, respectively.

In some experiments, the effects of the chimeras on the Mn^{2+}- and RGD peptide-induced expression of the 9EG7 epitope were also examined. For this analysis, MG-63 cells were transiently transfected with the indicated chimera and then harvested 15-48 hours after transfection. The cells were then incubated with either 1 mM RGD or RGE peptides (Life Technologies, Inc., MD) for 30 minutes at room temperature in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. For the $MnCl_2$ experiments, the cells were incubated for 30 minutes at room temperature in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mg/ml glucose) without $MgCl_2$ and $CaCl_2$. The cells were then stained with mAb 9EG7 and an antibody against the IL-2 receptor in the presence of $MnCl_2$, RGD or RGE peptides, and then analyzed by two-color flow cytometry for chimeric receptor expression and the expression of the 9EG7 epitope.

The effect of PD 098059 on the ability of the β1 chimera to inhibit 9EG7 expression was also examined. PD 098059, which is an inhibitor of mitogen-activated/extracellular-signal regulated protein kinase kinase (MEK), was added at a final concentration of 20 μM after electroporation of normal human fibroblasts with the control chimera or the β1 chimera. The cells then remained in the presence of PD 098059 until approximately 15 hours after transfection. Each dish of cells was then washed with PBS and replenished with 10 ml of serum-free DMEM. PD 098059 was added again to each sample at 20 μM. After a 30 minute incubation at 37°C under 5% CO_2, the cells were washed twice with cold PBS and processed for the analysis of 9EG7 expression and chimeric receptor expression by flow cytometry.

The effect of calphostin C on the ability of the β1 chimera to inhibit 9EG7 expression was also examined. Approximately 15 hours after transfection with the control chimera or the β1 chimera, each dish of normal human fibroblasts was washed with PBS and then replenished with 10 ml of serum-free DMEM. Calphostin C was added to one dish of each sample at a concentration of 0.5 μM. The samples were incubated for 15 minutes at 37°C under 5% CO_2 and then exposed to white light for an additional 15 minutes at room temperature. The cells were then harvested and examined for the expression of the 9EG7 epitope and the chimeric receptor as described above.

Cell attachment assay

2×10^6 cells from each transfection were resuspended in 10 ml of serum-free DMEM and plated onto 100 mm tissue culture dishes that had been coated with human fibronectin (10 μg/ml), which was a gift from Dr Paula J. McKeown-Longo (Albany Medical College). The cells were then incubated for 10 or 30 minutes at 37°C in 5% CO_2. In order to recover the unattached cells, the dishes were rotated on an orbital shaker for 30 seconds at 150 rpm and the medium containing the unattached cells was removed. The attached cells were removed using trypsin/EDTA, followed by trypsin inhibitor and two washes with PBS. A PE-conjugated antibody specific for the human IL-2 receptor (Becton Dickinson) was used in conjunction with flow cytometry to determine the chimeric receptor expression on the unattached cells, the attached cells, and a sample of the starting population of cells. In order to quantitatively recover and analyze unattached cells on the flow cytometer, 5×10^5 untransfected MG-63 cells were added to each sample containing the unattached cells.

In certain experiments, cells transfected with chimeric receptors were incubated with $MnCl_2$ or the monoclonal antibody to the β1 subunit, TS2/16 for 20 minutes at 37°C prior to the attachment assay. For the experiments analyzing the effects of PD 098059 on the ability of the β1 chimera to inhibit cell attachment, normal human fibroblasts were incubated with this MEK inhibitor after electroporation with the cDNA encoding the chimera. The cells then remained in the presence of PD 098059 until they were harvested at the end of the attachment assay (approximately 16 hours). For the experiments analyzing the effects of calphostin C on the ability of the β1 chimera to inhibit cell attachment, normal human fibroblasts were harvested approximately 15 hours after transfection and treated with 0.5 μM calphostin C in serum-free DMEM for 15 minutes at 37°C under 5% CO_2. The samples were then exposed to white light at room temperature for 15 minutes and then examined for their ability to attach to fibronectin-coated dishes as described above.

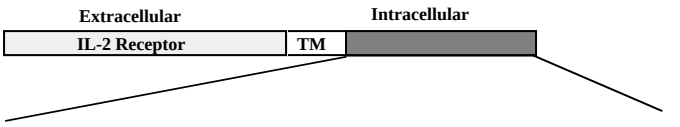
Soluble fibronectin binding

MG-63 cells transiently expressing the control chimera, the β1 chimera or mock-transfected cells were suspended at 4×10^6 cells/ml in TBS containing 1 mM $MnCl_2$. Each sample was incubated with 100 μg/ml human fibronectin at room temperature for 30 minutes and then washed twice. Antibodies specific for the IL-2 receptor (Becton Dickinson, CA) and human fibronectin (Collaborative Biomedical Products, MA) were used to analyze the effects of chimeric receptor expression on fibronectin binding by flow cytometry. To determine the extent of ligand binding, the fluorescence signal representing the cell-associated fibronectin (fibronectin still associated with the cells after harvesting) was subtracted from the fluorescence signal obtained after the addition of soluble fibronectin. The binding of soluble fibronectin was inhibited by the monoclonal antibody P4C10, which is specific for β1 integrins. In our assay, specific binding of soluble fibronectin to suspended cells was not detectable without the addition of $MnCl_2$.

RESULTS

Chimeric receptors containing integrin β1, β3 and β5 subunit cytoplasmic domains inhibit the expression of conformation-dependent epitopes on β1 integrins

In the present study, we examined whether the extracellular conformation of endogenous β1 subunits can be regulated through the interaction of the β cytoplasmic domain with cytosolic factors. We analyzed the expression of a number of β1 subunit-specific epitopes on fibroblast-like MG-63 cells in the presence of β cytoplasmic domains joined to a reporter domain consisting of the extracellular and transmembrane domains of the tac subunit of the interleukin-2 receptor (Fig. 1). The expression of the conformation-dependent 9EG7 (Lenter et al., 1993) and 12G10 (Mould et al., 1995) epitopes,



β1 Wild-type	KLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK
β3 Wild-type	KLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTFTNITYRGT
β5 Wild-type	KLLVTIHDRREFAKFQSERSRARYEMASNPLYRKPISTHTVDFTFNKFNKSYNGTVD
β3-d728-762	KLLITIHDRKEF
β3-723,726(*/A)	KLLITI**H**ARK**A**FAKFEEERARAKWDTANNPLYKEATSTFTNITYRGT
β3-747(Y/A)	KLLITIHDRKEFAKFEEERARAKWDTANNPL**A**KEATSTFTNITYRGT
β3-756(N/A)	KLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTFT**A**ITYRGT
β3-751-753(*/A)	KLLITIHDRKEFAKFEEERARAKWDTANNPLYKEA**AAA** FTNITYRGT
β5-756-758(*/A)	KLLVTIHDRREFAKFQSERSRARYEMASNPLYRKP**AAA** HTVDFTFNKFNKSYNGTVD
β5-752(Y/A)	KLLVTIHDRREFAKFQSERSRARYEMASNPL**A**RKPISTHTVDFTFNKFNKSYNGTVD

Fig. 1. Chimeric receptors containing integrin β cytoplasmic domains. The amino acid sequences of wild-type and mutant β cytoplasmic domains connected to the extracellular and transmembrane (TM) domains of the IL-2 receptor are shown. Mutations were made in the regions that are conserved among the β1, β3 and β5 cytoplasmic domains. For deletion mutants, the positions of the amino acids deleted are indicated using the numbering of the cytoplasmic domain of the full-length β subunit. For substitution mutants, the position and identity of the amino acid residues are indicated and shown in bold in the amino acid sequence provided. Not shown are the control receptor, which contains the transmembrane and extracellular domains of the IL-2 receptor and a single intracellular lysine residue, and the chimeric receptor containing the β4 cytoplasmic domain, which has little sequence homology with the other β cytoplasmic domains, although it contains NPXY motifs.

the constitutively expressed K20 (Amiot et al., 1986) epitope, as well as the epitopes for the inhibitory mAb 13 (Akiyama et al., 1989) and the activating mAb TS2/16 (Hemler et al., 1984) were examined by two-color flow cytometry using these β1-specific mAbs together with a mAb specific for the IL-2 receptor. The flow cytometric data was analyzed with gates set at each log of fluorescence intensity produced by the anti-IL-2 receptor mAb (Fig. 2A), in order to determine the effects of increasing chimeric receptor levels on the expression of the various β1 specific epitopes.

MG-63 cells have β1 integrins that express the 9EG7 and 12G10 epitopes in the absence of added ligand or activating agents. This expression is dramatically inhibited on MG-63 cells expressing high levels of the chimeric receptor containing the β1 cytoplasmic domain (Fig. 2B). In contrast, cells expressing high levels of the control receptor lacking an intracellular domain had levels of the 9EG7 and 12G10 epitopes similar to untransfected MG-63 cells (Fig. 2B). These results suggest that the extracellular conformation of the β1 subunit is regulated by cytosolic interactions involving the β1 cytoplasmic domain. Additionally, the inhibition of these epitopes was directly proportional to the expression level of the β1 chimeric receptor (Fig. 2B). Chimeric receptors containing the β3 and β5 cytoplasmic domains, but not the β4 or α5 cytoplasmic domains (Figs 3 and 4, and data not shown for 12G10), could also inhibit the expression of the 9EG7 and 12G10 epitopes. This suggests that the ability of the chimeras to reduce the expression of these conformation-dependent epitopes is dependent upon amino acid sequences that are conserved within the β1, β3 and β5 cytoplasmic domains.

As a control, we also examined the expression of the K20 epitope, which is specific for the β1 subunit and is not sensitive to functional changes in β1 integrin conformation (Lenter et al., 1993; Amiot et al., 1986). K20 expression was also reduced on cells expressing high levels of the β1 chimera, but to a much lesser extent (Fig. 2C). The cell surface expression of the α4, α5 and α6 subunits was reduced to the same extent as the K20 epitope (data not shown). The reduction in cell surface receptors appeared to be limited to integrins, since the expression of HLA-A was not affected by the expression of the β1 chimera (data not shown). This suggests that the cell surface expression of all β1 integrins was equally reduced on cells expressing high levels of the chimeras. We would predict that the expression of the 9EG7 and 12G10 epitopes would be reduced to the same degree as the K20 epitope if the decrease in the 9EG7 and 12G10 epitopes was due to the reduction in the cell surface expression of β1 integrins. This is clearly not the case, since the cells expressing high levels of the β1 chimera still had a 50% reduction in the expression of the 9EG7 epitope even after their expression was calculated relative to the expression of the K20 epitope (Fig. 3). Therefore, these results indicate that the conformation of the β1 subunit can be regulated by the interaction of β cytoplasmic domains with intracellular factors. Interestingly, the expression of the inhibitory epitope recognized by mAb 13 was affected similarly to the K20 epitope, whereas the expression of the epitope recognized by the TS2/16 activating antibody was significantly inhibited, albeit less than the 9EG7 and 12G10 epitopes (Fig. 2B). This suggests that the ability of activating antibodies to stimulate β1-dependent cell attachment can also be regulated, in part, by β cytoplasmic domains.

Elements in the C-terminal region of the β cytoplasmic domain are involved in the inhibition of the 9EG7 epitope by the chimeric receptors

The observation that the expression of the β1, β3 and β5 chimeras, but not the β4 chimera or α5 chimera, inhibited the basal expression of the 9EG7 and 12G10 epitopes suggests that certain amino acid sequences conserved among the β1, β3 and β5 cytoplasmic domains are involved in the ability of the chimeras to function as dominant inhibitors. Therefore, β3 chimeric receptors containing deletion and substitution mutations in regions conserved within the cytoplasmic domains of β subunits (Fig. 1) were tested for their ability to inhibit the 9EG7 epitope. Chimeric receptors containing the conserved membrane proximal region of the β cytoplasmic domain (β3-d728-762), previously found to bind to FAK and paxillin in vitro (Schaller et al., 1995), did not inhibit 9EG7 expression. Chimeric receptors containing amino acid substitutions within the putative FAK binding domain (β3-723,726 (*/A)) or within the NPXY (β3-747 (Y/A)) and NXXY (β3-756 (N/A)) motifs, and at the intervening TST

motif (β3-751-753 (*/A)), were found to have intermediate effects on the inhibition of 9EG7 expression compared to wild-type cytoplasmic domains (Fig. 4). The mutation in the NXXY motif was found to have the most significant effect on the ability of isolated β cytoplasmic domains to regulate β1 integrin conformation (Fig. 4).

Chimeric receptors containing integrin β1, β3 and β5 subunit cytoplasmic domains inhibit cell attachment to fibronectin

We also tested whether the chimeric receptors that inhibited the expression of the conformation-dependent 9EG7 and 12G10 epitopes could also inhibit cell attachment. For this analysis, chimeras containing the β1, β3, β4, β5 or α5 cytoplasmic domain were transiently expressed in MG-63 cells and then examined for their effects on cell attachment. Since the experiments were performed with transiently transfected cells, the ability of high levels of expression of the chimeric receptors to inhibit cell attachment was determined by comparing the levels of expression of the chimeric receptors on the starting, attached and unattached populations of cells by flow cytometry using antibodies specific for the IL-2 receptor. Cells expressing high levels of the β1, β3 and β5 chimeras did not attach to immobilized fibronectin (Fig. 5A). To further correlate the ability of the chimeras to inhibit cell attachment with their level of expression, we compared the mean fluorescence intensity (MFI) of cells that were attached or unattached with that of the starting population. The expression levels of the chimeras on attached cells were approximately half that of the starting population, while the unattached cells expressed twofold higher levels of chimeric receptors than the starting population (Fig. 5B). Thus, the β1, β3 and β5 chimeras act as inhibitors of cell attachment when expressed at high levels. Similar to our results involving the 9EG7 and 12G10 epitopes, the ability of the chimeras to inhibit cell attachment was dependent on the amino acid sequence of its β cytoplasmic domain, since the α5, β4, and the control chimera did not affect cell attachment to fibronectin (Fig. 5B). Furthermore, the β1, β3 and β5 chimeras can all inhibit β1 integrin-dependent cell attachment, since MG-63 cell attachment to fibronectin is completely inhibited by mAb 13 (data not shown).

Elements in the C-terminal region of the β cytoplasmic domain are required for the inhibition of cell attachment by the chimeric receptors

Cells expressing high levels of the β1, β3 and β5 chimeras were inhibited in both their expression of the 9EG7 and 12G10 epitopes (Fig. 2B) and their ability to attach to immobilized fibronectin (Fig. 5). This correlation led us to examine whether the regions within the β cytoplasmic domain that were observed to be involved in regulating integrin conformation were also involved in regulating cell attachment. For this analysis, cells expressing the β3

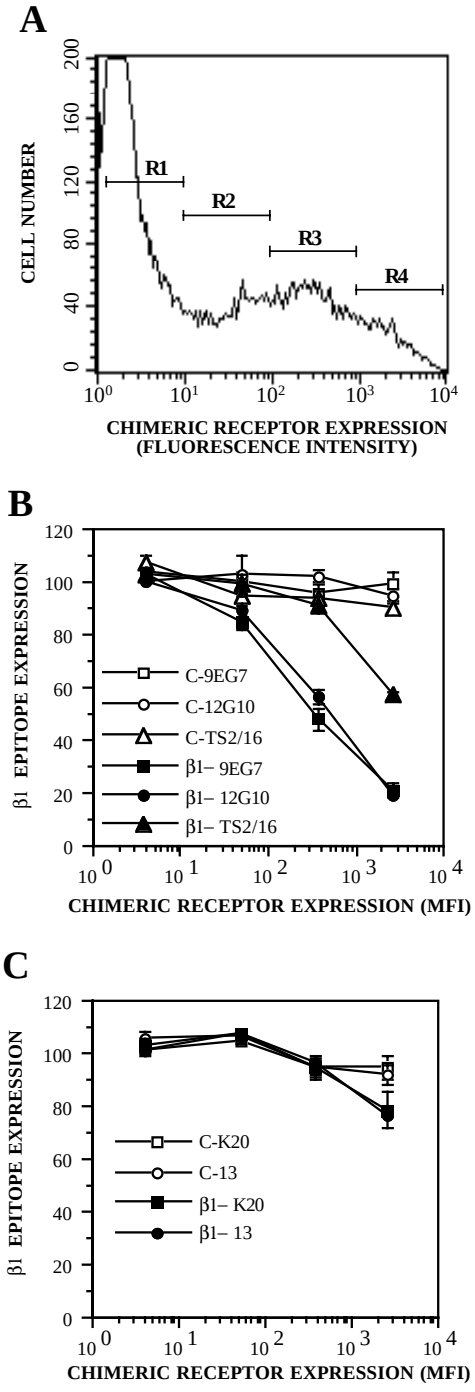


Fig. 2. Chimeric receptors containing the integrin β1 cytoplasmic domain inhibit the expression of the 9EG7, 12G10 and TS2/16 epitopes. (A) A representative flow cytometric histogram indicating the levels of chimeric receptor expression on transiently transfected MG-63 cells. Gates (R1, R2, R3, and R4) were placed around each fluorescence log in order to determine the effect of increasing levels of chimera expression on the expression of the 9EG7 epitope. MG-63 cells were transiently transfected with either the β1 chimera or control chimera, and the expression of the chimeric receptor and various β1-specific epitopes were analyzed simultaneously on individual cells from transfected and mock-transfected samples using two-color flow cytometry. The cell surface expression of the 9EG7, 12G10 and TS2/16 epitopes (B) or the K20 and 13 epitopes (C) as a function of increasing chimeric receptor expression is shown. In B and C, the samples C- and β1- refer to cells transiently expressing the control or the β1 chimera, respectively. The expression of each epitope on cells expressing chimeric receptors was compared to untransfected cells and is shown as the percentage of untransfected cells. The data represent the mean from three separate experiments ± s.e.m. MFI, mean fluorescence intensity.

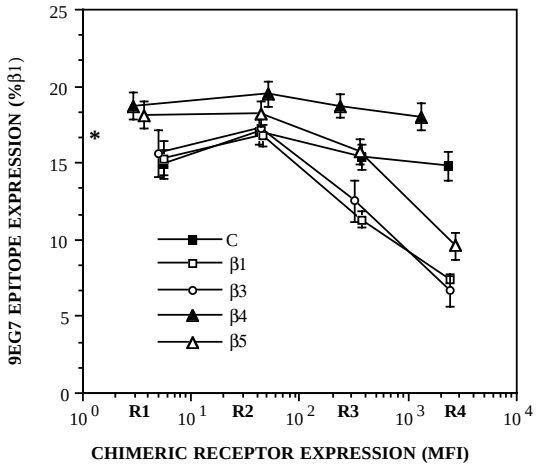


Fig. 3. Chimeric receptors containing integrin β1, β3 and β5 cytoplasmic domains inhibit the expression of the 9EG7 epitope. MG-63 cells were transiently transfected with either the β1, β3, β4, β5 or control chimera (C). Chimeric receptor and 9EG7 expressions were analyzed simultaneously on individual cells from transfected and mock-transfected samples using two-color flow cytometry. The gates shown in Fig. 2A were again used to determine the effect of increasing levels of chimeric receptor on the expression of the 9EG7 epitope. The expression of 9EG7 is presented as a percentage of total β1 integrin expression determined using monoclonal antibody K20. The asterisk refers to the expression of the 9EG7 epitope relative to total β1 expression on mock-transfected cells. The data represent the mean from three separate experiments ± s.e.m. MFI, mean fluorescence intensity.

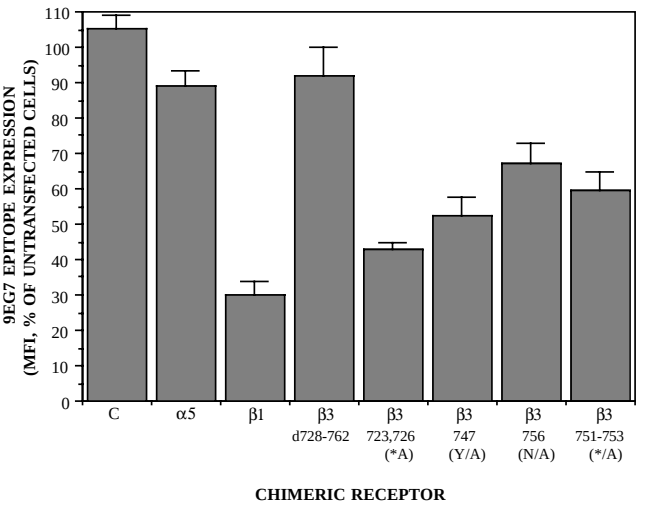


Fig. 4. The effects of β3 cytoplasmic domain mutant chimeras on the expression of the 9EG7 epitope. MG-63 cells were transiently transfected with the control chimeric receptor (C), the α5 chimera, the β1 chimera, or the following chimeras containing specific mutations within the β3 cytoplasmic domain: β3-d728-762; β3-723,726 (*/A); β3-747 (Y/A); β3-756 (N/A); and β3-751-753 (*/A). The effects of expressing these chimeras on the cell surface expression of the 9EG7 epitope were analyzed. Shown are the levels of the 9EG7 epitope on cells expressing high levels of the chimeras (10^3-10^4 fluorescence units). The expression of the 9EG7 epitope on cells expressing each chimeric receptor was compared to untransfected cells and is shown as the percenage of untransfected cells. The data represent the mean from three separate experiments ± s.e.m. MFI, mean fluorescence intensity.

chimeric receptor containing mutations in regions conserved within the cytoplasmic domain of β subunits (Fig. 1) were examined for their ability to attach to fibronectin. The conserved membrane proximal amino acids (β3-d728-762) were not sufficient for the dominant negative effect on cell attachment, and chimeric receptors containing mutations within the putative FAK binding domain [β3-723,726 (*/A)] still retained the ability to inhibit cell attachment (Fig. 6A). This suggests that the putative FAK binding region is not required for the ability of the chimeras to inhibit cell attachment. However, mutations within the highly conserved NPXY [β3-747 (Y/A)], NXXY [β3-756 (N/A)] and TST [β3-751-753 (*/A)] motifs abolished the ability of the β3 chimera to inhibit cell attachment (Fig. 6A). These data suggest that the ability of the chimeras to inhibit cell attachment requires that these conserved regions either participate in or regulate interactions between cytoplasmic proteins and β cytoplasmic domains.

To confirm that amino acid motifs conserved among these β cytoplasmic domains were involved in regulating this inhibitory effect, β5 chimeric receptors containing alanine substitutions within the NPXY [β5-752 (Y/A)] and TST [β5-756-758 (*/A)] motifs were constructed and tested for their ability to inhibit cell attachment (Fig. 1). These mutations were also found to reverse the ability of the β5 chimera to inhibit attachment (Fig. 6B). Thus, the dominant negative phenotype mediated by the different β chimeric receptors involves the same regions within their cytoplasmic domains, suggesting that the inhibitory effect is due to similar interactions with cytosolic factors.

Chimeric receptors containing the integrin β1 subunit cytoplasmic domain inhibit soluble fibronectin binding

The results demonstrating that the β1 chimera inhibited cell attachment and the basal expression of the 9EG7 and 12G10 epitopes led us to investigate whether the expression of the β1 chimera also affects the ability of endogenous β1 integrins to bind fibronectin. This was examined by two-color flow cytometry using antibodies specific for fibronectin and the IL-2 receptor, which allowed us to correlate the expression of the β1 chimera with soluble fibronectin binding in a population of cells expressing various levels of the chimera. As shown in Fig. 7, the binding of soluble fibronectin to MG-63 cells expressing high levels of the β1 chimera was reduced approximately 40% compared to mock-transfected cells and cells expressing the control chimera. This suggests that the β1 cytoplasmic domain plays a role in maintaining the ability of β1 integrins to bind soluble ligand, and that the inhibition in cell attachment by the β1 chimera may be, at least in part, due to a reduction in the ability of endogenous β1 integrins to bind fibronectin.

Extracellular activators of integrin function partially reverse the inhibition of cell attachment and 9EG7 epitope expression induced by the β1 chimeric receptor

Since Mn^{2+} and the activating antibody TS2/16 can stimulate β1 integrin-mediated cell attachment in other systems (Masumoto and Hemler, 1993), we tested whether these agents

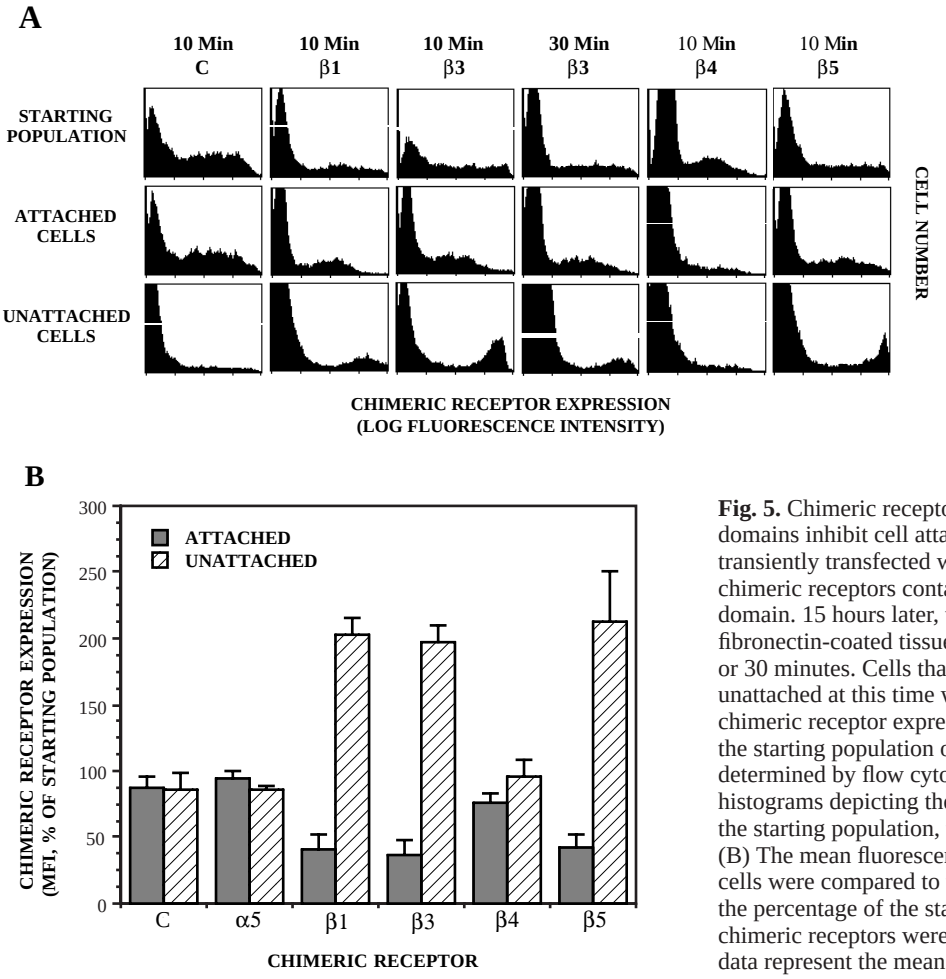


Fig. 5. Chimeric receptors containing integrin β cytoplasmic domains inhibit cell attachment to fibronectin. MG-63 cells were transiently transfected with either the control receptor (C) or with chimeric receptors containing either the β1, β3, β4 or β5 cytoplasmic domain. 15 hours later, the transfected cells were plated on fibronectin-coated tissue culture dishes and allowed to attach for 10 or 30 minutes. Cells that were attached and cells that remained unattached at this time were collected separately. The levels of chimeric receptor expression on attached cells, unattached cells and the starting population of cells (cells prior to attachment) were determined by flow cytometry. (A) Representative flow cytometric histograms depicting the levels of chimeric receptor expression on the starting population, the attached cells and unattached cells. (B) The mean fluorescence intensities of attached and unattached cells were compared to the starting population and are expressed as the percentage of the starting population. Only cells expressing chimeric receptors were analyzed (10-10^4 fluorescence units). The data represent the mean from three separate experiments ± s.e.m.

could rescue cell attachment inhibited by the chimeric receptors. As shown in Fig. 8, the expression level (MFI) of the β1 chimera on the attached population of cells was increased in the Mn^{2+}-treated and TS2/16-treated samples compared to untreated samples. However, the majority of high expressors still remained unattached.

Mn^{2+} and RGD peptides are known to increase the number of β1 integrins that express the 9EG7 and 12G10 epitopes (Lenter et al., 1993; Mould et al., 1998). On cells expressing high levels of the β1 chimera, RGD peptides and Mn^{2+} increased the expression of the 9EG7 epitope to basal levels found on untransfected cells, but did not increase the epitope to levels induced by RGD or Mn^{2+} on untransfected cells (Fig. 9). Interestingly, although Mn^{2+} increased 9EG7 expression to levels found on untransfected cells, Mn^{2+} was unable to restore cell attachment to levels observed for cells expressing the control chimera (Fig. 8). Therefore, changing the extracellular conformation of β1 integrins using extracellular activators is not sufficient to restore integrin function in cell attachment in the presence of intracellular inhibitors such as the chimeric receptors. Taken together, these results suggest that the chimeric receptors may be inhibiting cell attachment by negatively regulating β1 integrin conformation and by inhibiting post-ligand binding events that are also required for cell attachment.

The pharmacological agents calphostin C and PD 098059 do not reverse the dominant negative effect of the chimeric receptors

Ligation of αvβ3 or the expression of chimeric receptors containing the β3 cytoplasmic domain were previously shown to inhibit α5β1-mediated phagocytosis (Blystone et al., 1994, 1995). The pharmacological agents H7 and calphostin C, which inhibit protein kinase C (PKC), reversed this inhibition, suggesting that the chimeric receptors were inhibiting α5β1-mediated phagocytosis by activating PKC (Blystone et al., 1995). For this reason, we tested the ability of calphostin C to rescue the expression of the 9EG7 epitope and cell attachment inhibited by the chimeric receptors. Additionally, since constitutive activation of the Ras/Map kinase pathway was recently demonstrated to inhibit the high affinity ligand binding of αIIbβ3 (Hughes et al., 1997), we also examined whether the chimeric receptors were regulating β1 integrin conformation and function in cell attachment by constitutively activating this pathway.

Interestingly, calphostin C neither inhibited the expression of the 9EG7 epitope on untransfected cells nor rescued the expression of the 9EG7 epitope on cells expressing high levels of the β1 chimera (Table 1). Furthermore the treatment of cells with the pharmacological agent PD 098059, which is a specific MEK inhibitor (Dudley et al., 1995), also failed to rescue the

expression of 9EG7 inhibited by the chimeric receptors (Table 1), although PD 098059 inhibited the activation of Map kinase triggered by growth factors (data not shown).

We would predict that if calphostin C or PD 098059 reversed the inhibition of cell attachment by the β1 chimera, then more cells expressing high levels of the β1 chimera would attach to fibronectin, increasing the MFI of the attached population and decreasing the MFI of the unattached population. PD 098059 had no effect on the ability of the β1 chimera to inhibit cell attachment (Table 2). However, as previously shown (Vuori

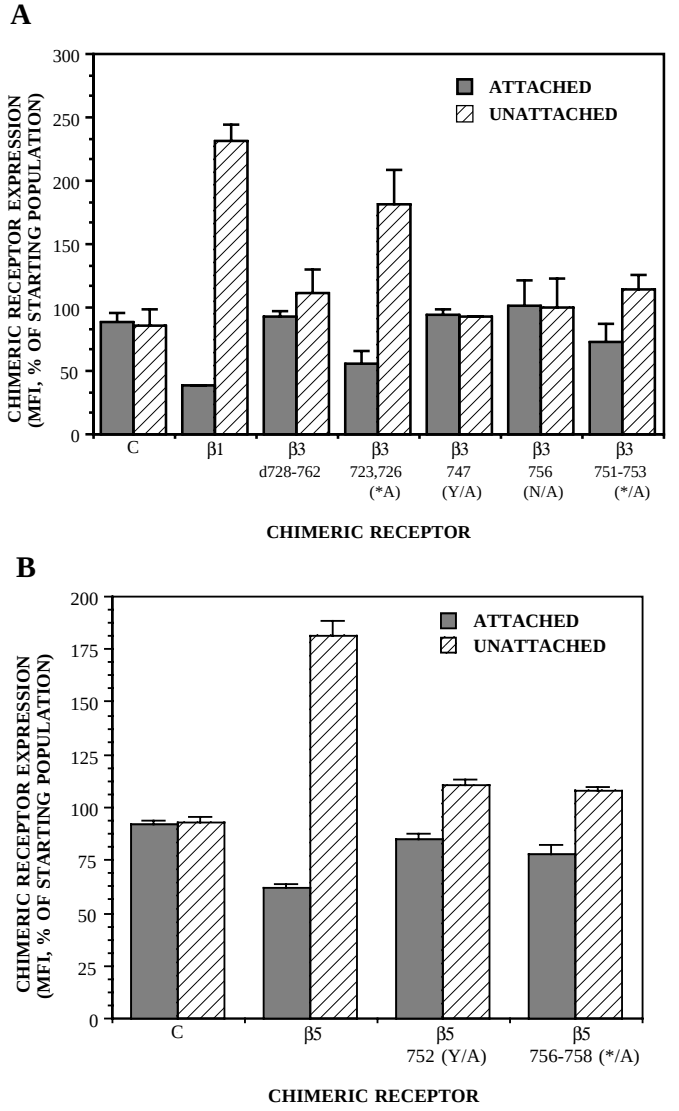


Fig. 6. The effects of β3 and β5 cytoplasmic domain mutant chimeras on cell attachment. (A) MG-63 cells were transiently transfected with the control chimeric receptor (C), the β1 chimera, or the following chimeras containing specific mutations within the β3 cytoplasmic domain: β3-d728-762; β3-723,726 (*/A); β3-747 (Y/A); β3-756 (N/A); and β3-751-753 (*/A). (B) MG-63 cells were transiently transfected with the control chimeric receptor (C), the β5 chimera, or the following chimeras containing specific mutations within the β5 cytoplasmic domain: β5-752 (Y/A) and β5-756-758 (*/A). To determine the effects of expressing these chimeras on cell attachment to fibronectin, the levels of chimeric receptor expression on attached cells, unattached cells and the starting population of cells (cells prior to attachment) were determined by flow cytometry. The mean fluorescence intensities of attached and unattached cells were compared to the starting population and are expressed as the percentage of the starting population. Only cells expressing chimeric receptors were analyzed (10-10^4 fluorescence units). The data represent the mean from three separate experiments ± s.e.m.

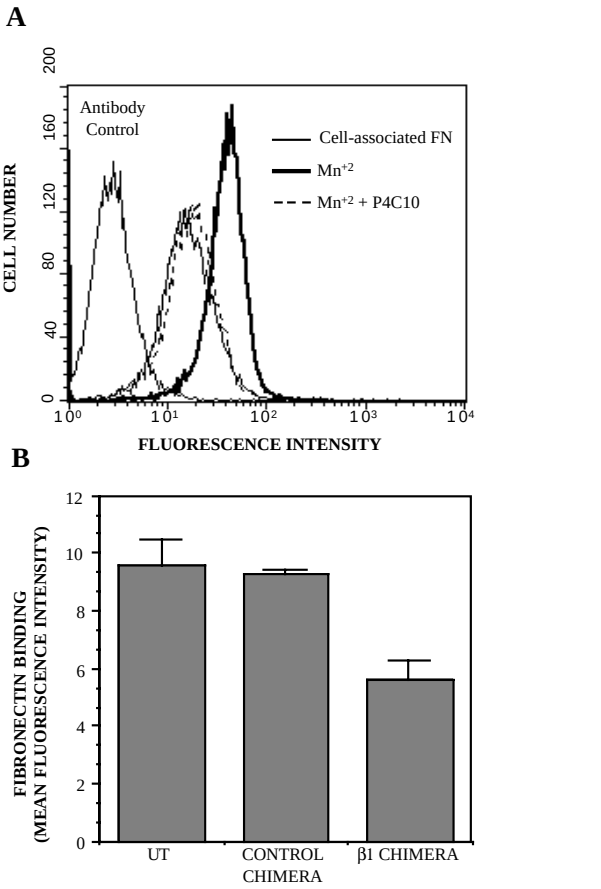


Fig. 7. Chimeric receptors containing the integrin β1 cytoplasmic domain inhibit the binding of soluble fibronectin. (A) Soluble human fibronectin was added to MG-63 cells suspended in TBS containing 1 mM $MnCl_2$ in the absence or presence of P4C10, which is a blocking antibody specific for β1 integrins. After 30 minutes at room temperature, fibronectin binding was analyzed by flow cytometry using an antibody specific for fibronectin. A representative flow cytometric histogram from one experiment is shown, which demonstrates that the binding of soluble fibronectin is inhibited by a blocking antibody to β1 integrins. The antibody control is labeled as shown. The thin solid line represents cell-associated fibronectin (fibronectin associated with the cells after harvesting). The thick solid line represents soluble fibronectin binding in the presence of $MnCl_2$ and the thin dashed line represents soluble fibronectin binding in the presence of both $MnCl_2$ and the blocking antibody to β1 integins, P4C10. (B) MG-63 cells were transiently transfected with either the control or the β1 chimera. The cells were incubated with soluble fibronectin in the presence of $MnCl_2$ as described in Materials and methods. The ability of the cells to bind soluble fibronectin was anlayzed by two-color flow cytometry using antibodies specific for human fibronectin and the IL-2 receptor. Soluble fibronectin binding on cells expressing high levels of the chimeric receptors was determined as described in the Materials and methods and is expressed as mean fluorescence intensity. UT, untransfected (mock) cells. The mean ± s.e.m. from triplicate samples of one representative experiment is shown.

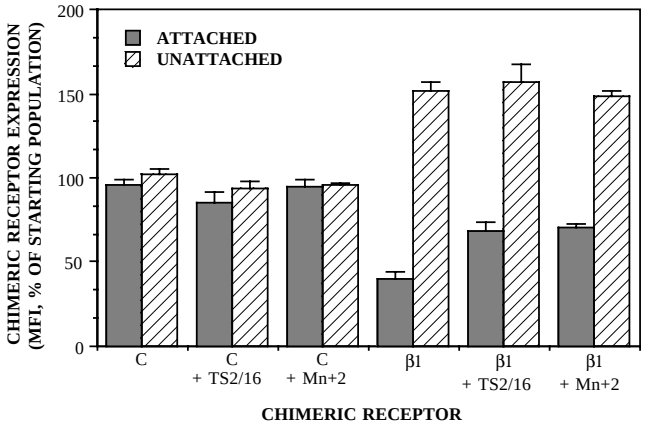


Fig. 8. The effects of Mn^{2+} and TS2/16 on the inhibition of cell attachment by the β1 chimera. MG-63 cells were transiently transfected with either the control (C) or β1 chimera. The ability of the cells to attach to immobilized fibronectin was analyzed after incubation of the cells with 1 mM Mn^{2+} or 20 μg/ml TS2/16. The levels of chimeric receptor expression on attached cells, unattached cells and the starting population of cells (cells prior to attachment) were determined by flow cytometry. The data with Mn^{2+}-treated samples represent the mean from three separate experiments ± s.e.m. The mean fluorescence intensities of attached and unattached cells were compared to the starting population and are expressed as the percentage of the starting population. Only cells expressing chimeric receptors were analyzed (10-10^4 fluorescence units). The data with the TS2/16-treated samples represent the mean from three separate experiments.

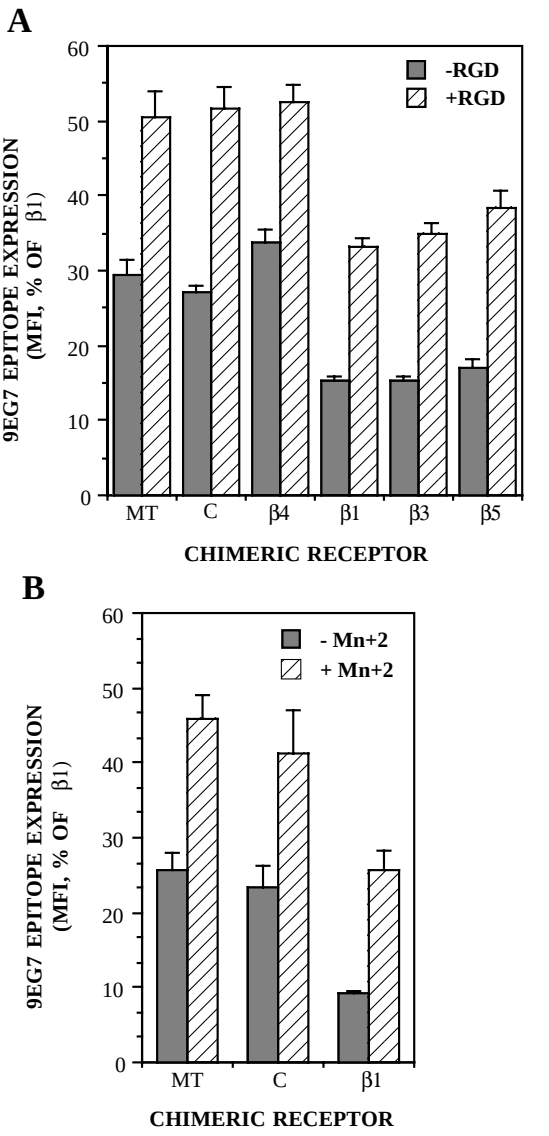


Fig. 9. Mn^{2+} and RGD peptide partially reverse the inhibition of 9EG7 epitope expression by the β1 chimera. MG-63 cells were transiently transfected with the indicated chimera. Chimeric receptor expression and the expression of the 9EG7 epitope after incubation of the cells with 1 mM RGD (A) or 1 mM Mn^{2+} (B) were analyzed simultaneously on individual cells from transfected and mock-transfected (MT) samples using two-color flow cytometry. The levels of 9EG7 on cells expressing high levels of the chimeras (10^3-10^4 fluorescence units) are shown. The total surface expression of the β1 subunit was determined using mAb K20 in order to ascertain the percentage of 9EG7 expression relative to total β1 expression. The data represent the mean from three separate experiments ± s.e.m. MFI, mean fluorescence intensity.

and Ruoslahti, 1993), calphostin C inhibited cell attachment to fibronectin. As a result of this overall inhibition of cell attachment, very few attached cells were available for analysis. However, the expression levels of the β1 chimera on the unattached and starting populations were very similar (Table 2). Therefore, calphostin C did not cause cells expressing high levels of the β1 chimera to attach to fibronectin. Thus, the inhibition of 9EG7 expression and cell attachment by the β1 chimera occurs by a mechanism which is distinct from that reported for the inhibition of α5β1-mediated phagocytosis and does not appear to involve the constitutive activation of Map kinase.

DISCUSSION

We have five major conclusions from our studies: (1) cytosolic interactions involving β cytoplasmic domains can regulate endogenous β1 integrin conformation, ligand binding and function in cell attachment; (2) isolated β cytoplasmic domains regulate β1 integrin conformation and function in cell attachment in a similar dose-dependent and β cytoplasmic domain-specific manner; (3) mutations in the conserved NPXY, NXXY and TST-like motifs inhibit the ability of isolated β cytoplasmic domains to regulate 'in trans' endogenous β1 integrin conformation and function in cell attachment; (4) the chimeric receptors are not inhibiting integrin conformation and cell attachment by constitutively activating signaling pathways which have previously been shown to inhibit integrin function; and (5) the presence of integrins in conformations recognized by mAb 9EG7 is not sufficient for cell attachment to fibronectin, but may be required.

Although experiments from other laboratories have provided evidence that β cytoplasmic domains are involved in the regulation of β1 integrin conformation (Puzon-McLaughlin et al., 1996; Belkin et al., 1997; Sakai et al., 1998; Wennerberg et al., 1998), our results are the first to indicate that this can

Table 1. The effects of calphostin C and PD 098059 on the inhibition of 9EG7 expression by the β1 chimeric receptor

Sample	9EG7 epitope expression (% of untransfected cells) Exp 1	Exp 2
Control chimera	80.0*	70.7
Control chimera+PD098059	70.0	74.9
Control chimera+calphostin C	75.8	92.4
β1 chimera	25.3	33.1
β1 chimera+PD098059	26.4	32.7
β1 chimera+calphostin C	26.8	36.9

*The mean fluorescence intensity produced with antibody 9EG7 for each sample of cells expressing high levels of chimeric receptor (last decade of fluorescence units) was compared to untransfected cells and is expressed as the % of untransfected cells.

occur by the interaction of integrin β cytoplasmic domains with intracellular factors. The expression of 9EG7 was initially examined on T lymphocytes and the K562 myeloid cell line, where its expression was not detected without prior incubation with Mn^{2+} or soluble ligands such as RGD peptides (Lenter et al., 1993; Bazzoni et al., 1995). For this reason, the 9EG7 epitope is referred to as a cation- ligand-influenced binding site or CLIBS (Bazzoni et al., 1995). In our studies, we show that the 9EG7 epitope is expressed on MG-63 cells at a basal level and that 9EG7 expression is enhanced in response to Mn^{2+} or RGD peptides. Chimeric receptors containing the β1, β3 or β5 cytoplasmic domain inhibited the basal expression of 9EG7. However, the addition of RGD peptides or Mn^{2+} to cells expressing the chimeric receptors increased 9EG7 expression to basal levels found on control cells, but not to levels found on RGD or Mn^{2+}-treated control cells. Therefore, 9EG7 expression may, to some extent, be influenced by cations and soluble ligands via mechanisms that are independent of cytosolic interactions. Since the chimeric receptors similarly inhibit the expression of the 9EG7 and 12G10 epitopes, we conclude that the conformation of β1 integrins can be regulated by both extracellular mechanisms and cytosolic interactions involving the β1 cytoplasmic domain.

The ability of the chimeric receptors to inhibit both the expression of these epitopes and cell attachment in a similar dose-dependent and β cytoplasmic domain-specific manner correlates the expression of the 9EG7 and 12G10 epitopes with β1 integrin function in cell attachment. In addition, previous studies have demonstrated that α5β1-mediated cell adhesion can be enhanced by Mn^{2+} (Bazzoni et al., 1995), which stabilizes the β1 integrin conformation recognized by the 9EG7 and 12G10 mAbs (Lenter et al., 1993; Mould et al., 1998). This also suggests that integrins which can bind 9EG7 and 12G10 are in a conformation that favors cell attachment. Interestingly, the 9EG7 epitope is expressed highest on the α5β1 and α4β1 integrins, where its expression correlates with integrin function of these particular integrin heterodimers (Bazzoni et al., 1998). However, it should be noted that merely having integrins in this conformation is not sufficient for cell attachment. This is supported by our observation that Mn^{2+} restored 9EG7 expression to basal levels on cells expressing the β1 chimera, but it did not restore the attachment of these cells to control levels. This indicates that increasing the expression of the 9EG7 epitope is not sufficient to rescue the inhibition of cell attachment by intracellular inhibitors, such as the chimeras. This is also supported by the previous observation that cytochalasin B did not affect the expression of the 15/7 epitope, which also represents a CLIBS on the β1 subunit (Yednock et al., 1995), but did inhibit cell attachment (Bohnsack et al., 1995).

The interaction between β1 integrins and cytosolic factors that influence cell attachment appear to occur mainly via the β1 cytoplasmic domain, since a chimeric receptor containing the cytoplasmic domain of the α5 subunit did not affect cell attachment. Previous studies have shown that chimeric receptors containing the β1 or β3 cytoplasmic domain also function as trans-dominant inhibitors of cell spreading,

Table 2. The effects of calphostin C and PD 098059 on the inhibition of cell attachment by the β1 chimeric receptor

		Calphostin C			
		Control chimera	Control chimera calphostin C	β1 chimera	β1 chimera calphostin C
Experiment no. 1	Starting	144.1*	115.7	337.1	306.0
	Attached	102.2	**86.2**	88.3	**115.1**
	Unattached	134.1	103.4	374.0	242.6
Experiment no. 2	Starting	136.0	119.4	119.8	165.0
	Attached	116.0	**73.0**	70.0	**45.0**
	Unattached	127.5	106.0	240.3	162.2
		MEK inhibitor (PD 098059)			
		Control chimera	Control chimera PD 098059	β1 chimera	β1 chimera PD 098059
Experiment no. 1	Starting	226.0	283.0	354.0	279.0
	Attached	213.0	272.0	121.0	94.0
	Unattached	249.0	294.0	611.0	467.0
Experiment no. 2	Starting	136.0	119.0	199.8	165.4
	Attached	116.0	94.0	70.0	53.4
	Unattached	127.5	114.0	240.3	209.5

*The mean fluorescence intensities of chimeric receptor expression for the attached, unattached and starting population of cells are shown for two separate experiments. Approximately 3-5,000 cells expressing chimeric receptors were analysed for each sample. Calphostin C inhibited cell attachment for all samples tested. As a result, <300 cells were analysed for the attached samples that were treated with calphostin C (numbers in bold).

fibronectin matrix assembly and cell migration (LaFlamme et al., 1994). However, higher doses of the chimeric receptors are required to inhibit cell attachment and β1 integrin conformation compared to cell spreading, suggesting that different protein interactions with the β cytoplasmic domain may be involved in these different dominant negative effects. Although most of our current studies were performed using MG-63 cells, similar results were obtained with normal human fibroblasts which have a very similar integrin complement compared with MG-63 cells (data not shown).

The conserved NPXY, NXXY and TST motifs within β cytoplasmic domains were found to affect the ability of the chimeras to inhibit the expression of the 9EG7 epitope and cell attachment. Mutations in these motifs in the β1 cytoplasmic domain of heterodimeric integrins inhibit cell attachment and the expression of the 9EG7 and 12G10 epitopes (Sakai et al., 1998; Wennerberg et al., 1998). In the context of the heterodimeric receptors, these mutations may inhibit these processes by structural alterations in the integrin itself that affect ligand binding without necessarily affecting cytoplasmic interactions. Our results extend these earlier studies by demonstrating that these conserved motifs play a role in cell attachment and β1 integrin conformation by regulating cytosolic protein interactions with the β cytoplasmic domain.

These conserved motifs have also been found to be important in regulating β2 and β3 integrin function in a number of processes. For example, disruption of the NPXY motif inhibited β3-mediated cell attachment (Filardo et al., 1995) and αIIbβ3-mediated cell spreading and high affinity ligand binding, whereas mutations in the NXXY motif had lesser effects (O'Toole et al., 1995; Ylanne et al., 1995). Alanine substitutions in the analogous TST-like motif in the β2 subunit cytoplasmic domain inhibited β2 integrin-mediated cell attachment (Hibbs et al., 1991). Additionally, we have previously shown that mutations in the NPXY motif completely inhibited the ability of clustered β3 cytoplasmic tails to trigger FAK phosphorylation, whereas mutations in the NXXY and TST motifs inhibited FAK phosphorylation to a lesser extent (Tahiliani et al., 1997). In this current study, the inhibition of cell attachment was more sensitive to mutations in the NPXY, NXXY and TST motifs compared with the inhibition of 9EG7 expression. This might reflect differences in the sensitivities of the two assays. Alternatively, basal 9EG7 expression might require protein interactions that are distinct from those required for post-ligand binding events. Therefore, the chimeric receptors could potentially be inhibiting two steps: (1) the expression of β1 integrin conformations favorable to ligand binding and cell attachment, and (2) post-ligand binding events necessary to maintain cell attachment. Thus, distinct cytoplasmic factors may influence β1 integrin conformation and post-ligand binding events needed for cell attachment. This is consistent with our observation that the potent PKC inhibitor, calphostin C, inhibited cell attachment, but did not inhibit the expression of the 9EG7 epitope.

It has been suggested that chimeric receptors may interact with cytoplasmic proteins and thereby activate signaling pathways that are inhibitory to endogenous integrin function. This mechanism appears to be involved in the ability of the chimeric receptors and the ligation of αvβ3 to inhibit α5β1-mediated phagocytosis (Blystone et al., 1994, 1995). In this case, the addition of the pharmacological agents H7 and calphostin C was able to rescue the inhibitory effects on phagocytosis (Blystone et al., 1994, 1995). However, in our studies these agents did not rescue cell attachment or the expression of the 9EG7 epitope, indicating that the mechanisms by which the chimeric receptors inhibit these processes are distinct from those involved in the inhibition of α5β1-mediated phagocytosis. Also, the constitutive activation of the Ras/Map kinase pathway has previously been shown to inhibit the high affinity ligand binding of the PAC-1 mAb to an αIIbβ3 integrin engineered to be constitutively active (Hughes et al., 1997). However, inhibiting this pathway with the specific MEK inhibitor, PD 098059, did not affect chimeric receptor-mediated inhibition of 9EG7 binding or cell attachment. Therefore, the chimeric receptors do not inhibit these processes by constitutively activating Map kinase. These findings, together with the requirement for high expression levels of the chimeras and the observation by other laboratories that the mutation of similar motifs in the context of heterodimeric receptors similarly affect integrin function in these processes, suggest that the regulatory effect of the chimeras may be due to their ability to interact with and sequester cytoplasmic proteins that would otherwise associate with endogenous integrins. This sequestration of cytoplasmic factors by the chimeras might result in the inability of endogenous integrins to cluster and/or connect to the cell's cytoskeletal and signal transduction systems.

The C-terminal region of integrin β cytoplasmic domains is involved in regulating several integrin-mediated functions, including cell attachment, cell spreading, FAK phosphorylation and conformational changes of the β subunit. This region is known to interact with several different cytosolic proteins. These motifs appear to be involved in the interaction of several proteins with β cytoplasmic domains (LaFlamme et al., 1997). For example, the NXXY motif is required for the interaction between integrin cytoplasmic domain-associated protein-1 (ICAP-1) and the β1 cytoplasmic domain (Chang et al., 1997), and between β3-endonexin and the β3 cytoplasmic domain (Eigenthaler et al., 1997). However, interactions of ICAP-1 and β3-endonexin are specific for the β1 and β3 cytoplasmic tails, respectively (Chang et al., 1997; Hannigan et al., 1996). The TST motif is located within the region of the β1, β2 and β3 cytoplasmic domains that contains a binding site for integrin-linked kinase (ILK) (Hannigan et al., 1996). The NPXY motif appears to be involved in the binding of both talin and α-actinin with the β cytoplasmic domain (Pfaff et al., 1998; Otey et al., 1993). It is not yet known whether any of these protein interactions are involved in the ability of the chimeric receptors to regulate β1 integrin conformation and function in cell attachment. However, some of these interactions are not likely to be candidates because of their specificity for individual β cytoplasmic domains. There are likely to be both cytoplasmic domain-specific and promiscuous integrin binding proteins that recognize distinct and overlapping regions. Future studies will be aimed at identifying specific interactions that regulate specific aspects of integrin function. Our chimeric receptor approach will be a useful tool in these studies to further define the mechanisms by which β cytoplasmic domains regulate integrin function.

We thank Drs Randy Morse, Jane Sottile, Allison Berrier and Denise Hocking for helpful comments during the preparation of this

manuscript, Dr Kenneth Yamada for generously providing mAb 13, the Cellular Immunology Laboratory at the Albany Medical Center for help with flow cytometry, and Robert Martinez for his excellent technical assistance. This work was supported in part by American Heart Association, New York State Affiliate Postdoctoral Fellowship 970150 to A. M. M., National Institutes of Health grant GM51540 to S. E. L. and American Heart Association, New York State Affiliate grant 960148 to S. E. L. S. M. H. is supported by National Institutes of Health grant T32-HL-07194. M. J. H. was supported by grants from the Wellcome Trust.

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