Interchangeable α Chain Cytoplasmic Domains Play a Positive Role in Control of Cell Adhesion Mediated by VLA-4, a β_1 Integrin

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Summary

Integrins can exist in a range of functional states, depending on the cell type and its state of activation. Although the mechanism that controls activity is unknown, it has been suggested that for some integrins, α chain cytoplasmic domains may exert either a negative effect or no effect on adhesion function. To address this issue for VLA-4 (an $\alpha^4\beta_1$ heterodimer), we constructed an α^4 cytoplasmic deletion mutant and chimeric α chains composed of the extracellular domains of α^4 and the cytoplasmic domains of α^2, α^4, or α^5. Upon stable transfection of wild-type α^4, VLA-4 heterodimer was obtained that mediated (*a*) poor adhesion to CS1 peptide, fibronectin, or vascular cell adhesion molecule 1 (VCAM-1) (in K562 cells); (*b*) poor adhesion to CS1 peptide but moderate adhesion to VCAM-1 (in MIP101 cells); and (*c*) moderate adhesion to both CS1 peptide and VCAM-1 (in PMWK cells). Chimeric α^4 constructs and wild-type α^4 yielded similar results in these cell lines. In contrast, truncation of the α^4 cytoplasmic domain (after the conserved GFFKR motif) caused an almost complete loss of adhesive activity in all three cell lines. Thus, several interchangeable α chain cytoplasmic domains play a fundamentally positive role in determining the state of constitutive activity for VLA-4. The α chain cytoplasmic domain is also required for agonist-stimulated adhesion, since phorbol ester stimulated the cell adhesion mediated by wild-type and chimeric α chains, but not by the cytoplasmic deletion mutant. The inactivity of both wild-type VLA-4 (in K562 cells), and truncated VLA-4 (in all three cell lines) was overcome by the addition of a stimulatory anti-β_1 monoclonal antibody. Thus, the α cytoplasmic domain–dependent cellular mechanism controlling both constitutive and agonist-stimulated VLA-4 activity could be bypassed by external manipulation of the integrin.

The integrin family is comprised of at least 20 heterodimers, each with different ligand-binding specificities formed by the pairing of one of 14 α chains with one of eight β chains. The $\alpha^4\beta_1$ integrin VLA-4 has been implicated in a wide variety of biological processes. It is involved in cell adhesion to extracellular matrix via the alternatively spliced CS1 domain of fibronectin (FN)[1] (1–4), and in cell–cell adhesion through the vascular cell adhesion molecule-1 (VCAM-1) on activated endothelium (3, 5, 6). VLA-4, which is normally expressed on T and B lymphocytes, monocytes, and eosinophils, is involved in inflammation, migration, and recirculation of lymphocytes (7, 8). Eosinophil adhesion to activated endothelium as seen in asthmatic and allergic conditions appears to be mediated by VLA-4 interaction with VCAM-1 (9). VLA-4/VCAM-1 interaction is likely to be important in lymphocyte adhesion to inflamed endothelium in rheumatoid synovium (10–12). Abs against the $\alpha^4\beta_1$ integrin were shown to prevent the onset of experimental autoimmune encephalomyelitis, suggesting that VLA-4 may be involved in inflammatory diseases of the central nervous system, such as multiple sclerosis (13). Differentiation of B lymphocytes in germinal centers (14), hematopoietic stem cells in bone marrow (15–17), and T lymphocytes in thymus (18), all involve interaction of VLA-4 with VCAM-1 or FN. Additionally, $\alpha^4\beta_1$ is expressed on neural crest–derived cells and interaction with the CS1 domain of FN is involved in migration of those cells from the neural crest during embryogenesis (19, 20). The VLA-4/VCAM-1 interaction has also been demonstrated during the formation of myotubes during muscle development (21). Because adhesion through $\alpha^4\beta_1$ is involved in so many biological processes, it follows that regulation of the adhesion mediated by $\alpha^4\beta_1$ (and other integrins) is of fundamental importance (22).

Integrins are versatile, exhibiting different levels of functional activity depending on the cell type and state of activation (23–25). For example, on platelets and leukocytes, many

[1] *Abbreviations used in this paper:* FN, fibronectin; pBS, pBluescript-KS$^+$; VCAM, vascular cell adhesion molecule.

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integrins exist in a relatively nonfunctional condition, but show increased binding capabilities after cell activation with a variety of agonists (23, 24, 26–30). In contrast, on many other cell types, integrins demonstrate a high level of functional activity, with relatively little capability for further upregulation (23, 24). Elucidation of the mechanism(s) by which the functional activity of integrins is controlled is an area of intense research, and of fundamental importance. Nonetheless, this mechanism is poorly understood, although it has been variously suggested that membrane lipid composition (31, 32), divalent cations (24, 33–35), integrin phosphorylation (36–38), and glycosylation (39) may alter functional activity of integrin heterodimers. Other studies have suggested a role for the cytoplasmic domains of the integrin chains in the control of functional activity. Deletion of the β_2, but not the α^L, cytoplasmic domain resulted in loss of the ability to upregulate function by the phorbol ester PMA (40, 41), implicating the β but not α chain cytoplasmic domain in the control of functional regulation. Additionally, it has been demonstrated that deletion of the α^{IIb} cytoplasmic tail or exchange with the α^5 cytoplasmic domain converted an inactive $\alpha^{IIb}\beta_3$ heterodimer to a highly functional state (42). This suggested that the α^{IIb} cytoplasmic tail plays a negative regulatory role, and thus, alteration of that domain resulted in upregulation of function. Heretofore, there has been minimal analysis of the role of β_1-associated α chain cytoplasmic domains in the regulation of integrin-mediated adhesion.

To analyze the role of the α chain cytoplasmic domains in the control of both constitutive and agonist-stimulated activity of β_1 integrins, we have constructed a series of mutant α chains. The cytoplasmic domain of α^4 was either deleted or exchanged with the cytoplasmic domains of α^2 or α^5, and stable transfectants expressing these mutant α^4 chains or wild-type α^4 were assayed for adhesive function. For transfections, we chose three cell lines (K562, MIP101, and PMWK) in which wild-type α^4 was expressed to yield relatively inactive, partly active, or fully active VLA-4. Thus we were able to directly compare and determine the extent to which exchange or deletion of the cytoplasmic domain affects the variable levels of constitutive VLA-4 activity in different cell types.

Materials and Methods

Abs, Antisera, Cell Lines, and Purified Ligands. mAbs used in this study were anti-α^4, B-5G10 (43), and HP 1/2 (44); anti-β_1, A-1A5 (45) and TS2/16 (46); and J-2A2 (47) was used as a negative control Ab. Peptides corresponding to the COOH-terminal 21 amino acids of α^2, and the COOH-terminal 21 amino acids of α^4 were coupled to KLH and injected into rabbits for generation of antisera as previously described (48). The primary melanoma PMWK (49) was a gift from Dr. H.R. Byers (Massachusetts General Hospital, Boston, MA). The 40-kD fragment of FN-40, which contains the CS1 region, was purified as described (50). Recombinant soluble VCAM-1 (51), the form with seven Ig domains, (sVCAM-1) was the gift of Dr. Roy Lobb (Biogen Inc., Cambridge, MA). CS1 peptide conjugated to BSA (CS1-BSA) was the gift of Dr. T. Shimo-Oka (Iwaki Glass Co., Tokyo, Japan).

Construction of Chimeric α Chains, Transfection, and Cell Culture. Mutations introducing a HindIII site into α^2 and α^4 cDNAs were as described (52). The X4C2 chimeric α subunit was first constructed in pBluescript-KS$^+$ (pBS) by excising the 3′ 1.3 kb of the α^4 cDNA using $KpnI^{2527}$ and $XbaI^{pBS\ polylinker}$ and then inserting the 516-bp $KpnI^{2527}$-$HindIII^{3043}$ fragment of α^4 and the 1.9-kb $HindIII^{3512}$-$XbaI^{pBS\ polylinker}$ fragment of α^2. For construction of the X4C5 chimera, the 0.7-kb $HindIII^{3087}$-$XbaI^{pBS\ polylinker}$ fragment from the α^5 cDNA was used instead of the 1.9-kb fragment of α^2. The α^4 cDNA containing the introduced HindIII site (X4C4) encodes an α^4 protein with a single conservative amino acid change from alanine to leucine at amino acid number 969. The α^4 cytoplasmic deletion mutant (X4CO) was created by replacing codons for Q^{975} and Y^{976} (CAA, TAC) with termination codons (TAA, TAG) after the highly conserved GFFKR motif in the cytoplasmic domain of the integrin α^4 cDNA using a PCR mutagenesis procedure (53). Sequencing of the product confirmed that only the intended mutations were introduced into the cDNA. The constructs were cloned into the pFNeo (54, 55) or pSRαNeo expression vector using the SalI and XbaI sites present in the pBS polylinker. The latter vector is a composite of pSRα (56) and pFNeo, prepared by Dr. Hamid Band (Brigham and Women's Hospital, Boston, MA; manuscript in preparation). K562 (erythroleukemia), MIP101 (colon carcinoma), and PMWK (primary melanoma) were chosen as α^4 recipients because they do not normally express VLA-4. K562 cells were transfected via electroporation at 960 μF and 270 mV using a gene pulser (Bio-Rad Laboratories, Cambridge, MA). Constructs were transfected into MIP101 and PMWK cells using the Lipofectin reagent (Bethesda Research Laboratories, Bethesda, MD). After selection with 2.0-mg/ml G418 (Gibco, Grand Island, NY), resistant cells were enriched for α^4 expression using the B-5G10 mAb and immunomagnetic beads (Dynal, Inc., Great Neck, NY). Stably transfected cells were cultured in RPMI 1640 supplemented with 10% FCS L-glutamine, antibiotics, and 1.0 mg/ml G418 (complete media).

Flow Cytometry and Immunoprecipitations. Indirect immunofluorescence was performed as described (3). For immunoprecipitation, lactoperoxidase and ^{125}I were used for cell surface labeling followed by lysis with 1% Triton X-100 in Tris-buffered saline. Immunoprecipitation and SDS-PAGE were carried out as described (57).

Adhesion Assays. Adhesion assays were essentially as described (58). Briefly, cells were labeled by incubation with the fluorescent dye BCECF-AM (Molecular Probes, Inc., Eugene, OR), and then 5×10^4 cells in RPMI with 0.1% BSA (assay media) were added to each well of 96-well microtiter plates (Flow Laboratories, Inc., McLean, VA) that had been coated with protein ligands and blocked with 0.1% BSA. After 20-min incubation at 37°C, unbound cells were removed (two to three washes with assay media). Cells remaining attached to the plate were analyzed using a fluorescence concentration analyzer machine (IDEXX Co., Portland, ME), or Cytofluor 2300 (Millipore Corp., Bedford, MA). Background binding (assessed using BSA-coated wells) was typically <5% of the total, and results are reported as the mean of triplicate determinations ± SD. TS2/16 (1 μg/ml) or PMA (10 ng/ml) was added to indicated samples at the start of the assay. For assays in which blocking Abs were used, cells were incubated with blocking or control Abs for 30 min before the time of plating.

Results

Chimeric α Chains Associate with β_1 and Are Expressed on the Cell Surface. To examine the role of the α chain cytoplasmic domain in control of integrin function, a series of

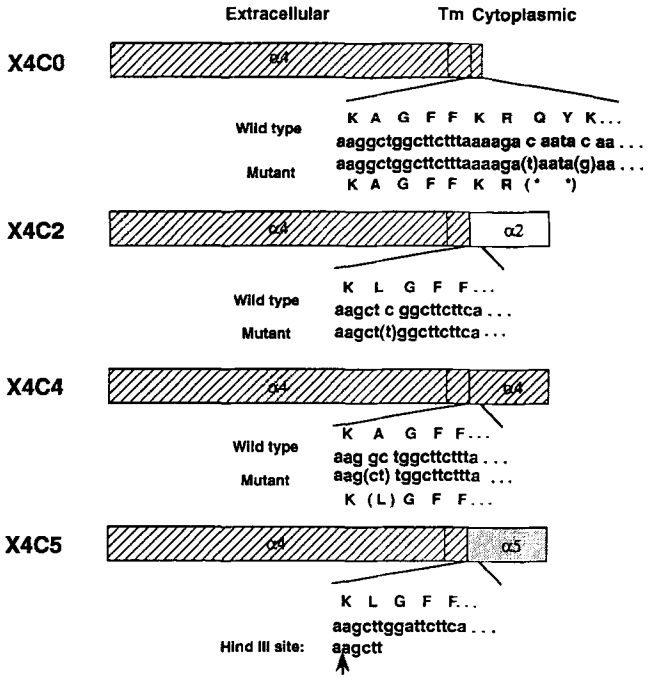

Figure 1. Schematic diagram of truncated and chimeric α chain constructs. The extracellular and transmembrane domains for each construct were derived from the α^4 (*diagonal stripes*) cDNA, the cytoplasmic domains were derived from α^2 (*open*), α^4 (*diagonal stripes*), or α^5 (*stippled*) cDNAs (see Materials and Methods). (*Lower case letters*) Wild-type and mutated nucleotide sequence. Single letter amino acid code (*upper case letters*) is used to denote the wild-type and mutant protein sequence beginning from the lysine immediately inside the putative transmembrane sequence. Mutations in both the nucleotide as well as the protein sequence are in parentheses.

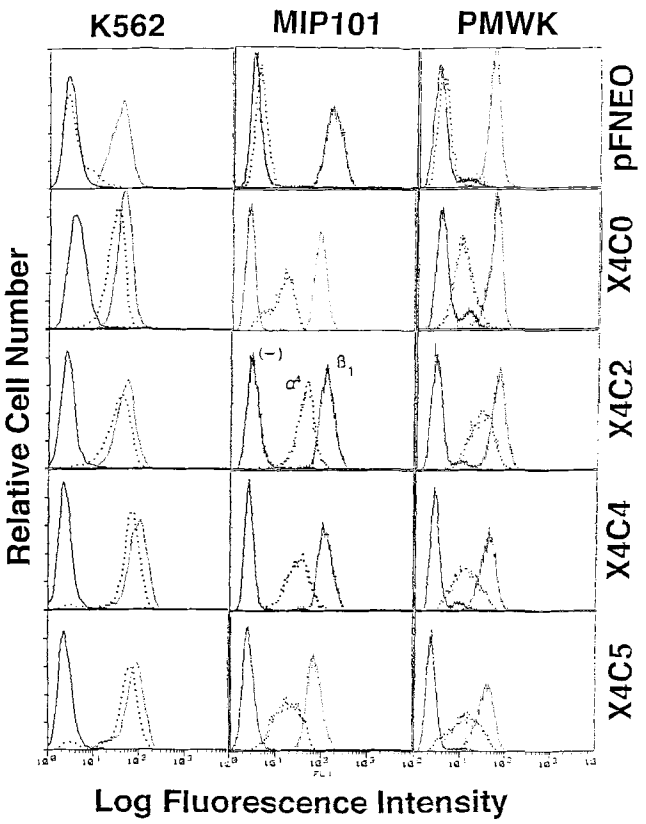

Figure 2. Flow cytometry profiles of cells transfected with chimeric or truncated α^4 constructs. K562 (*column 1*), MIP101 (*column 2*), and PMWK (*column 3*) cell lines transfected with vector alone (*row 1*), or the vector containing the X4C0 (*row 2*), X4C2 (*row 3*), X4C4 (*row 4*), X4C5 (*row 5*) constructs were analyzed for surface expression of α^4 and β_1 by flow cytometry. The J2A2 mAb (*solid line*) was used as a negative control antibody (−), B5G10 (*large dotted line*) was used to determine surface levels of α^4, and A-1A5 (*small dotted line*) was used to determine surface levels of β_1. Fluorescence intensity is shown in logarithmic scale, and 3,000–5,000 cells were measured to yield each histogram.

truncated and chimeric α^4 molecules were created (Fig. 1) and stably expressed at the cell surface of K562, MIP101, and PMWK cells (Fig. 2). Notably, the chimeric molecules (X4C2 and X4C5) were expressed at roughly similar levels as unaltered α^4 (X4C4), whereas α^4 lacking a cytoplasmic domain (X4C0) was expressed at variably lower levels. Expression of β_1 in K562 cells was increased slightly upon transfection of the α chain constructs, although no significant differences in β_1 expression were seen in the MIP101 or PMWK cell lines because of the large number of α chains endogenously expressed.

β_1 can form a stable heterodimer with each of the chimeric α chains, as shown by coprecipitation of the α chimera with β_1-specific mAbs (Fig. 3, lanes *e*, *i*, *m*, and *q*) as well as coprecipitation of β_1 with the α^4-specific mAb B-5G10 (Fig. 3, lanes *f*, *j*, *n*, and *r*). Also coprecipitating with β_1 was α^5, seen as a faint band at ~150 kD (Fig. 3, lanes *a*, *e*, *i*, *m*, and *q*). The presence of this other integrin partly accounts for the level of total β_1 being greater than the level of β_1 associated with α^4. Identity of the chimeric α^4 chains was confirmed using antisera recognizing specific cytoplasmic tails. Antisera raised against a peptide from the cytoplasmic domain of α^2 precipitated chimeric proteins from X4C2 transfected cells (Fig. 3, lane *h*) but not from pFNEO, X4C4, X4C5, or X4C0 transfected cells (Fig. 3, lanes *d*, *l*, *p*, and *t*). Similarly, antisera raised against a peptide from the α^4 cytoplasmic domain precipitated proteins from cells transfected with X4C4 (Fig. 3, lane *k*) but not from pFNEO, X4C2, X4C5, or X4C0 constructs (Fig. 3, lanes *c*, *g*, *o*, and *s*). These results demonstrated that substitution of the cytoplasmic domain of α^4 did not alter heterodimer assembly or expression. Although the X4C0 mutant was expressed in association with β_1, deletion of the α^4 cytoplasmic domain consistently resulted in lower expression (Fig. 2), perhaps because of slightly altered heterodimer assembly or stability. Exchange or deletion of the cytoplasmic domain did not alter the ability of α^4 to be processed into its various structural forms (59). In K562 cells, wild-type and modified α^4 chains were all equally cleaved into $\alpha^{4/80}$ and $\alpha^{4/70}$ fragments (Fig. 3). The $\alpha^{4/70}$ fragment (derived from the COOH-terminal portion of α^4) of X4C0 migrates at a lower apparent molecular weight (Fig. 3, lanes *q* and *r*) than the same fragment of X4C4 (Fig. 3,

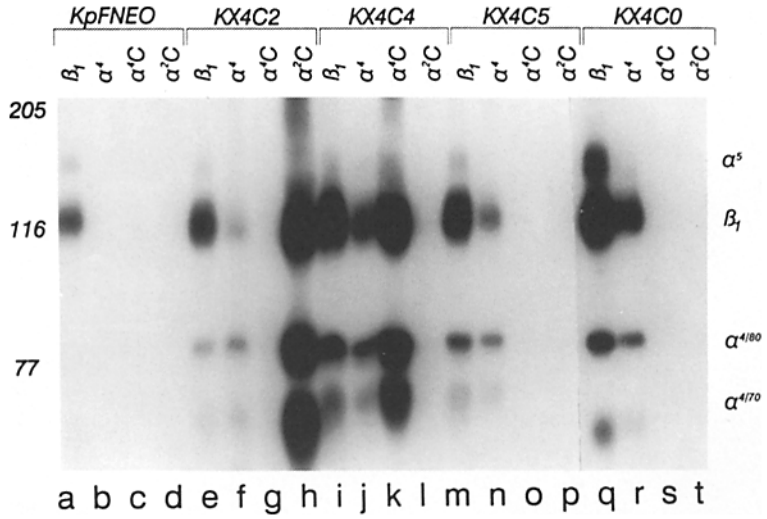

Figure 3. Biochemical characterization of chimeric or truncated α chains in K562. Transfectants KpfNEO (lanes *a–d*), KX4C2 (lanes *e–h*), KX4C4 (lanes *i–l*), KX4C5 (lanes *m–p*), and KX4C0 (lanes *q–t*) were surface labeled with ^{125}I and extracted with 1% Triton X-100 in TBS. Immunoprecipitations were carried out with mAb A-1A5 (β_1) in lanes *a*, *e*, *i*, *m*, and *q*; mAb B-5G10 (α^4) in lanes *b*, *f*, *j*, *n*, and *r*; rabbit antisera to the cytoplasmic domain of α^4 (α^4C) in lanes *c*, *g*, *k*, *o*, and *s*; or rabbit antisera to the cytoplasmic domain of α^2 (α^2C) in lanes *d*, *h*, *l*, *p*, and *t*, and analyzed by SDS-PAGE (7% acrylamide) followed by autoradiography. Molecular weight standards (*left*); labeled protein species (*right*).

lanes *i–k*) consistent with deletion of the COOH-terminal 25 amino acids. In MIP101 and PMWK transfectants, the appearance of the $\alpha^{4/150}$ and $\alpha^{4/180}$ structural forms (60), was not altered by cytoplasmic domain modification (data not shown). The $\alpha^4\beta_7$ complex, examined by flow cytometry using the Act-1 mAb (61), was not detected on K562, MIP101, or PMWK transfectants, consistent with α^4 being entirely associated with β_1.

Variable Constitutive Activity of VLA-4 in Different Cell Types. K562, MIP101, and PMWK transfectants expressing X4C4 were assayed for their basal level of adhesion to sVCAM-1 and to CS1-BSA. Although surface expression of VLA-4 on each of these transfectants was similar, they exhibited widely variable adhesive properties (Fig. 4 *A*). Without stimulation, K562-X4C4 transfectants showed very little binding to either CS1-BSA or sVCAM-1, the MIP101-X4C4 transfectants displayed low level adhesion to CS1-BSA yet moderate adhesion to sVCAM-1, and the PMWK-X4C4 transfectants exhibited substantial adhesion to both ligands. Adhesion of each cell line was differentially amplified after stimulation with the TS2/16 mAb (previously shown to enhance β_1 integrin mediated adhesion [23, 24, 62, 63]). Cell adhesion to sVCAM-1 was enhanced by 9.9, 3.0, and 1.4-fold (K562, MIP101, and PMWK, respectively), and cell adhesion to CS1-BSA was stimulated by 26.8, 6.6, and 1.4-fold (K562, MIP101, and PMWK, respectively) (Fig. 4 *B*). Thus we conclude that the K562 cell line had low constitutive activity but had a high potential for stimulation, the MIP101 cell line had an intermediate level of constitutive activity and moderate potential for stimulation, and the PMWK cell line had high constitutive activity with a low capacity for further stimulation. In several other experiments (data not shown), the magnitude of stimulation within each cell line was variable but the trend was consistent. Thus α^4 derived from a single cDNA expressed in different cellular environments resulted in $\alpha^4\beta_1$ with different levels of constitutive activity. As previously demonstrated, endogenous $\alpha^4\beta_1$ can exist in a range of functional states depending on the cellular environment, and the degree of stimulation is inversely proportional to the basal level of activity (24). This is analogous with results obtained with α^2-transfected cells demonstrating differential ability to bind to collagen and laminin (23).

Because PMWK cells showed substantially more consitutive adhesion to VLA-4 ligands than did K562 cells, it was considered that these cell types could differ in their general overall adhesivity, independent of VLA-4 function. However, this was not the case because adhesion of PMWK cells was not greater than K562 cells on poly-L-lysine (Fig. 5 *B*), and both showed similarly low adhesion to BSA (Fig. 5 *A*). Fur-

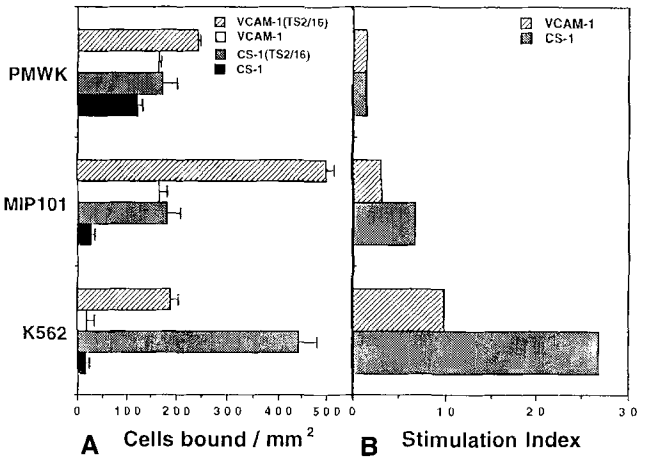

Figure 4. Adhesion of X4C4 transfected cell lines to CS1-BSA and sVCAM-1 is variable and affected by stimulation to different degrees. (*Left*) Adhesion of X4C4 transfectants to CS1-BSA and sVCAM-1, both at a coating concentration of 2.5 μg/ml, was assayed in the absence or presence of mAb TS2/16 (1 μg/ml) as described in Materials and Methods. CS1-BSA (*filled*), CS1-BSA plus TS2/16 (*gray crosshatch*), sVCAM-1 (*unfilled*), and sVCAM-1 plus TS2/16 (*diagonal stripes*). (*Right*) Stimulation index was calculated as (adhesion in the presence of TS2/16)/(adhesion in the absence of TS2/16), and plotted for both CS1-BSA (*gray crosshatch*) and sVCAM-1 (*diagonal stripes*).

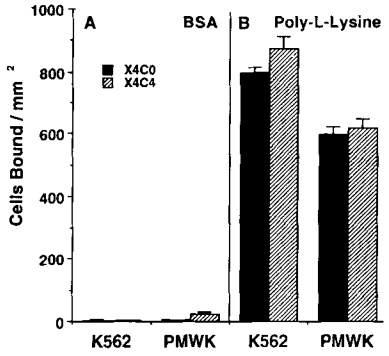

Figure 5. Comparison of $\alpha^4\beta_1$-transfected K562 and PMWK cell adhesion to control surfaces. Adhesion of K562 and PMWK transfectants was determined as described in Materials and Methods, except that background binding was not subtracted. K562 and PMWK cell lines transfected with X4C0 (*filled*) or X4C4 (*diagonal stripes*) were examined for their adhesion to plastic surfaces coated with 0.1% heat-denatured BSA (*A*) or 5 μg/ml poly-L-lysine (*B*).

thermore, in these control experiments, there was essentially no difference between X4C0 and X4C4 contructs, despite their marked differences in mediating adhesion to VLA-4 ligands (see Figs. 6–8).

Functional Activity of $\alpha^4\beta_1$: Dependence on the identity of the Cytoplasmic Domain. It has been suggested that deletion or substitution of the α chain cytoplasmic domain can release an integrin from an inactive conformation and allow it to display adhesive function (42). To determine how the identity of the α chain cytoplasmic tail affected the level of constitutive function of the $\alpha^4\beta_1$ integrin heterodimer, cells transfected with α^4 chimeras were tested for adhesion to sVCAM-1 and CS1-BSA in a standard adhesion assay (58). Dose-dependent adhesion of all transfectants to sVCAM-1, CS1-BSA, or to a 40-kD fragment of FN containing the CS1 region (FN-40) was VLA-4 specific, as evidenced by nearly complete inhibition by the α^4-specific antibody HP1/2 (data not shown). Additionally, mock transfectants displayed only minimal binding to either ligand.

Adhesion of K562 transfectants was uniformly low at the lower doses of FN-40 (0–20 μg/ml) and sVCAM-1 (0–2.5 μg/ml), regardless of the cytoplasmic domain (Fig. 6, *A* and *B*). Thus replacement or removal of the cytoplasmic tail of α^4 did not lead to increased constitutive activity of VLA-4. At higher doses of FN-40 (>20 μg/ml, data not shown) or sVCAM-1 (>2.5 μg/ml) a moderate level of adhesion was observed, but still there was no consistent difference between

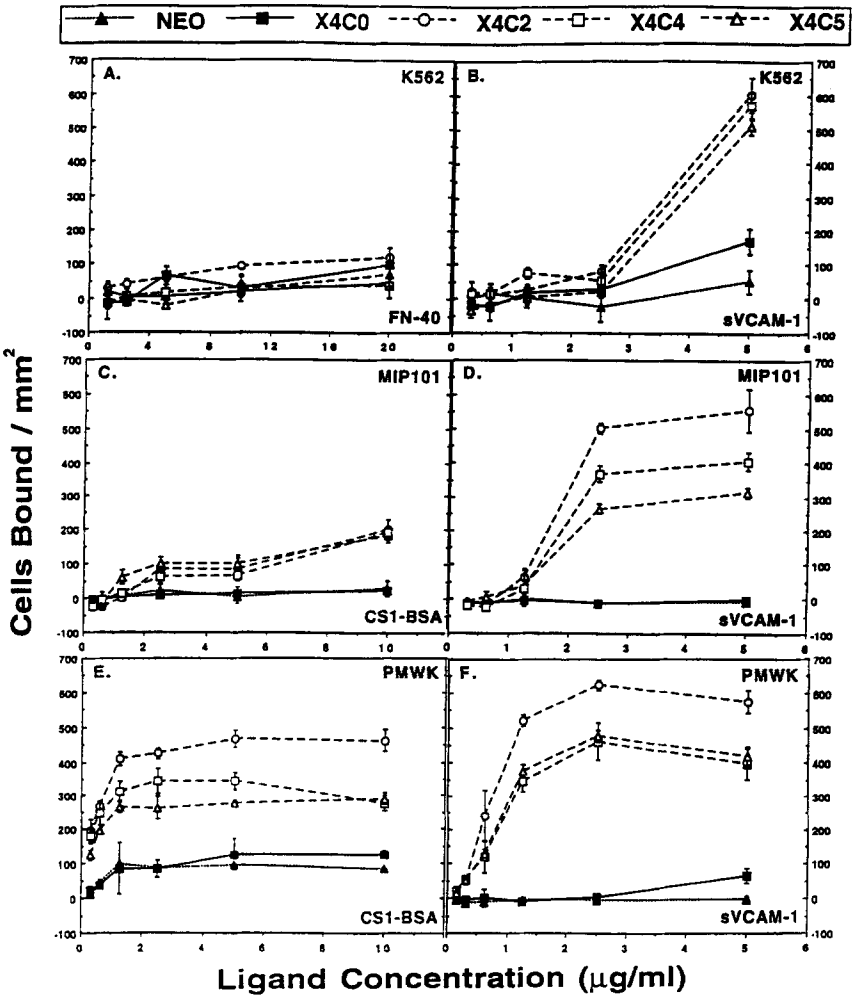

Figure 6. Dose-dependent adhesion of transfectants to ligand. Adhesion of transfectants to a range of ligand concentrations was determined as described in Materials and Methods. Adhesion of K562 transfectants to FN-40 (*A*) and sVCAM-1 (*B*), MIP101 transfectants to CS1-BSA (*C*) and sVCAM-1 (*D*), and PMWK transfectants to CS1-BSA (*E*) and sVCAM-1 (*F*) are results of representative experiments. Adhesion of each individual transfectant demonstrated variability, however results presented in a single panel were derived from a single assay.

cells transfected with the different chimeric α^4 constructs. However, at the higher ligand doses (sVCAM-1 5 μg/ml) a lower level of adhesion was mediated by X4C0 than by X4C4, X4C2, or X4C5 in K562 cells. It appears unlikely that the slightly lower level of surface expression of the X4C0 construct could account for the greatly diminished adhesion at the high ligand level. When tested for adhesion to CS1-BSA (data not shown), the panel of K562 transfectants yielded results very similar to those obtained using FN-40.

For MIP101 and PMWK transfectants, the functions of the X4C4, X4C2, and X4C5 constructs were again not markedly different from one another (Fig. 6, *C–F*). Multiple other experiments (data not shown) confirmed that each of these constructs supported adhesion essentially to a similar extent, with minor variations perhaps due to slightly different expression levels.

In marked contrast to the chimeric constructs, the truncated α^4 (X4C0) supported essentially no adhesion above that seen with mock-transfected MIP101 and PMWK cells (Fig. 6, *C–F*). Although cell surface expression of X4C0 was at a slightly lower level in those cells (Fig. 2), the nearly complete absence of adhesion appeared to be greater than could be explained by lower expression.

To test whether variation in expression levels caused the differences seen in levels of adhesion, cell surface expression of chimeric α^4 chains and α^4-mediated adhesion were examined in parallel, and then flow cytometry results were used to normalize adhesion results. Upon dividing cell adhesion (cells bound/mm^2) by α^4 expression (mean fluorescence intensity units [MFI]), we obtained a normalized result that clearly showed that adhesion mediated by $\alpha^4\beta_1$ was not altered by exchange of the cytoplasmic tail (Fig. 7). As indicated, whether constitutive activity was low (K562), intermediate (MIP101), or high (PMWK), similar normalized results were obtained using X4C2, X4C4, or X4C5.

However, even after normalizing for expression differences, the X4C0 construct yielded substantially lower adhesive activity in both MIP101 and PMWK cells. In K562 cells, the adhesive activity of VLA-4 was already at such a low level, that truncation of the cytoplasmic domain could not result in a further decrease in activity.

To verify that the normalization of adhesion to cell surface expression was valid over a range of expression levels, we examined a series of six MIP101-X4C4 clones and lines, and compared their adhesion to either CS1-BSA or sVCAM-1, with their surface expression of α^4. It was apparent that over a wide range of expression levels, adhesion to sVCAM-1 (Fig. 8 *A*) and to CS1-BSA (Fig. 8 *B*) correlated well with cell

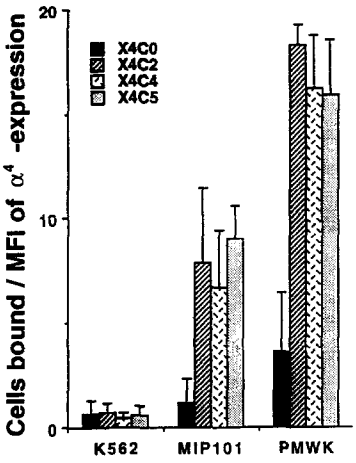

Figure 7. Normalization of adhesion for cell surface expression of chimeric and truncated α^4 chains in transfected cell lines. Adhesion assays and flow cytometry analyses were as described in Materials and Methods. These assays were done concurrently for greater accuracy in normalization. For K562, the mean of three experiments for CS1-BSA (5.0 μg/ml) is presented, whereas for MIP101 and PMWK the mean of four experiments (two for sVCAM-1 at 2.5 μg/ml and two for CS1-BSA at 2.5 μg/ml) are presented. Adhesion was measured as cells bound/mm^2 and α^4 expression was calculated by subtraction of the mean fluorescence intensity of a negative control mAb (J2A2) from the mean fluorescence intensity of an α^4 mAb (B-5G10).

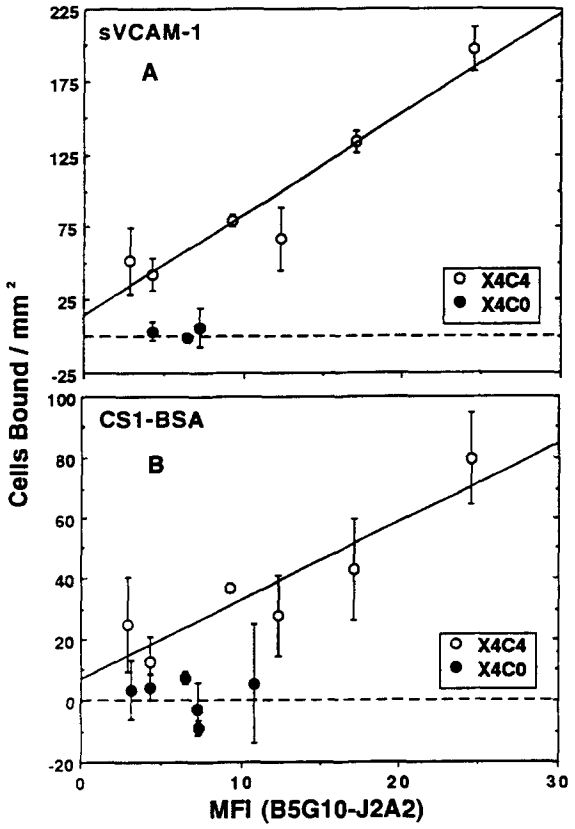

Figure 8. Normalization of cell adhesion for expression of X4C4 in MIP101 cell clones with different levels of α^4 expression. Adhesion to sVCAM-1 (5.0 μg/ml) and CS1-BSA (5.0 μg/ml) and cell surface expression were determined concurrently as described in Materials and Methods. In each graph cell adhesion (*sVCAM-1*, *top* and *CS1-BSA*, *bottom*) is plotted on the ordinate, whereas the α^4 specific mean fluorescence intensity (MFI^{B5G10}-MFI^{J2A2}) is plotted on the abscissa. The data from six X4C4 transfected MIP101 clones and lines result in a linear correlation defined by y = 13.770 + 6.9345X, R^2 = 0.913 for sVCAM-1, and y = 7.0733 + 2.5843X, R^2 = 0.831 for CS1-BSA. Adhesion of several MIP101-X4C0 cell lines is also plotted (●).

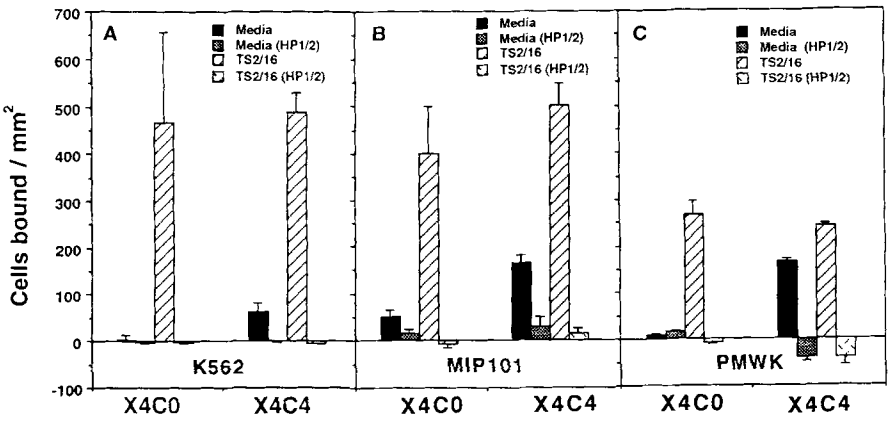

Figure 9. Adhesion mediated by X4C4 and X4C0 after stimulation with mAb TS2/16. Adhesion of X4C0$^+$ and X4C4$^+$ cell lines was examined in assay media alone (*filled*), assay media in the presence of 1 μg/ml α^4-blocking mAb HP1/2 (*gray crosshatch*), assay media including 1 μg/ml TS2/16 (*diagonal stripes*), or assay media including 1 μg/ml TS2/16 and 1 μg/ml HP1/2 (*tire tread*). (*A*) K562 transfectant adhesion to sVCAM-1 (10 μg/ml). (*B*) MIP101 transfectant adhesion to sVCAM-1 (5.0 μg/ml). (*C*) PMWK transfectant adhesion to sVCAM-1 (2.5 μg/ml).

surface expression of α^4. In addition, MIP101-X4C0 cells showed notably lower levels of adhesion to both CS1-BSA and to sVCAM-1 than would be predicted based on α^4 expression (Fig. 8, *A* and *B*), providing further evidence that the loss of cell adhesion was due to the deletion of the α^4 cytoplasmic domain, and not to low surface expression of X4C0.

Ab Stimulation of α Chain Cytoplasmic Deletion Mutants. To determine whether α^4 cytoplasmic deletion mutants had an inherent defect that prevented them from binding ligand under any circumstance, we tested their ability to adhere to sVCAM-1 after stimulation by the anti-β_1 mAb TS2/16 (23, 24, 62). Whereas there was minimal unstimulated activity for the X4C0 construct in K562 (Fig. 9 *A*), MIP101 (Fig. 9 *B*), or PMWK cells (Fig. 9 *C*), TS2/16 stimulation caused a greatly elevated level of adhesion in each case. Also, the low (Fig. 9 *A*) or intermediate (Fig. 9 *B*) activity or the X4C4 construct was greatly elevated by TS2/16 stimulation. In PMWK cells (Fig. 9 *C*) X4C4 was already active, and therefore only slightly stimulated. For each cell line, adhesion (unstimulated or stimulated) was entirely blocked by HP1/2, indicating that it was VLA-4 mediated. When adhesion to CS1-BSA was examined, similar results were obtained (data not shown). The nearly identical activities of X4C0 and X4C4 in the presence of TS2/16 emphasizes again (as also seen in Fig. 5) that there is no difference in the overall adhesive properties of the transfected cells. Also, this result emphasizes that when VLA-4 is fully stimulated, there is no difference in the specific adhesive contributions of X4C4 and X4C0.

Phorbol Ester Stimulation of Adhesion: Affect of α Chain Cytoplasmic Domain. As it is known that cell stimulation with a variety of agonists results in enhanced β_1 integrin-mediated adhesion (22, 64, 65), we sought to ascertain the role of the α chain cytoplasmic domain in agonist-stimulated adhesion. Adhesion of X4C0 and X4C4 transfectants to both CS1-BSA and sVCAM-1 was assayed in the presence of PMA (a model cellular agonist) or TS2/16, and results are shown in Fig. 10. Predictably, each of the cell lines transfected with X4C4 exhibited increased cell adhesion to either CS1-BSA or sVCAM-1 in response to either PMA and TS2/16. Notably, there was variable enhancement of cell adhesion in response to PMA, with the K562-X4C4 transfectant showing a high degree of stimulation, MIP-X4C4 exhibiting an intermediate increase in adhesion, and PMWK-X4C4 displaying only slightly elevated adhesion. In this regard, stimulation with PMA resembles that seen with TS2/16 in Fig. 4. In contrast to the X4C4 transfectants, none of the X4C0-bearing cell lines exhibited enhanced adhesion in response to PMA, although they all demonstrated markedly increased adhesion in response to TS2/16. These data suggest that the cytoplasmic domain of the α chain is critically involved in the stimulation of adhesion by a cellular agonist such as PMA.

To further examine the role of α chain cytoplasmic domains in agonist-stimulated adhesion, cell lines expressing chimeric α chains were assayed for sensitivity to PMA and to TS2/16. To help control variability in absolute levels of adhesion, results were expressed in three ratios: (*a*) adhesion in the presence of PMA divided by adhesion in normal media; (*b*) adhesion in the presence of TS2/16 over adhesion in the presence of assay media; and (*c*) adhesion in the presence of PMA over adhesion in the presence of TS2/16 (maximal adhesion). These three ratios were determined for each separate experiment, and the mean and SD of ratio values from multiple experiments are presented in Table 1. In all three cell lines, the PMA/media and PMA/TS2/16 ratios were notably diminished for X4C0- compared to X4C4-transfected cells (values, *). Thus truncation of the α cytoplasmic domain clearly resulted in loss of response to PMA. Also, the PMA/media and PMA/TS2/16 ratios from X4C2, X4C5, and X4C4 transfectants were not significantly different in any of the three cell types. Therefore substitution of the α^2 or α^5 tail did not alter the affect of either PMA or TS2/16 on cell adhesion. Together, these results imply that the α cytoplasmic domain is critically involved in agonist-stimulated adhesion, but the identity of the domain is not crucial. The PMA/media ratio for adhesion to sVCAM-1 did not appear to be decreased for the X4C0 transfected MIP101 or PMWK cell lines. However this may be due to the very low levels of initial binding seen by these cells. When the PMA/TS2/16 ratios for these cell lines are compared, it is clear that PMA stimulation did not enhance adhesion to the levels seen with the X4C4 transfected cell lines.

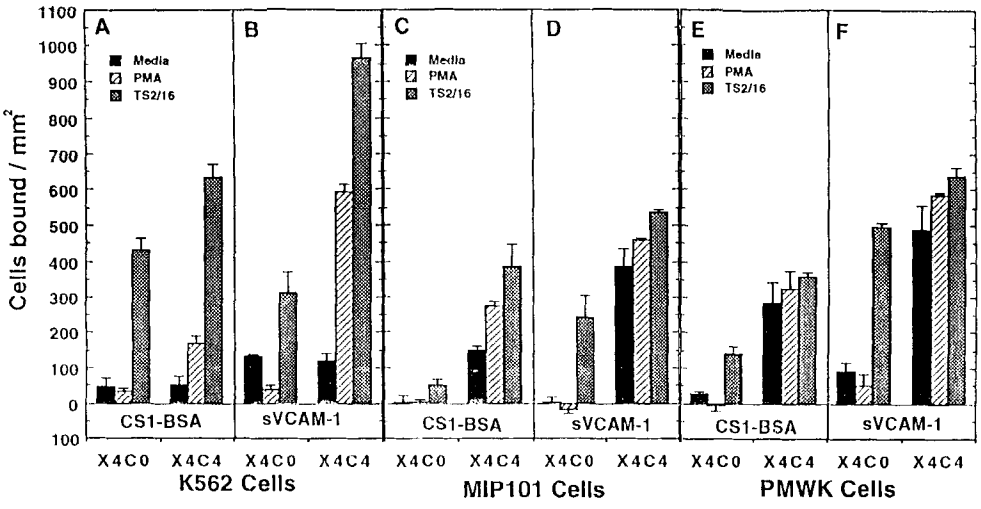

Figure 10. Agonist-stimulated adhesion of X4C0 and X4C4 transfected cell lines. K562 (*A* and *B*), MIP101 (*C* and *D*), and PMWK (*E* and *F*) transfectants were assayed for adhesion to CS1-BSA or VCAM-1 in assay media (*filled*), in the presence of 10 ng/ml PMA (*diagonal stripes*), or in the presence of 1 μg/ml TS2/16 (*gray crosshatch*), as described in Materials and Methods. Coating concentrations were as follows: CS1-BSA at 10 μg/ml (*A*), 5 μg/ml (*C*), 2.5 μg/ml (*E*); and sVCAM-1 at 5 μg/ml (*B*), 2.5 μg/ml (*D* and *F*).

Discussion

This study clearly demonstrates a role of the α chain cytoplasmic domain in regulation of constitutive avidity and agonist-stimulated activity of a prototype β_1 integrin heterodimer, VLA-4. First, we established that the same α^4 cDNA expressed in three different cell types yielded three distinct levels of activity. Second, we found that exchange of the α^4 cytoplasmic tail with that of α^2 or α^5 had no effect on the level of constitutive activity of the $\alpha^4\beta_1$ integrin

Table 1. *PMA and TS2/16 Stimulation of Transfected Cell Lines*

Cell line	CS1-BSA				sVCAM-1			
	n	PMA Media	TS2/16 Media	PMA TS2/16	*n*	PMA Media	TS2/16 Media	PMA TS2/16
K562								
X4C0	8	*0.52 ± 0.40	**7.60 ± 6.50**	***0.11 ± 0.14**	6	***0.72 ± 0.69**	3.74 ± 2.83	*0.31 ± 0.33
X4C2	8	9.78 ± 11.56	15.58 ± 15.37	0.61 ± 0.37	6	3.71 ± 2.71	4.60 ± 3.63	0.88 ± 0.16
X4C4	8	13.25 ± 14.14	30.69 ± 22.27	0.45 ± 0.24	6	2.99 ± 1.89	5.43 ± 3.47	0.67 ± 0.31
X4C5	8	4.27 ± 2.85	22.68 ± 23.92	0.26 ± 0.09	6	2.30 ± 2.48	7.14 ± 11.94	0.56 ± 0.30
MIP101								
X4C0	3	***0.30 ± 0.42**	5.78 ± 5.94	***0.02 ± 0.04**	7	1.29 ± 1.00	15.83 ± 14.06	*0.29 ± 0.47
X4C2	3	1.70 ± 0.19	3.26 ± 1.16	0.59 ± 0.31	3	1.05 ± 0.29	1.87 ± 0.67	0.63 ± 0.28
X4C4	3	1.74 ± 0.23	4.66 ± 4.14	0.56 ± 0.31	3	1.59 ± 0.56	3.06 ± 2.40	0.68 ± 0.27
X4C5	3	1.48 ± 0.37	3.63 ± 2.65	0.52 ± 0.27	3	0.96 ± 0.12	1.53 ± 0.57	0.67 ± 0.19
PMWK								
X4C0	7	***0.51 ± 0.87**	3.42 ± 3.14	***0.16 ± 0.23**	6	2.47 ± 1.78	**22.16 ± 18.96**	***0.11 ± 0.12**
X4C2	7	2.24 ± 1.72	2.29 ± 1.19	0.97 ± 0.37	6	1.13 ± 0.06	1.19 ± 0.10	**0.95 ± 0.05**
X4C4	7	1.48 ± 0.41	1.83 ± 0.87	0.87 ± 0.16	6	1.35 ± 0.32	1.53 ± 0.39	0.89 ± 0.05
X4C5	7	1.67 ± 0.51	1.72 ± 0.52	0.99 ± 0.17	6	1.28 ± 0.48	1.37 ± 0.57	0.95 ± 0.11

Adhesion assays were as described in Materials and Methods. The PMA/media ratio was determined by dividing the adhesion (cells bound/mm²) in the presence of PMA by the adhesion in the presence of assay media alone. The (TS2/16)/(media) ratio was calculated by dividing adhesion in the presence of TS2/16 by adhesion in assay media, and the (PMA)/(TS2/16) ratio was computed by dividing the adhesion in the presence of TS2/16 by the adhesion in the presence of PMA. Ratios from multiple experiments were averaged and presented as the mean ± SD. Values in bold-face type demonstrate significant differences ($p < 0.01$, Student's *t* test) when compared with values for X4C4 transfectants.
* Discussed in text.

whether the activity was low (K562 cells), intermediate (MIP101 cells), or high (PMWK cells). Third, deletion of the α^4 cytoplasmic domain led to greatly diminished adhesion relative to that observed using X4C4 or the chimeras (X4C2, X4C5). This diminished activity (X4C0 mutant) was especially obvious in both the MIP101 and PMWK cell lines, and was not merely a result of lower expression. Fourth, cell stimulation with a model cellular agonist (PMA) was able to upregulate integrin-mediated adhesion regardless of which cytoplasmic tail (α^2, α^4, or α^5) was used, whereas truncation of the cytoplasmic tail eliminated the capability of PMA to stimulate cell adhesion. A fifth major finding in this work is that the anti-β_1 mAb TS2/16 could stimulate adhesive function of all three cells bearing the X4C0 construct. Thus, whereas the absence of a cytoplasmic domain may disable the "natural" mechanism for generating a functionally active integrin, this mechanism can be bypassed by direct action of the TS2/16 mAb on the integrin. Notably, the natural constraints on integrin function in K562 cells expressing X4C4 were similarly bypassed through the addition of TS2/16.

Although results presented here strongly suggest that several interchangeable α chain cytoplasmic domains play a positive role in regulating cell adhesion, studies involving the α cytoplasmic domains of other integrins have yielded confusing and dissimilar results. The cytoplasmic domain of α^{IIb} appeared to play a negative regulatory role because deletion or exchange of the α^{IIb} tail with that of α^5 caused the affinity of the $\alpha^{IIb}\beta_3$ complex for its ligand to increase (42). In a separate study, the cytoplasmic tail of α^L was truncated with no apparent alteration of the adhesive properties of the $\alpha^L\beta_2$ heterodimer (40, 41), suggesting that the α chain cytoplasmic domain has no effect on the level of constitutive avidity of the β_2 integrin. There are several possible reasons why our results are contrary to those found with the α^{IIb} and α^L integrin subunits. First, the cells transfected in those studies were different than those in our study, and it is becoming increasingly evident that integrins behave differently in distinct cellular environments (23, 24, 66, 67). Second, in the α^{IIb} study, the α^{IIb} cytoplasmic tail was truncated before the highly conserved GFFKR sequence, whereas we have deleted α^4 after that sequence. Third, the cytoplasmic tail of α^5 was placed onto the α^{IIb} extracellular domain, forcing the unnatural association of the α^5 and β_3 cytoplasmic domains, which could be responsible for the increase in ligand binding. Fourth, it is possible that integrin α subunit cytoplasmic domains are regulated in distinct ways depending on whether they are associated with β_1, β_2, or β_3. This could explain why in three studies of α cytoplasmic domains, involving three different β chain partners, three distinct answers regarding regulation of constitutive activity were obtained.

We do not know the mechanism whereby integrins are maintained in different states of constitutive activity in different cell types. Also, it is not clear how PMA stimulation causes a rapid upregulation in integrin function. Nonetheless, we propose that the regulation of constitutive activity and agonist-stimulated activity could have similar mechanistic elements because (*a*) neither is influenced by exchange of α^4 with α^2 or α^5 cytoplasmic tails; (*b*) both are suppressed upon α^4 cytoplasmic domain deletion; and (*c*) in both cases, the negative effects of cytoplasmic domain deletion can be overcome by the addition of the anti-β_1 mAb TS2/16. Thus, we hypothesize that PMA, on a rapid scale, introduces a change into cells (such as K562) that is already constitutively present in cells such as PMWK.

It is quite clear that VLA-4–mediated cell adhesion varies in different cell types, is suppressed upon cytoplasmic tail deletion, and is upregulated upon PMA treatment. However, it is not clear whether any of these changes are due to variations in post-ligand binding events necessary for cell adhesion, or involve direct alterations in integrin-ligand binding affinity.

In one model, cytoplasmic tail deletions or PMA treatment could alter integrin interaction with the cytoskeleton or other key proteins, and thus influence the overall cellular adhesiveness without changing ligand binding affinity. Consistent with this, agents that disrupt the cytoskeleton are known to cause diminished cell adhesion (62, 68, 69). Also, PMA stimulation of LFA-1 ($\alpha^L\beta_2$) function correlated with coclustering of LFA-1 with the cytoskeletal protein talin (70, 71), and PMA-treated macrophages showed increased cytoskeletal anchoring (38). Furthermore, in one study, PMA stimulated increased cell adhesion to FN without altering the apparent affinity for soluble FN binding (64).

On the other hand, α cytoplasmic domain–dependent changes in cell adhesion may be due to alterations of ligand-binding affinity. For example, others have demonstrated that agonist-stimulated cell adhesion correlated with integrin conformational changes (72, 73), and cytoplasmic tail mutations resulted in altered ligand-binding constants (42). Although it bypasses normal cellular regulation mechanisms, the anti-β_1 Ab TS2/16 and other similar Abs can stimulate cell adhesion, most likely by causing an increase in integrin affinity (23, 74, 75), thus providing another precedent for upregulated adhesion through modulation of integrin affinity.

To determine whether regulation through α chain cytoplasmic domains does or does not involve alteration in integrin-ligand binding affinity, direct affinity measurement will be required. However, multiple attempts at direct ligand binding of CS1 peptide to VLA-4 have thus far proven unsuccessful because of high levels of nonspecific binding.

Regardless of whether α chain cytoplasmic domains influence integrin affinity, it is clear that their role is not highly specific since three different sequences (α^2, α^4, and α^5) were interchangeable. Whereas the details of the roles of α chain cytoplasmic domains remain to be elucidated, we suggest that α chain–specific association with other proteins, or specific phosphorylation events are unlikely, given the extent of sequence divergence. Most likely, the role of these α chains in regulating cell adhesion is closely connected with the role of the β_1 cytoplasmic domain. Previous work has indicated that the cytoplasmic domains of β_1 (76) and β_2 (40, 41) are also very important for regulating integrin contributions to

normal and agonist-stimulated cell adhesion. Notably, although the specific sequences of three different α chain cytoplasmic domains are interchangeable with respect to integrin-mediated cell adhesion, in other functional contexts, involving cell migration and collagen gel contraction, the α^4 cytoplasmic domain appeared to be quite distinct from those of α^2 and α^5 (52).

In conclusion, our data establishes that the α chain cytoplasmic domain plays a critical positive role in determining the level of adhesive function of VLA-4 and perhaps other β_1 integrins. Although its exact identity is not critical, the presence of an α chain cytoplasmic domain is essential for the integrin to manifest the full activity permitted by the cellular environment in which it is expressed. We postulate that the α chain cytoplasmic domain is involved in "inside-out" signaling, such that cellular factors acting at least partly through the α chain cytoplasmic domain are altering the external conformation of the $\alpha^4\beta_1$ heterodimer and therefore controlling the level of both constitutive and agonist stimulated integrin function.

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