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Influence of $\beta_1$ Integrin Intracytoplasmic Domains in the Regulation of VLA-4-Mediated Adhesion of Human T Cells to VCAM-1 under Flow Conditions

Maria Alessandra Rosenthal-Allieri, Michel Ticchioni, Jean Philippe Breittmayer, Yoji Shimizu, and Alain Bernard

The VLA-4 integrin supports static cell-cell, cell-matrix adhesion, and dynamic interactions with VCAM-1. Although functions for well-conserved $\beta_1$ integrin cytoplasmic domains in regulating static cell adhesion has been established, the molecular basis for $\beta_1$ integrin-dependent arrest on VCAM-1 under flow conditions remains poorly understood. We have transfected the $\beta_1$ integrin-deficient A1 Jurkat T cell line with $\beta_1$ cDNA constructs with deletions of the NPXY motifs and specific mutations of tyrosine residues. Deletion of either NPXY motif impaired static adhesion induced by CD2 or CD47 triggering or direct $\beta_1$ integrin stimulation. In contrast, PMA-induced adhesion to VCAM-1 was unaffected by deletion of the NPIY motif and only slightly impaired by deletion of NPKY. Moreover, deletion of the NPIY motif resulted in enhanced rolling and reduced arrest on VCAM-1 under shear flow conditions. In contrast, deletion of the NPKY motif did not alter arrest under flow. Although tyrosine to phenylalanine substitutions within two NPXY motifs did not alter static adhesion to VCAM-1, these mutations enhanced arrest on VCAM-1 under flow conditions. Furthermore, although deletion of the C-terminal 5 AA of the $\beta_1$ cytoplasmic domain dramatically impaired activation-dependent static adhesion, it did not impair arrest on VCAM-1 under flow conditions. Thus, our results demonstrate distinct structural requirements for VLA-4 function under static and shear flow conditions. This may be relevant for VLA-4 activity regulation in different anatomic compartments, such as when circulating cells arrest on inflamed endothelium under shear flow and when resident cells in bone marrow interact with VCAM-1-positive stromal cells.

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VLA-4 is a transmembrane $\alpha_\beta_1$ heterodimeric receptor that mediates cell-cell and cell-matrix interactions (17, 18). It is widely expressed on mononuclear leukocytes and plays a major role in several cellular functions, including cell spreading and migration and recruitment of leukocyte subsets into inflammatory sites. The VLA-4 ligand VCAM-1 is expressed on vascular endothelium at sites of inflammation and on other selected sites, such as bone marrow stroma, where interaction with VLA-4 mediates stem cell attachment and mobilization (19–24). VLA-4 mediates static adhesion in the bone marrow hemopoietic compartment by interacting with VCAM-1, which is constitutively expressed on bone marrow stromal cells (25). Moreover, interaction between leukemic cell VLA-4 and stromal fibronectin is crucial for minimal residual disease and acute myeloid leukemia progression (26). Whereas most leukocyte integrins support stationary cell-cell or cell-matrix adhesion, VLA-4 can also mediate versatile dynamic interactions with VCAM-1 under shear flow (12, 14). The interaction of VLA-4 integrin with VCAM-1 can support all of the adhesive steps required for the arrest of lymphocytes on inflamed vascular endothelium (12, 14, 27). Integrins sharing the $\alpha_\beta_4$-chain, such as $\alpha_\beta_1$ and $\alpha_\beta_7$, retain basal recognition of ligand at adhesive contacts under shear stress and can facilitate the spontaneous firm arrest of leukocytes on VCAM-1-expressing endothelium, bypassing the requirement for chemokine stimulation (28–30). Thus, VLA-4 mediates cell adhesion in distinct environmental conditions.

The molecular basis for support of $\beta_1$ integrin-dependent rolling and spontaneous arrest on VCAM-1 under flow conditions are poorly understood. In the current study, we used the $\beta_1$-negative Jurkat T cell line A1 (31) to investigate the role of specific intracytoplasmic motifs of the $\beta_1$ subunit of the VLA-4 molecule to
identify the specific domains critical for both static adhesion and dynamic adhesion under conditions of shear flow. We show that specific intracytoplasmic β1 integrin sequences, including two highly conserved motifs (NPXY and NPVKY) and the carboxyl-terminal end of the β1 tail are differentially involved in the regulation of β1 integrin adhesion under dynamic or static conditions. These differences may reflect different roles played by VLA-4, such as in inflammation or in hematopoietic progenitor maturation and mobilization.

Materials and Methods

Reagents and Abs

The CD29 (K20) and CD2 (D66 and X11) mAbs were purchased in our laboratory and were described elsewhere (32–35). α1 and α2 mAbs were purchased from Immunotech; CD47 (B6H12) was produced in our laboratory from the American Type Culture Collection cells (American Type Culture Collection). TS2/16, which stimulates direct β1 integrin activation, was a kind gift from Dr. Sanchez Madrid (Hospital de la Princesa, Madrid, Spain). PMA was purchased from Sigma-Aldrich; VCAM-1, fibronectin (FN),1 and TNF-α were obtained from R&D Systems.

Cells

The Jurkat T cell line JE6.1 (American Type Culture Collection) and the β1 integrin-deficient Jurkat T cell line A1 (31) were both cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 5% FCS (Invitrogen Life Technologies), 50 μg/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM pyruvate (Invitrogen Life Technologies). The A1 cell line lacks expression of the β1 integrin subunit and is unable to adhere to β1 integrin ligands, including VCAM-1 and FN (31). The transformed human umbilical endothelial cell line EA.hy926 was kindly provided by Dr. Cora Jean Edgell (University of North Carolina, Chapel Hill, NC) and cultured in DMEM (Invitrogen Life Technologies) supplemented with 20% FBS and 0.1% BSA for 1 h at room temperature. Cells were labeled with green fluorescent fluorescein diacetate and orange fluorescent tetramethylrhodamine were used for control A1-β1 and A1-β1NPXY, respectively. Cell adhesion was evaluated without stimulation and after a 20-min incubation at 37°C with various stimuli at optimal concentration, namely, PMA (10 ng/ml) and TS2/16 stimulating CD29 mAb at 10 μg/ml as indicated for static adhesion. Cells were mixed in equal amounts at 1 × 106 cells/ml final concentration and adhered to a dish in the dark, then washed twice before use. For some experiments, cells were injected through the flow chamber on TNF-α-activated EA cells adherent to Lab-Tek chamber slides. All flow experiments were performed at 37°C. Temperature was maintained by warming the microscope plate (Warming Plate; Miniblot). Each flow experiment has been done at least in triplicate. In rolling experiments, lymphocyte interactions with coated sVCAM-1 in different fields were video recorded for 1 min using the Axiovid camera. Rolling velocity was measured by determining the number of frames (each 1/30th of 1 s) it took 30 cells to cross a 350-μm length field.

Detachment assay

A1-β1 control cells and A1-β1NPXY or A1-β1NPKY cells were stained with fluorescence dyes. Green fluorescent fluorescein diacetate and orange fluorescence tetramethylrhodamine were used for control A1-β1 and A1-β1NPXY, respectively. Cell adhesion was evaluated without stimulation and after a 20-min incubation at 37°C with various stimuli at optimal concentration, namely, PMA (10 ng/ml) and TS2/16 stimulating CD29 mAb at 10 μg/ml as indicated for static adhesion. Cells were mixed in equal amounts at 1 × 106 cells/ml final concentration and adhered to a dish in the dark, then washed twice before use. The amount of cells resting adherent on 10 different fields at each shear stress conditions was counted. Comparisons between the A1-β1 control cells and the various transfectants were performed using a variance analysis test, with p ≤ 0.05 as the statistically significant value.

Fluorescent dyes

For flow experiments, cells were stained with fluorescent dyes purchased from Molecular Probes. Green fluorescent fluorescein diacetate (Cell-Tracker Green CMFDA) is a green tracer with excitation at 522 nm and emission at 533 nm; orange fluorescent tetramethylrhodamine (CellTracker Orange CMTMR) is a red tracer with excitation at 541 nm and emission at 574 nm. Transfected cells were labeled in the culture medium at a concentration of 5 × 106 cells/ml with the different probes (0.5–2 μg/ml) for 30 min at 37°C in the dark, then washed twice before use.

Statistical analysis

Results from static adhesion assays of at least three independent experiments in triplicate wells were pooled and expressed as mean ± SD. Whole data were analyzed by the Epilinfo 6.0 software (Centers for Disease Control and Prevention) using the ANOVA parametric test for variance comparison and by the Kruskal-Wallis test, with p ≤ 0.05 as the statistically significant value. For each condition, results of transfectants were compared with 95% confidence limits for each well. Specific adhesion was calculated for each well as: (fluorescence before wash – control well fluorescence)/fluorescence after wash – control well fluorescence) × 100. Data are expressed as mean ± SD of specific adhesion per three replicate wells.

Flow chamber and laminar flow adhesion assay

The flow chamber (Immunetics) used has been previously described elsewhere (36–38). Briefly, the chamber allows stabilized laminar flow be-

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Footnotes:

1 Abbreviation used in this paper: FN, fibronectin.
classical T cell activation stimuli such as PMA plus CD3 mAb and suboptimal mitogenic concentrations of CD3 mAb did induce Ca^{2+} release from intracellular stores (data not shown).

We stably transfected A1 cells with the human β1A cDNA, which resulted in re-expression of VLA-4 and VLA-5 on the cell surface (Fig. 1). There was no change in expression of surface molecules capable of activating integrins, such as CD2, CD3, CD28, CD47, or CD99 (data not shown).

The A1-β1 reconstituted cells recovered the adhesion properties of VLA-4 and VLA-5 to fixed VCAM-1 or FN in a static cell adhesion assay (Fig. 2, A and B, respectively). In comparison to wild-type JE6.1, adhesion to VCAM-1 was specifically blocked by the β1 mAb 4B4 and increased by stimulation via integrin activators, namely, PMA, mAb TS2/16, which directly activates β1 integrin chain, CD2 mAb pair, or CD47, acting by inside-out mechanisms (Fig. 2A). Similar results were obtained on FN-coated plates (Fig. 2B), in accord with previous results using transiently transfected A1 cells expressing human β1 (31).

Moreover, A1-β1-reconstituted cells were able to arrest on coated VCAM-1 in a flow chamber assay. The number of arrested cells per square millimeter was similar to JE6.1 and modulated by integrin-activating pairs of CD2-specific mAb pairs or a CD47-specific mAb. Thus, our results demonstrate a role for the NPXY motifs in the β1 integrin tail in adhesion induced by the activating β1-specific mAb and inside-out signals mediated by CD2 and CD47. In addition, there is a role for the NPKY motif, but not the NPIY motif, in PMA-induced adhesion to VCAM-1 specifically.

The results obtained in the static adhesion assay on VCAM-1 were confirmed by using the detachment assay (Fig. 5). A1-β1APNY1 cells (Fig. 5, A and B) and A1-β1NPKY cells (Fig. 5, C and D) were stimulated with PMA (Fig. 5, A and C, respectively) and TS2/16 (Fig. 5, B and D, respectively) and allowed to adhere in the Lab-Tek chamber slide. When increasing shear stresses of washing medium were administered, significant differences (see Fig. 5 legend) appeared between the NPXY-deleted cells and their control. The strength of adhesion of A1-β1APNY1 was lower than that of control cells after stimulation with the TS2/16 mAb (Fig. 5B), but not with PMA (Fig. 5A). For the A1-β1NPKY cells, the strength of adhesion was lower than that of control cells after stimulation with both PMA (Fig. 5C) and TS2/16 (Fig. 5D).

Deleting of the β1 NPIY intracytoplasmic motif, but not NPKY, affects the firm arrest of T cells on VCAM-1 under flow conditions

Since static adhesion experiments showed that VLA-4 adhesion regulation was dependent on NPXY motifs, we next performed experiments to determine whether firm arrest mediated by VLA-4 was also related to these motifs. Under flow conditions at a shear stress of 1 dyn/cm² as described in Materials and Methods, the number of A1-β1APNY1 cells that arrested on coated VCAM-1 was significantly lower then A1-β1 control cells. However, no differences were observed at a higher shear stress (2 dyn/cm²) (Fig. 5D).

FIGURE 1. Transfectant phenotype. Expression of β1, α4, and α5 integrins by flow cytometry on the β1A integrin-deficient Jurkat A1 cell line and on A1 cells transfected with the wild-type form of the β1A integrin cDNA and various β1A integrin cDNAs containing specific deletions or amino acid substitutions. Cells were labeled with the anti-β1 integrin mAb TS2/16, anti-CD49d (VLA-4), and anti-CD49c (VLA-5) mAbs (open histograms) at the concentration of 10 μg/ml on ice for 30 min and then washed three times. The binding of the primary Ab as detected with secondary Ab conjugated to FITC and indirect immunofluorescence was assessed by flow cytometry. Cells labeled with isotype-matching Abs represent the negative control (filled histograms).
an increase of A1-β1ΔNPIY was obtained. However, adhesion of A1-β1ΔNPIY cells was significantly lower than A1-β1 control cells both in basal conditions and after stimulation with TS2/16 and CD47 mAbs. At 2 dyn/cm², a very moderate increase of firm adhesion was obtained after stimulation with TS2/16 and CD47 mAbs, but not in A1-β1ΔNPIY cells (Fig. 6A). A1-β1ΔNPIY-deleted cells rolled faster than A1-β1 control cells (Fig. 6B). When cells were allowed to roll on TNF-α-activated human endothelial cells, firm adhesion of A1-β1ΔNPIY cells was significantly lower than that of A1-β1 cells, both in basal conditions and after stimulation with TS2/16 mAb (Fig. 6C).

In marked contrast to A1-β1ΔNPIY-deleted cells, no difference was observed in the firm arrest of A1-β1NPKY transfectants on VCAM-1 when compared with A1-β1 cells at either shear stress tested (Fig. 6D). These results were confirmed when A1-β1NPKY-deleted T cells were injected into the flow chamber on TNF-α-activated EA cells (data not shown). A significant increase of firm adhesion was obtained after TS2/16 stimulation at both 1 and 2 dyn/cm² (Fig. 6E). Thus, our results demonstrate a role for the NPIY motif, but not the NPKY motif, in the regulation of T cell adhesion to VCAM-1 under shear flow conditions.

Deletion of the carboxyl-terminal five amino acids totally abolishes static adhesion but allows efficient firm arrest to VCAM-1 under flow conditions

Deletion of the carboxyl-terminal five amino acids of the cytoplasmic domain of the β1 tail has previously been shown to completely inhibit basal and stimulated adhesion of Jurkat T cells to FN (31). Stable A1 transfectants expressing the β1(793) deletion were also unable to adhere to VCAM-1 in a static adhesion assay, when compared with A1-β1 cells (Fig. 7, A, C, and E), following either PMA stimulation (Fig. 7A) or direct β1 integrin activation by the mAb TS2/16 (Fig. 7C). Only very weak stimulation with an optimal concentration of PMA and of coated VCAM-1 was detected. Similarly, stimulation of A1-β1(793) cells with integrin-activating the CD2 mAb pair or CD47-specific mAb did not enhance adhesion above unstimulated controls, both on VCAM-1 and FN-coated surfaces (Fig. 7, B, D, and F, respectively). Similar results for static adhesion assays were obtained on FN-coated plates (Fig. 7, B, D, and F) in the presence of increasing concentrations of PMA (Fig. 7B), TS2/16 (Fig. 7D), or activating β1 integrin costimuli (Fig. 7F).

Although the β1(793) deletion had a dramatic effect on static T cell adhesion to both VCAM-1 and FN, A1-β1(793)-activated human endothelial cells at either shear stress tested (Fig. 7G). When whole data from at least three different experiments performed were statistically analyzed, the number of arrested cells was comparable between A1-β1(793) cells and control A1-β1 cells at both flow rates tested. The same pattern was observed

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Adhesion of β1-A1-reconstituted cells to coated ligands. A, Static adhesion to VCAM-1-coated at 2.5 μg/ml of JE6.1, A1, and A1 cells retransfected with wild-type β1A. Cells were prepared for static adhesion assay as indicated in Materials and Methods. They were studied in basal condition (M) following stimulation with PMA, activating β1 integrin TS2/16 mAb, blocking (4B4) or nonrelevant (K20) mAb, and with β1 integrin costimuli such as the CD2 mAb pair D66 plus X11 or CD47 B6H12 mAb, all used at 10 μg/ml. Mean specific adhesion of triplicate wells ± SD from at least three independent experiments are presented. B, For adhesion to FN, cells were studied in the same conditions as in A. FN was used at 5 μg/ml. C, Arrest of JE6.1, A1-β1A, and A1 cells on coated VCAM-1 in a flow chamber assay. Cells were previously stained with green and orange calcein, respectively, as described in Materials and Methods, and injected in the flow chamber at a flow rate of 1 dyn/cm² for 5 min. The chamber was then washed with medium for an additional 5 min at the same shear stress before counting the number of firmly arrested cells on 10 independent areas. Cells were preincubated with TS2/16 or 4B4 mAbs for 10 min at 37°C or left unstimulated. Cumulative data from a minimum of three different experiments are presented.

6A). The same significant impairment of A1-β1ΔNPIY firm arrest was observed at a very low shear stress of 0.5 dyn/cm² (data not shown). When cells were stimulated with TS2/16 or CD47 mAbs,
when cells were stimulated with TS2/16 at 10 μg/ml (data not shown).

Mutation of tyrosine residues of the NPY and NPKY motifs increases the firm arrest of T cells on VCAM-1 under flow conditions, but has no effect in static adhesion

The tyrosine residues in the NPIY(Y783) and NPKY(Y795) motifs have been implicated in downstream signaling activities and integrin functions, such as cell spreading (45), tissue invasion (46), and bacterial internalization (47). To elucidate the importance of these motifs in adhesion, transfectants were generated expressing mutant A1-ΔNPIY cells with tyrosine to phenylalanine substitution in each NPXY motif (A1-Y783F and A1-Y795F) or in both motifs (A1-Y783,795F).

In a static adhesion assay, A1-ΔNPIY cells with tyrosine mutations were compared with A1 wild-type control cells for each condition by the ANOVA or Kruskal-Wallis tests, with p ≤ 0.05 as a statistically significant value. * No statistically significant difference; M, basal condition.

Discussion

We used the A1-ΔNPIY T cell line to define the structural requirements in the β1 integrin cytoplasmic domain that regulate VLA-4-mediated adhesion under both static conditions and in the presence of shear flow. The A1 cell line represents a unique cellular reagent for these studies, since the lack of endogenous A1 integrin expression in this cell line allowed us to stably express wild-type and mutant A1 integrin subunits without the complications of endogenous wild-type β1 integrin expression. Our studies reveal that integrin function under static and shear flow conditions are differentially regulated by three regions of the A1 integrin tail (Table II): 1) the membrane-proximal NPIY motif expressing the double tyrosine mutant Y783,795F exhibited higher levels of arrest at both low and high shear rates (Fig. 8C). The same pattern of firm adhesion increase was observed when tyrosine-mutated cells were stimulated with TS2/16 mAb, in comparison to basal conditions. In particular, A1-Y783F and A1-Y795F firm arrest was significantly higher than in A1-ΔNPIY cells at 2 dyn/cm². Moreover, firm arrest of A1-Y783,795F cells was significantly higher than A1-ΔNPIY cells at both 1- and 2-dyn/cm² shear stress (data not shown).
FIGURE 5. Effect of PMA and TS2/16 mAb on the resistance to detachment by shear flow of A1-β1ΔNPIY (A and B) and A1-β1ΔNPKY (C and D) transfectants. A1-β1 and A1-β1ΔNPKY-deleted cells were stained with fluorescence dyes as described in Materials and Methods. Cells were studied without stimulation and after a 20-min incubation at 37°C with 10 ng/ml PMA (A and C) or 1 µg/ml TS2/16 (C). Significant difference were determined by ANOVA (p ≤ 0.05). A representative experiment is presented from at least three independent assays.

FIGURE 4. Static adhesion of A1-β1ΔNPKY cells to VCAM-1 and FN. A1-β1 control cells (■) and A1-β1ΔNPKY cells (□) were prepared for static adhesion assays as indicated in Materials and Methods. Following a 20-min stimulation at 37°C with different effectors, cells were allowed to adhere on ligand-coated 96-well microplates. As a negative control, adhesion to 0.1% BSA was performed. Results of static adhesion on coated VCAM-1 at a concentration of 2.5 µg/ml (A and C) after stimulation with PMA (A) and TS2/16 (C). Results of static adhesion on FN at a concentration of 5 µg/ml (B and D), with increasing concentrations of PMA (B) and TS2/16 (D). Results from inside-out stimulation on VCAM-1 (E) and FN (F) with the CD2 mAbs pair D66 and X11 or CD47 mAb B6H12, both used at 10 µg/ml and PMA at 1 ng/ml. Global results of A1-β1ΔNPKY cell static adhesion assays of at least three different experiments in triplicate were pooled and statistical analysis was performed. ANOVA and Kruskal-Wallis tests were used, with p ≤ 0.05 as the statistically significant value. *, No statistically significant difference; M, basal condition.
FIGURE 6. Attachment of A1-β1ΔNPXY cells under flow conditions. Mutant β1 cells and their wild-type control were stained with green and orange fluorescent dyes, respectively, in HBSS medium in the presence of 1 mM Ca²⁺ and Mg²⁺, as indicated in Materials and Methods, and mixed in equal amounts at the final concentration of 10⁶ cells/ml. A, A1-β1ΔNPPIY cells were injected into the flow chamber at different shear stress and compared with A1-β1 wild-type T cells. The number of arrested cells per square millimeter was calculated as mean ± SD of 10 different fields and global results are presented from at least three independent experiments. Results were compared by the ANOVA statistical test, with p ≤ 0.05 as the statistically significant difference. B, Rolling velocity of A1-β1ΔNPPIY cells in comparison to A1-β1 wild-type cells was manually measured at a shear stress of 1 dyn/cm² as indicated in Materials and Methods by using an AxioCAM video camera and expressed as mean ± SD of 30 cells in motion analyzed from three different experiments. Results were compared by the ANOVA statistical test, with p ≤ 0.05 as the statistically significant difference. C, Results of cumulative data of three independent experiments of firm adhesion of A1-β1ΔNPPIY and A1-β1 control cells on TNF-α-activated EA cells at 1 dyn/cm² in the absence of stimulation (Medium) or after TS2/16 activation for 20 min at 37°C at the concentration of 10 μg/ml. D, Results of flow experiments with A1-β1ΔNPKY cells compared with A1-β1 wild-type cells. Cells were prepared and allowed to roll in the flow chamber as indicated in A. Results are representative of cumulative data of at least three different experiments. E, Results of flow experiments on coated VCAM-1 of A1-β1ΔNPKY cells stimulated with TS2/16 mAb as in A and C. For all of the experiments, the Kruskal-Wallis or the ANOVA tests were used to perform statistical analysis from cumulative data. A value of p ≤ 0.05 indicates a statistically significant difference. * No statistically significant difference.

regulates VLA-4-mediated adhesion under both static and shear conditions, 2) the carboxyl-terminal region of the β₁ tail has a specific role in regulating static adhesion, but not arrest under shear flow, and 3) the tyrosines in the NPXY motifs do not play a major role in regulating static adhesion but appear to negatively regulate adhesion under shear flow.

The β₁ integrin NPIY motif appears to be particularly critical for optimal VLA-4 function, since deletion of this motif inhibits both activation-dependent VLA-4-mediated adhesion under static conditions and firm arrest under flow conditions. The shear flow rates used in our experiments likely mimic physiological inflammatory conditions in microvessels, when microaneurysms induced by inflammation slow the shear stress to these rates (48).

It is interesting to note that deletion of the NPIY motif altered firm arrest at 1 dyn/cm², but not at 2 dyn/cm². This was also observed in a more physiological model, on human activated endothelial EA cells. This may be related to a potential role of the NPIY motif in regulating affinity states of VLA-4, since Chen et al. (28) demonstrated that low-affinity VLA-4 states preferentially mediate transient tethering and rolling of PBL on VCAM-1, whereas high-affinity VLA-4 states preferentially support spontaneous firm arrest of both PBL and Jurkat T cells on VCAM-1. It is possible that cells arresting at higher shear stress conditions (2 dyn/cm²) is mediated by high-affinity VLA-4, whereas cell arrest at 1 dyn/cm² is mediated predominantly by intermediate-/low-affinity VLA-4. The reduced firm adhesion of the A1-β1ΔNPPIY cells was also confirmed on VCAM-1-coated Lab-Tek slides at very low shear stress, such as 0.5 dyn/cm². This suggests that the NPIY motif might be particularly critical in regulating intermediate-/low-affinity states of VLA-4 rather than high-affinity states.

The NPIY motif has also been implicated in the binding of the cytoskeleton protein talin to the β₁ cytoplasmic domain (49), and talin interaction with integrin tails is proposed to play a critical role in integrin activation (42, 50). Thus, the inability of the A1-β1ΔNPPIY cells to adhere to VCAM-1 following integrin-activating signals may be due to disruption of the interaction between...
talin and the integrin $\beta_1$ subunit. In addition, our results with adhesion under shear flow suggest a role for talin in optimal firm arrest, but only under low shear flow rates. Integrin activation by TS2/16 or cosignal stimulation by CD47 mAb was not affected in A1- $\beta_1$NPPIY cells, since an increase in firm arrest was observed at levels comparable to those of A1- $\beta_1$ control cells.

The C-terminal region of the integrin $\beta_1$ tail that contains the NPKY motif was found to be particularly important for static adhesion but not for adhesion under shear flow. This was most evident with the A1- $\beta_1$(793) cells, which exhibited defective static adhesion to both VCAM-1 and FN under static adhesion conditions, but had surprisingly normal patterns of cell arrest under shear flow conditions. In addition, like A1- $\beta_1$NPPIY, A1- $\beta_1$NPKY cells showed reduced adhesion after stimulation with the integrin-activating mAb TS2/16 and complete absence of response to other inside-out stimuli that activate $\beta_1$ integrins. The impairment in the adhesion properties of the NPXY-deleted cells seen in both static and detachment assays was statistically significant, although small in some cases, rather than an “all or none” effect. The existence of different pools among Jurkat T cells of cells bearing different states of VLA-4 affinity for VCAM-1 could explain these smaller differences observed with VCAM-1 compared with the effects observed on FN, where the affinity states of VLA-4 may be more homogeneous.

**FIGURE 7.** Adhesion of A1- $\beta_1$(793) cells to VCAM-1 and FN. Static adhesion of A1- $\beta_1$(793) cells (■), in comparison to A1- $\beta_1$ control cells (○), to VCAM-1-coated 96-well microplates at 2.5 $\mu$g/ml, with PMA at various concentrations (A), in the presence of the direct activating $\beta_1$ integrin mAb TS2/16 (C) and costimulatory signals CD2 mAb pairs D66 and X11 or CD47 B6H12 mAb (E), both used at 10 $\mu$g/ml and PMA used at 1 ng/ml. Results of static adhesion on FN coated at 5 $\mu$g/ml A1- $\beta_1$(793) cells stimulated with PMA (B) in the presence of TS2/16 and (D) and costimulatory signals (F). G, Equal amounts of A1- $\beta_1$ wild-type and A1- $\beta_1$(793) cells stained with green and orange fluorescent dyes, respectively, as indicated in Materials and Methods, were mixed and injected into the flow chamber system on VCAM-1-coated slides at various shear stresses. For static and dynamic assays, results represent cumulative data of means ± SD of at least three independent experiments, analyzed by Kruskal-Wallis or ANOVA tests, with $p < 0.05$ as a statistically significant value. *, No statistically significant difference; M, basal condition.

**FIGURE 8.** Firm arrest of A1- $\beta_1$(Y783F), A1- $\beta_1$(Y795F), and A1- $\beta_1$(Y783,795F) cells to VCAM-1 under flow conditions. A1- $\beta_1$(Y783F) (A), A1- $\beta_1$(Y795F) (B), and A1- $\beta_1$(Y783,795F) (C) cells were prepared as described in Materials and Methods and allowed to roll on VCAM-1-coated chamber slides in a flow chamber at different flow rates. Results of cumulative data (means ± SD) of at least three different assays are indicated. The Kruskal-Wallis statistical test was used to compare the number of arrested cells between the transfectants tested. *, No statistically significant difference.
Like A1-β1(793) cells, arrest of cells expressing the A1-β1ΔNPKY under shear flow conditions was comparable to A1 cells expressing wild-type β1. Our results also show that TS2/16 stimulation is affected following deletion of the NPXY motifs or deletion of the five C-terminal amino acids of the β1 integrin cytoplasmic tail. Although TS2/16 is thought to activate β1 integrin primarily via alterations in the β1 extracellular domain, recent studies have shown that TS2/16-induced adhesion is inhibited when Rap1 signaling is impaired (51). Thus, our results are consistent with a potential requirement for intracellular signaling in regulating integrin activation induced by the TS2/16 mAb.

In other studies, the carboxyl-terminal amino acids of the β1 tail were found to be critical for β1 integrin-dependent adhesion, as well as migration and metastasis. In an in vivo model, Stroeken et al. (46) showed that expression of a β1(793) construct in β1 integrin-deficient double knockout ESB lymphoma cells (ESB-DKO) impaired invasion and metastasis in vivo when injected in mice and greatly reduced adhesion in vitro. Since firm cell arrest on endothelium is critical for invasion in vivo, our results suggest that the carboxyl-terminal end of the β1 integrin tail may be particularly critical for regulating integrin function subsequent to firm arrest, such as transendothelial migration.

The mechanism by which the carboxyl-terminal end of the β1 integrin tail reduces integrin function remains unclear. Studies with chimeric integrins expressed in Chinese HO cells (45) suggest that the β1(793) mutant is still capable of interacting with talin. Our results suggest that structural integrity at this region of the β1 tail is particularly critical for static adhesion regulated by inside-out signaling, but not firm adhesion under hydrodynamic shear flow conditions.

The third structural feature of the integrin β1 tail is that tyrosine residues 783 and 795 in the NPXY motifs appear to play a critical role in regulating adhesion under shear flow, but not under static conditions. When either of these tyrosine residues is substituted with phenylalanine, adhesion at high shear stress rates was actually enhanced when compared with wild-type A1-β1 cells. In contrast, adhesion under static conditions was unaffected by these mutations, consistent with previous results (31). Similar results were observed when both tyrosines were substituted with phenylalanine, although now enhanced firm arrest was also observed at low shear rates.

Although the role of tyrosine phosphorylation of the β1 tail remains unresolved, our results nevertheless suggest a potential function for phosphorylation of these tyrosine residues in regulating adhesion under shear flow. A role for these tyrosines in migration has also been suggested, since a tyrosine to phenylalanine substitution at Y795 impairs the migration of mouse GD25 fibroblasts (52). Furthermore, the Y783/795F double mutation markedly affects cell migration and cytoskeletal architecture, but not adhesion (52).

However, when expressed in β1-deficient lymphoid ESB-DKO cells, the Y783/795F double mutation impaired adhesion and migration in vitro, but not in vivo in the liver (46). Other studies have also implicated these tyrosine residues in integrin affinity modulation (40), as tyrosine to alanine substitutions at these sites impairs LIBS mAb binding. However, tyrosine to phenylalanine substitutions did not affect binding of this mAb to β1 and β2 integrins in this study, suggesting a structural role for these tyrosine residues in this system. Some of these differences may be due to the different cell types used and potential differences in integrin-associated proteins that may regulate integrin function via binding to this region of the β1 tail. Our data show that the deletion of the individual tyrosine residues is markedly different from deletion of the entire motif. Deletion of the entire NPXY motif may result in important structural changes in the conformation of the cytoplasmic tail of β1 integrin that may not occur when individual tyrosines are mutated. Moreover, NPXY tyrosines could be implicated in complex signaling pathways that regulate adhesion-dependent responses. The substitution of these amino acids may lead to the disruption of a possible negative control pathway for firm adhesion mediated by β1 integrin.

Leukocyte trafficking from blood into peripheral tissues occurs through a sequential process that involves tethering, rolling, firm adhesion, and transendothelial migration. The VLA-4 integrin has been implicated in all of these phases of trafficking. In addition, the functional activity of VLA-4 is dynamically regulated, because circulating lymphocytes maintain their integrins in a nonadhesive state to avoid nonspecific adhesion to blood vessels. When inflammation occurs, VCAM-1 is expressed on endothelial cells in postcapillary venules and activated T lymphocytes, expressing high-affinity integrins, are capable of tethering and arrest. In tissue sites, VLA-4 also mediates adhesion. For example, the adhesion of hemopoietic progenitors in bone marrow is mediated in part by VLA-4 binding to VCAM-1 expressed on bone marrow stromal cells. Our structural analysis in this report demonstrates that distinct regions of the integrin β1 tail regulate VLA-4 function under static and shear flow conditions. These findings have relevance to our understanding of how VLA-4 mediates adhesion in distinct anatomic compartments, such as in the bone marrow or in blood vessels. Furthermore, our work suggests a potential structural basis for modulating VLA-4-mediated adhesive functions in tissue sites while maintaining the ability of VLA-4 to mediate firm arrest and attachment under conditions of vascular shear flow.

Acknowledgments

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**Table II. Summary of the functions of β1 integrin cytoplasmic tail mutants**

<table>
<thead>
<tr>
<th>Constructions</th>
<th>Unstimulated</th>
<th>PMA</th>
<th>Direct β1 stimulation</th>
<th>Inside-out stimulation</th>
<th>Firm Arrest under Flow Conditions</th>
<th>Functional Type</th>
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*Summary of the functions of β1 integrin cytoplasmic tail mutants. +, Normal function; +, weak function; –, minimal, no function; +++, increased function.*
Disclosures

The authors have no financial conflict of interest.

References


