The CD81 Tetrastatin Facilitates Instantaneous Leukocyte VLA-4 Adhesion Strengthening to Vascular Cell Adhesion Molecule 1 (VCAM-1) under Shear Flow*  

Received for publication, April 7, 2003, and in revised form, September 2, 2003  
Published, JBC Papers in Press, October 7, 2003, DOI 10.1074/jbc.M303601200

Sara W. Feigelson‡, Valentin Grabovsky‡, Revital Shamri‡, Shoshana Levy§, and Ronen Alon‖

From the ‡Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel 76100 and the §Department of Oncology, Stanford University CCBR 1a, Stanford, California 94305

This paper is available online at http://www.jbc.org

Leukocyte integrins must rapidly strengthen their binding to target endothelial sites to arrest rolling adhesions under physiological shear flow. We demonstrate that the integrin-associated tetrastatin, CD81, regulates VLA-4 and VLA-5 adhesion strengthening in monocytes and primary murine B cells. CD81 strengthens multivalent VLA-4 contacts within subsecond integrin occupancy without altering intrinsic adhesive properties to low density ligand. CD81 facilitates both VLA-4-mediated leukocyte rolling and arrest on VCAM-1 under shear flow as well as VLA-5-dependent adhesion to fibronectin during short stationary contacts. CD81 also augments VLA-4 avidity enhancement induced by either chemokine-stimulated G_{i} proteins or by protein kinase C activation, although it is not required for G_{i} protein or protein kinase C signaling activities. In contrast to other proadhesive integrin-associated proteins, CD81-promoted integrin adhesiveness does not require its own ligand occupancy or ligation. These results provide the first demonstration of an integrin-associated transmembranal protein that facilitates instantaneous multivalent integrin occupancy events that promote leukocyte adhesion to an endothelial ligand under shear flow.

To emigrate from the bloodstream to specific sites of inflammation or antigen presentation, circulating leukocytes must rapidly develop firm adhesion to vessel walls in response to both adhesive and stimulatory endothelial signals (1). These tissue-specific “traffic signals” orchestrate a selective multistep cascade of attachment (tethering), rolling, and arrest of recruited leukocyte subsets under disruptive shear forces (2). The firm adhesions are mediated exclusively by subsets of leukocyte integrins, which include α_{v}β_{2}, VLA-4 (α_{4}β_{1}), and LFA-1 (3, 4). These integrins exhibit basal recognition of ligand under shear flow and enable leukocyte capture, rolling, and arrest on their respective endothelial ligands when present at sufficiently high densities (5, 6). This basal integrin adhesiveness can be dramatically augmented by leukocyte exposure to appropriate endothelial chemokines in situ, which enhance integrin avidity through their respective G-protein-coupled receptors on tethered leukocytes (7, 8).

The mechanism of cellular regulation of both basal and chemokine-stimulated integrin adhesiveness to endothelial ligands is still obscure. Integrins exist in heterogeneous affinity states, constantly regulated through peripheral inflammatory or survival signals (9). However, the ability of VLA-4 to tether and support their rolling on the endothelial ligand, VCAM-1, under shear flow does not require high affinity to soluble ligands (9). In addition, previous studies suggested that VLA-4 and LFA-1 associations with the intracellular actin-cytoskeleton control integrin adhesiveness to ligand under shear flow and are independent of the intrinsic affinity of these integrins to their respective ligands (5, 6). Accordingly, the ability of leukocyte integrins to self-cluster upon ligand binding has been predicted to facilitate the generation of high avidity tethers under disruptive shear forces even without acquisition of conformations with high affinity to monovalent ligands (10).

Evidence for specific integrin-associated partners that modulate integrin affinity or integrin clustering in leukocytes encountering endothelial or matrix ligands has been missing (11). Members of the tetrastatin family (transmembrane 4 superfamily (TM4SF)) of proteins, which consist of four highly conserved transmembrane domains (12), associate with specific integrins on the cell membrane and have been postulated to regulate integrin activities in leukocytes (11, 13) as well as in other cell types (14–16). The tetrastatins constitute a growing family of proteins implicated in cell signaling, motility, homotypic aggregation, viral entry, protein folding, and tumor metastasis (17). The tetrastatins can either constitutively or inducibly associate with key signaling effectors of integrin function such as phosphatidylinositol 4-kinase (18) and diacylglycerol (DAG)-dependent PKC isoforms (19). Several tetrastatins, including CD81, CD82, and CD151 as well as other integrin partners, augment integrin-dependent cell adhesion (20–23). However, these activities are observed after prolonged contacts that involve cytoskeletal remodeling events downstream to the nascent integrin-dependent contacts. Recently, the β_{2} and β_{1}-integrin-associated protein pentaspan, CD47, was shown to indirectly augment rapid VLA-4 adhesion strengthening to VCAM-1 under shear flow (24). This CD47-mediated augmentation required, however, the co-occupancy of CD47 with its endothelial ligand and did not affect the inherent activity of VCAM-1.

* This work was supported in part by the Minerva Foundation, Germany, the Crown Endowment Fund for Immunological Research, and the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by National Institutes of Health Grants AI45900 and CA34233.

† Incumbent of The Tauro Career Development Chair in Biomedical Research. To whom correspondence and reprint requests should be addressed: Dept. of Immunology, the Weizmann Institute of Science, Rehovot 76100, Israel. E-mail: ronalon@wicc.weizmann.ac.il.

1 The abbreviations used are: VCAM, vascular cell adhesion molecule; DAG, diacylglycerol; PKC, protein kinase C; mAb, monoclonal antibody; ICAM, intercellular adhesion molecule 1; PMA, phorbol myristate acetate; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; FN, fibronectin; MHC, major histocompatibility complex; PE, R-phycocerythrin.
CD81-regulated Integrin Avidity in Leukocytes

VLA-4 adhesion to VCAM-1 (24). To date, functional evidence that a membranal integrin-associated molecule can directly up-regulate integrin avidity to ligand at rapid adhesive contacts independent of its own occupancy or ligation, has not been demonstrated.

We now report that the CD81 tetraspanin is a key regulator of multivalent β1 integrin contacts that are critical for rapid adhesion strengthening in monocytes and primary murine B cells interacting with VCAM-1 under shear flow. This specialized activity does not require ligation or external occupancy of CD81 by ligand. CD81-facilitated VLA-4 adhesiveness results in the potentiation of rolling and arrest of CD81-expressing leukocytes on VCAM-1 as well as on immobilized integrin-binding mAb under shear flow. It also enables CD81-expressing leukocytes to integrate proadhesive inside-out signals from both chemokines and phospholipid esters much more efficiently than CD81-null cells. CD81 is a first example of a membranal integrin-associated molecule acting as a facilitator of an exceptionally fast stabilization of multivalent integrin contacts under shear flow.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Recombinant 7-domain human VCAM-1, sVCAM-1, was provided by Dr. R. Lobb (Biogen, Cambridge, MA). Affinity purified human full-length ECM-1 was a gift of Dr. T. Springer (Harvard T. University, Boston, MA). Affinity purified human VLA-5 binding FN fragment, FN-120, was from Invitrogen (Paisley, UK). The SF-1 chemokine was purchased from R&D Systems (Minneapolis, MN). Mg(CD), bovine serum albumin (fraction V), poly-l-lysine, and Ca2+,-Mg2+-free Hanks’ balanced salt solution were from Sigma. Human serum albumin (fraction V) and PMA were purchased from Calbiochem (San Diego, CA).

Laminar Flow Adhesion Assays

Preparation of Adhesive Substrates—Purified ligands or mAbs were dissolved in PBS buffered with 20 mM bicitarbate, pH 8.5, and incubated on a polystyrene plate (60 × 15-mm Petri dish; BD Biosciences) for 2 h at 37°C or overnight at 4°C. The plate was then washed three times with PBS and blocked with 0.2% gelatin (in PBS) for 2 h at 4°C. For SDF-1 co-immobilization on the adhesive substrate, ligands were coated in the presence of normal or heat-denatured chemokine (R&D Systems) (2 μg/ml) and a carrier protein (human serum albumin, 2 μg/ml) as previously described (10).

Analysis of Cell Tethers and Adhesion Strength—A polystyrene plate on which purified ligand or integrin-specific mAb had been adsorbed was assembled on the lower wall of the flow chamber (260-μm gap) as previously described (9, 28). Cells were washed with cation-free H/H balanced salt solution containing 2 mg/ml bovine serum albumin and 10 mM Hepes, pH 7.4, supplemented with 1 mM CaCl2 and 1 mM MgCl2, at a concentration of 1 × 107 cells/ml at a 1 cell:8 beads ratio, followed by a 3-fold dilution in binding medium. The cellular side scatter, distinguishing between bead-bound and bead-free cells, was then analyzed immediately in a FACScan flow cytometer (BD Pharmingen). Binding specificity was confirmed by the complete absence of cell binding by control mAb-coated beads.

Flow Cytometry and Immunofluorescence Cell Staining

Staining and fluorescence activated cell sorter analysis were performed as previously described (9). For α4 integrin immunostaining, U937 monocytic cells were washed in PBS, fixed in 3% paraformaldehyde in PBS (30 min, room temperature), and incubated with anti-α4 B5G10 mAb for 30 min at 4°C. Cells were then washed once with PBS + 5 mM EDTA and twice with PBS, 0.1% bovine serum albumin and then stained with Alexa Fluor-546-conjugated anti-mouse Ab (Molecular Probes). Cells were attached to poly-l-lysine-coated glass slides and coverslips were mounted with elvanol overnight. Fluorescence microscopy was performed with a confocal microscope (Nikon Eclipse TE300 with the Laser scanning system 2000, Bio-Rad).

Results

CD81 Enhances the Avidity of α4 Integrin Measured during Rapid Contact of Mouse B Lymphocyte and Human U937 Cells with Immobilized VCAM-1—To directly address the role of (Roche Diagnostics) and prelabeled with human serum albumin-conjugated Protein G-Sepharose (Dynal Biotech Ltd., Upsalla, Sweden). Lysates were immunoprecipitated with antibodies (10 μg/ml) for 60 min followed by the addition of Protein G beads overnight at 4°C. Proteins were electrophoresed to nitrocellulose membranes and reacted with appropriate antibodies followed by peroxidase-labeled secondary antibodies. For Western blot studies, 1 × 107 cells (treated or untreated) were solubilized in 100 μl of lysis buffer (as above, but with 1% Nonidet P-40 instead of Brij 58) and 10 μl of lysates were separated by SDS-PAGE in reducing buffer. Blots were developed using enhanced chemiluminescence (ECL, Sigma).

Antibody-coated Microbeads Assay

Sheep anti-mouse IgG magnetic M-280 Dynabeads (Dynal Biotech Inc., Lake Success, NY) were coated with various concentrations (0.004–4 μg/ml) of HP1/2 or isotype matched control mAb, according to the manufacturer’s instructions. Cells and antibody-coated beads were mixed for 30 s in binding medium, consisting of H/H medium (Hanks’ balanced salt solution containing 2 mg/ml bovine serum albumin and 10 mM Hepes, pH 7.4) supplemented with 1 mM CaCl2 and 1 mM MgCl2, at a concentration of 1 × 107cells/ml at a 1 cell:8 beads ratio, followed by a 3-fold dilution in binding medium. The cellular side scatter, distinguishing between bead-bound and bead-free cells, was then analyzed immediately in a FACScan flow cytometer (BD Pharmingen). Binding specificity was confirmed by the complete absence of cell binding by control mAb-coated beads.
CD81 in integrin adhesion strengthening, we compared the ability of B splenocytes derived from normal and cd81−/− mice to develop integrin-dependent adhesion to the endothelial VLA-4 ligand, VCAM-1. CD81-expressing B splenocytes, although expressing slightly lower levels of α4 integrins (Fig. 1A, top left) generated much higher VLA-4 avidity to VCAM-1 than their CD81-null counterparts (Fig. 1A, right). In contrast, resting mouse wild type T splenocytes, which lack surface CD81 (Fig. 1B, bottom left), exhibited comparable VLA-4 avidity to VCAM-1 as that developed by T splenocytes derived from cd81−/− mice (Fig. 1B, right). This finding suggests that in B splenocytes, the CD81 tetrspanin enhances VLA-4 adhesion strengthening to VCAM-1 at rapid contact sites.

The female infertility of cd81−/− mice (29) limited the availability of cells and thus the extensive molecular characterization of the role of CD81 in regulating VLA-4 properties in murine leukocytes. To extend these results to an in vitro human system, we utilized U937 promonocytic leukemia cells, which do not express endogenous CD81. The human monocyte line was reconstituted with CD81 by stable retroviral infection with a vector encoding CD81 and a GFP reporter (herein CD81), or with GFP alone (herein null). Clones identical in expression of α4 integrin expression (Fig. 2A) were used for further characterization. Notably, both cells lacked α4β2 (Fig. 2A) indicating that all α4 expression in these cells is exclusively associated VLA-4 (α4β2).

Reminiscent of B splenocytes (Fig. 1A), CD81-expressing monocytes developed greater shear-resistant VLA-4-dependent adhesion to high density VCAM-1 than their CD81-null counterparts after a 1-min static contact with the integrin ligand (Fig. 2B). Interestingly, CD81 did not contribute to VLA-4-dependent adhesion strengthening to low density VCAM-1 (Fig. 2B). CD81-augmented VLA-4 adhesiveness to VCAM-1 was α4-specific as it was entirely blocked by preincubation of the cells with either an α4 blocking mAb (Fig. 2B) or the VLA-4-specific ligand, Bio1211 (30). This result ruled out a contribution of a direct co-adhesive interaction between CD81 and VCAM-1 to the VLA-4-dependent adhesion to VCAM-1. Pretreatment of CD81-expressing or null U937 cells with pharmacological inhibitors of PKC, protein-tyrosine kinase, and phosphatidylinositol 3-kinase did not affect the ability of their VLA-4 to develop adhesion strengthening at these rapid static contacts. Thus, the proadhesive effects of CD81 on VLA-4 adhesiveness did not involve activities of these kinases shortly prior to or within the time frame of the integrin contact with ligand. Furthermore, external ligation of CD81 with a stimulatory mAb (31) did not enhance VLA-4-dependent U937 adhesion VCAM-1 developed at 1-min static contacts at any ligand density tested (Fig. 2C). This result rules out the possibility that external CD81 ligation could, on its own, induce rapid generation of high VLA-4 avidity to VCAM-1.

Strikingly, CD81 also dramatically up-regulated the ability of VLA-4 in U937 cells to support cell capture and rolling on VCAM-1 under persistent physiological shear flow (Fig. 2D). Reminiscent of its proadhesive role at stationary VLA-4/VCAM-1 contacts, the contribution of CD81 to enhanced VLA-4 adhesiveness to low density VCAM-1 was diminished (Fig. 2D). The ability of VLA-4 to capture cells and mediate their successive rolling adhesions on VCAM-1 under shear flow involves generation of critical avidity to VCAM-1 within adhesive contacts lasting fractions of seconds (9). Thus, CD81 appeared to augment the ability of VLA-4 to generate critical binding avidity to high and medium density VCAM-1 at subsecond contacts without affecting the capacity of the integrin to recognize and transiently adhere to low density VCAM-1 (Fig. 2D, inset). As observed at stationary contacts, CD81 ligation with a stimulatory mAb failed to up-regulate VLA-4-mediated capture, rolling, or arrest on VCAM-1 under shear flow, suggesting that CD81 promotes VLA-4 avidity to high density ligand independent of its own ligation.

CD81 regulates VLA-4 adhesion strengthening without altering integrin ligand binding properties or preformed clustering. Artificial stimulation of integrin affinity to ligand often rescues adhesive defects caused by impaired inside-out integrin signaling (9). Suspension of CD81-null U937 cells with a vector encoding CD81 and a GFP reporter.
were left untreated or incubated with mAb (5 ng/ml) for 15 min at 37 °C. The mean ± range of two independent fields is shown.

Procedures.

Expression of CD81 in U937 monocytes.

Bio1211 (30), was identical in CD81-expressing and -null U937 cells, as was VLA-4-mediated adhesion to low density VCAM-1 on CD81-expressing or -null cells to equally stabilize adhesion (Fig. 2, B and C). Thus, the major defect of VLA-4 in CD81-null cells is affinity-independent. Consistent with this conclusion, direct binding to a monovalent VLA-4 binding peptide, the LDV derivative of mAb to α4-specific HP1/2, completely eliminated all cell adhesion to both VCAM-1 densities, as depicted by the open triangle. The mean ± range of two independent fields is shown. C, mAb-induced CD81 ligation does not increases VLA-4 avidity to VCAM-1. CD81-expressing U937 cells were left untreated or incubated with mAb (5 ng/ml for 15 min at 37 °C) specific for CD81 (5A6) or L-selectin (DREG-200) or treated with activating mAb to β1 (TS2/16) and their strength of VLA-4-dependent adhesion to VCAM-1 (0.75 µg/ml) was analyzed. The mean ± range of two independent fields is depicted. D, tethering, rolling, and spontaneous arrest of CD81-null and CD81-expressing U937 cells interacting with VCAM-1 under continuous shear flow. Tethered cells, determined in two fields at 1 dyn/cm², were grouped into three categories, as described under “Experimental Procedures.” Inset depicts enlarged bars for tether categories of both cell types on the lowest tested density VCAM-1. Results in B and D are representative of four experiments. Results in C are of three independent experiments.

We next considered that CD81 might promote VLA-4 avidity by enriching pre-existent high affinity VLA-4 subsets within the nascent contact site (9). Recent ultrastructural and cryostatographic studies conducted on isolated integrins and integrin-ligand complexes suggest that integrins of the β2 and β3 subfamilies can also regulate their adhesive bonds through rapid post-ligand stabilization events even without concomitant microclustering (32–34). Thus, ligand occupancy by VLA-4 could stabilize its bonds with VCAM-1 in an analogous manner. To evaluate these possibilities, we tested the ability of VLA-4 on CD81-expressing or -null cells to equally stabilize adhesion to a surface-bound α4-specific mAb, HP1/2. This mAb binds α4 integrin independent of the VLA-4 affinity to native ligands (9). Strikingly, VLA-4 expressed by CD81-null monocytes failed to stabilize cell adhesion on immobilized α4-specific mAb or on its corresponding Fab fragment, both at short static contacts or under shear flow (Fig. 3, A and B). Similarly, the fraction of cell binding to microbeads coated with the α4-specific mAb was 10–20-fold higher in CD81-expressing cells compared with their null counterparts. These results were surprising in light of the fact that VLA-4 binds this mAb at an affinity orders of magnitude higher than it binds VCAM-1 (35). Furthermore, microkinetic analysis of integrin interactions to immobilized α4-specific mAb revealed that CD81 dramatically augmented the ability of transient α4-dependent tethers lasting 0.2–0.5 s to develop firm irreversible arrests to the immobilized α4-specific mAb within these subsecond time frames (Fig. 3B, right panels). VLA-4-mediated tethers to lowest densities of the α4-binding mAb were, however, comparable in both CD81-null and CD81-expressing cells, reminiscent of the observations with VCAM-1 (Fig. 2, B and C). Thus, the major defect of VLA-4 adhesion strengthening on CD81-null U937 monocytes appeared to stem from an improper ability of VLA-4 molecules to instantaneously derive high avidity to ligand upon occupancy by a critical number of adjacent ligand or mAb molecules. Confocal microscopy analysis of VLA-4 revealed, however, normal uniform surface distribution on these and CD81-expressing monocytes in Mg2+ to artificially activate VLA-4 affinity did not rescue, however, the inability of VLA-4 to support high avidity adhesion to VCAM-1 at stasis. Strength of adhesion was analyzed as described in the legend to Fig. 1. Antibody blocking of CD81-expressing cells with a 4-specific HP1/2 mAb (10 ng/ml) completely eliminated all cell adhesion to both VCAM-1 densities, as depicted by the open triangle. The mean ± range of two independent fields is shown. C, mAb-induced CD81 ligation does not increases VLA-4 avidity to VCAM-1. CD81-expressing U937 cells were left untreated or incubated with mAb (5 ng/ml for 15 min at 37 °C) specific for CD81 (5A6) or L-selectin (DREG-200) or treated with activating mAb to β1 (TS2/16) and their strength of VLA-4-dependent adhesion to VCAM-1 (0.75 µg/ml) was analyzed. The mean ± range of two independent fields is depicted. D, tethering, rolling, and spontaneous arrest of CD81-null and CD81-expressing U937 cells interacting with VCAM-1 under continuous shear flow. Tethered cells, determined in two fields at 1 dyn/cm², were grouped into three categories, as described under “Experimental Procedures.” Inset depicts enlarged bars for tether categories of both cell types on the lowest tested density VCAM-1. Results in B and D are representative of four experiments. Results in C are of three independent experiments.

We next considered that CD81 might promote VLA-4 avidity by enriching pre-existent high affinity VLA-4 subsets within the nascent contact site (9). Recent ultrastructural and cryostatographic studies conducted on isolated integrins and integrin-ligand complexes suggest that integrins of the β2 and β3 subfamilies can also regulate their adhesive bonds through rapid post-ligand stabilization events even without concomitant microclustering (32–34). Thus, ligand occupancy by VLA-4 could stabilize its bonds with VCAM-1 in an analogous manner. To evaluate these possibilities, we tested the ability of VLA-4 on CD81-expressing or -null cells to equally stabilize adhesion to a surface-bound α4-specific mAb, HP1/2. This mAb binds α4 integrin independent of the VLA-4 affinity to native ligands (9). Strikingly, VLA-4 expressed by CD81-null monocytes failed to stabilize cell adhesion on immobilized α4-specific mAb or on its corresponding Fab fragment, both at short static contacts or under shear flow (Fig. 3, A and B). Similarly, the fraction of cell binding to microbeads coated with the α4-specific mAb was 10–20-fold higher in CD81-expressing cells compared with their null counterparts. These results were surprising in light of the fact that VLA-4 binds this mAb at an affinity orders of magnitude higher than it binds VCAM-1 (35). Furthermore, microkinetic analysis of integrin interactions to immobilized α4-specific mAb revealed that CD81 dramatically augmented the ability of transient α4-dependent tethers lasting 0.2–0.5 s to develop firm irreversible arrests to the immobilized α4-specific mAb within these subsecond time frames (Fig. 3B, right panels). VLA-4-mediated tethers to lowest densities of the α4-binding mAb were, however, comparable in both CD81-null and CD81-expressing cells, reminiscent of the observations with VCAM-1 (Fig. 2, B and C). Thus, the major defect of VLA-4 adhesion strengthening on CD81-null U937 monocytes appeared to stem from an improper ability of VLA-4 molecules to instantaneously derive high avidity to ligand upon occupancy by a critical number of adjacent ligand or mAb molecules. Confocal microscopy analysis of VLA-4 revealed, however, normal uniform surface distribution on these and CD81-expressing monocytes in Mg2+ to artificially activate VLA-4 affinity did not rescue, however, the inability of VLA-4 to support high avidity adhesion to VCAM-1 at stasis. Strength of adhesion was analyzed as described in the legend to Fig. 1. Antibody blocking of CD81-expressing cells with the 4-specific HP1/2 mAb (10 ng/ml) completely eliminated all cell adhesion to both VCAM-1 densities, as depicted by the open triangle. The mean ± range of two independent fields is shown. C, mAb-induced CD81 ligation does not increases VLA-4 avidity to VCAM-1. CD81-expressing U937 cells were left untreated or incubated with mAb (5 ng/ml for 15 min at 37 °C) specific for CD81 (5A6) or L-selectin (DREG-200) or treated with activating mAb to β1 (TS2/16) and their strength of VLA-4-dependent adhesion to VCAM-1 (0.75 µg/ml) was analyzed. The mean ± range of two independent fields is depicted. D, tethering, rolling, and spontaneous arrest of CD81-null and CD81-expressing U937 cells interacting with VCAM-1 under continuous shear flow. Tethered cells, determined in two fields at 1 dyn/cm², were grouped into three categories, as described under “Experimental Procedures.” Inset depicts enlarged bars for tether categories of both cell types on the lowest tested density VCAM-1. Results in B and D are representative of four experiments. Results in C are of three independent experiments.
CD81 Regulates Integrin Avidity in Leukocytes

CD81 Facilitates VLA-5 but Not LFA-1 Avidity at Rapid Contacts—CD81 has been shown to associate preferentially with \( \alpha_4 \) integrins rather than with \( \alpha_5 \) or \( \beta_2 \) integrins in several cellular systems (36, 37). Because both CD81-expressing and CD81-null U937 cells express VLA-5 at equivalent levels (Fig. 4A), we tested whether CD81 could also augment rapid VLA-5 (\( \alpha_4\beta_1 \)) binding to a VLA-5 binding FN-derived fragment and to surface-immobilized anti-VLA-5 mAb. These experiments revealed that CD81 augmented VLA-5 adhesiveness to both native ligand and integrin-binding mAb (Fig. 4B). Thus, rather than selectively associating with \( \alpha_4 \) integrins in U937 cells, CD81 may associate with the common \( \beta_2 \) integrin subunit of both VLA-4 and VLA-5 integrins. Indeed, communoprecipitation studies confirmed strong CD81 association with the common \( \beta_2 \) integrin subunit in U937 monocytes but much less association with the \( \beta_1 \) integrin subunit (Fig. 4C and D). Thus, \( \beta_2 \) integrins appeared to be excluded from CD81 complexes with \( \beta_1 \) integrins. In accordance with such exclusion, although LFA-1 expression was identical in both cell types, CD81 did not alter LFA-1 adhesiveness to ICAM-1 (Fig. 4E). Thus, the ability of CD81 to stimulate VLA-4 clustering and avidity to ligand may depend on its extracellular or membranal association with the VLA-4 \( \beta \) subunit.

CD81 Facilitates Chemokine-triggered VLA-4 Avidity to VCAM-1 without Affecting Intrinsic Inside-out Chemokine Signaling—Chemokine signals through G-protein-coupled receptors transduced to transiently adherent leukocytes can rapidly trigger adhesiveness of their vascular integrins under shear flow (10). We therefore wished to determine whether the CD81-augmented ability of VLA-4 on U937 monocytes to generate high avidity to VCAM-1 also amplifies further avidity stimulation by G-protein-coupled receptor-mediated inside-out signaling. CD81-expressing and -null cells, expressing comparable levels of the SDF-1 receptor CXCR4, exhibited a similar magnitude of SDF-1-induced signaling to ERK1/2 (Fig. 5A), consistent with comparable levels of G protein signaling activity of the G-protein-coupled receptors (38). Accordingly, at low density VCAM-1, VLA-4 on both CD81-expressing and -null U937 cells was similarly activated by immobilized SDF-1, as reflected by a sharp increase in VLA-4-dependent tethering to VCAM-1 (Fig. 5B). Thus, on very low VCAM-1 density, where ligand-induced VLA-4 rearrangement is minimal (Figs. 2B and 5B), SDF-1-stimulated VLA-4 adhesiveness was independent of CD81. In contrast, at a slightly higher VCAM-1 density, the presence of CD81 facilitated SDF-1 signals to VLA-4 (Fig. 5C). Thus, CD81-augmented VLA-4 avidity strengthening and SDF-1 triggering of VLA-4 clustering are independently regulated and complement each other at subsecond adhesive contacts.

Direct Extracellular Stimulation of VLA-4 Bypasses CD81-facilitated Integrin Avidity Strengthening—Because high affinity recognition of VLA-4 did not rescue its defective ability to generate high avidity in CD81-null cells, we next investigated the ability of an alternative artificial extracellular modulation of VLA-4 to rescue the defective avidity of the integrin in CD81-null cells. The \( \alpha_4 \)-specific mAb, B5G10, is a non-blocking \( \alpha_4 \) mAb and does not stimulate integrin affinity to ligand (39), but could trigger robust comparable VLA-4-dependent U937 adhesion to VCAM-1 in both CD81-expressing and -null cells (Fig. 6) without altering pre-ligand integrin clustering. Interestingly, the B5G10 mAb triggered VLA-4 adhesiveness even below the threshold VCAM-1 density (Fig. 6) required for CD81 to up-regulate VLA-4 avidity (Fig. 2). Notably, the mAb stim...
CD81-regulated Integrin Avidity in Leukocytes

Fig. 4. CD81 facilitates VLA-5 integrin avidity but not LFA-1 avidity at rapid contacts. A and E, flow cytometry analysis of cell surface expression of VLA-5 (A) on CD81-deficient (upper panels) or CD81-expressing (lower panels) U937 cells, followed by secondary PE-labeled anti-mouse Ig. B, spontaneous adhesion of CD81-expressing or -null cells to VLA-5 binding FN-derived fragment FN-120 (0.2 µg/ml) at stasis. Strength of adhesion was analyzed as described in the legend to Fig. 1. The mean ± range of two independent experiments is depicted. Results are representative of two independent experiments each. C, β1 but not β2 integrins preferentially associate with CD81 in U937 monocytes. β1 and β2 integrins and the control selectin ligand, P-selectin glycoprotein ligand-1 (PSGL-1) (KPL-1) were immunoprecipitated from CD81-expressing U937 cells with TS2/16, TS1/18, and KPL-1 mAbs, respectively, and probed for co-association with CD81 by immunoblotting with α-CD81 mAb (5A6). D, flow cytometry of cell surface expression of β1 integrins (bold black line) or β2 integrins (bold gray line) on CD81-expressing U937 cells, using mAbs TS2/16 or TS1/18, respectively, followed by secondary PE-labeled anti-mouse Ig. E, spontaneous LFA-1-dependent adhesion of CD81-expressing or -null cells to high density ICAM-1 (5 µg/ml) was analyzed as described in the legend to Fig. 1.

PMA could trigger robust VLA-4-dependent U937 binding to the surface-bound anti-α4 integrin mAb, HP1/2 (Fig. 7B). Strikingly, this PMA stimulation of α4 integrin binding to immobilized mAb took place only in CD81-expressing cells (Fig. 7B). All stimulatory activities of PMA were abolished by the broad DAG-dependent PKC inhibitor, bisindolylmaleimide I, or with a selective inhibitor to the DAG-dependent PKCβ isofrom, LY379196 (25). Despite the poor PMA responsiveness of VLA-4 in CD81-null cells observed both on VCAM-1 and mAb (Fig. 7, A and B), CD81-null cells expressed identical levels of all major DAG-dependent PKC isoforms found in CD81-expressing cells (Fig. 7D). These results, taken together with PMA inhibition studies (above) and the expression pattern of the major PKC isoforms found in our U937 cells, collectively suggest that the major DAG-dependent PKC mediating the preferential VLA-4 avidity stimulation in CD81-expressing monocytes is PKCβII.

Recent evidence suggested that conventional DAG-dependent PKC isoforms directly associate with CD81 upon PMA-induced activation and translocation to the membrane (19). Because PMA stimulation of VLA-4 was mediated by PKCβII, we asked whether this isofrom is preferentially associated with CD81 in PM-treated U937 cells. Surprisingly, we could not detect PMA-triggered PKCβII association with CD81 in these monocytes (Fig. 7E), but instead identified MHC class I, as a
scaffold for this PKC in both CD81-expressing and -null U937 cells (Fig. 7E). PKC activation, although having no affect on U937 cell adhesion to mAbs directed to membranal proteins not associated with CD81, such as PSGL-1 and LFA-1 (Fig. 4E), increased cellular adhesion to a surface-bound MHC-I-specific mAb. Consistent with its role as a PKC scaffold both in CD81-expressing and -null U937 cells, PKC-triggered U937 cell adhesion to the MHC-I-specific mAb was comparable in both CD81-expressing and -null cells. It seems, therefore, that MHC-I rather than CD81 recruits the major DAG-dependent PKC, \( \zeta \)-II, in U937 monocytes to the membrane. CD81 is complexed, however, with MHC-I and VLA-4 (Fig. 7E). This CD81-mediated enhanced responsiveness of VLA-4 to phorbol ester stimulation could reflect constitutive exposure of VLA-4 to endogenously activated PKC homeostatically recruited to the leukocyte surface. Indeed, cultured CD81-expressing U937 monocytes treated overnight with a DAG-dependent PKC blocker exhibited reduced VLA-4-dependent adhesion to both ligand and mAb. These results suggest that DAG-dependent PKC activities potentiate the ability of VLA-4 to instantaneously generate high avidity to VCAM-1 in CD81-expressing rather than in CD81-null monocytes.

**DISCUSSION**

To date, no integrin-associated membranal proteins have been shown to directly modulate integrin adhesiveness during leukocyte encounters with endothelial ligands under shear flow. In this report, we demonstrate that the CD81 tetraspanin is an essential regulator of U937 monocytes and B cell VLA-4 adhesiveness developed at subsecond adhesive contacts. Both VLA-4 and VLA-5 but not \( \alpha_4 \beta_2 \) (LFA-1) integrins undergo exceptionally rapid ligand-induced stabilization events preferentially in CD81-expressing cells. Corresponding to their regulation by CD81, \( \alpha_1 \beta_2 \) but not \( \beta_2 \) integrins are present in membranal complexes with CD81. Although preformed clustering, affinity, and intrinsic adhesiveness to highly diluted ligand of VLA-4 appear largely intact in CD81-deficient cells, the presence of CD81 is obligatory for simultaneously ligand-occupied
VLA-4 molecules to generate rapid adhesion strengthening in leukocytes interacting under shear flow with VCAM-1. The CD81-facilitated integrin avidity to ligand results in augmented VLA-4-dependent leukocyte capture, rolling, firm arrest, and shear-resistant adhesion to VCAM-1 under shear flow. CD81 also dramatically facilitates integrin avidity stimulation by DAG-dependent PKC although it is not essential for integrin stimulation by G protein-triggered chemokine signals. This is the first demonstration of a homeostatic role of an integrin-associated tetraspanin in up-regulating both spontaneous and agonist-triggered integrin avidity at dynamic endothelial and extracellular matrix contacts.

A key regulator of the novel cross-talk found in this study between VLA-4 and CD81 was the DAG-dependent PKC$_{\beta II}$ based on the involvement of both endogenous and exogenously activated PKC in CD81-augmented VLA-4 avidity (Fig. 7). Our results, however, ruled out the attractive possibility that CD81 acts as a specialized scaffold for PKC (19), bridging activated PKC and $\beta_1$ integrin members such as VLA-4 and VLA-5. In the monocytes investigated by us, CD81 does not recruit the major DAG-dependent, PKC isoform (PKC$_{II}$) responsible for phorbol ester-triggered VLA-4 avidity. Rather, the $\beta_1$ integrin-associated partner, MHC-I, appears to recruit equivalent levels of PKC$_{\beta II}$ in both CD81-expressing and CD81-null cells. Preferential recruitment of PKC activity to the vicinity of $\beta_1$ integrin targets by Rack1, a receptor for activated protein kinase C (40), reported to facilitate integrin associations with activated PKCs (41), was also excluded. Both CD81-expressing and CD81-null U937 cells pretreated with cell-permeable Rack1-derived peptide blockers of membranal PKC recruitment (40) failed to inhibit either constitutive or PMA-stimulated VLA-4 avidity. We therefore conclude that PKC is recruited to the membrane of monocytes independently of CD81, possibly at MHC-I/$\beta_1$ integrin containing domains. The recruited PKC phosphorylates one or more of the cytoplasmic integrin partners involved in VLA-4 avidity stimulation at VCAM-1 or anti-integrin mAb bearing sites.

What could then be the mechanism of such CD81-facilitated, PKC-regulated, affinity-independent VLA-4 avidity strengthening? Ligand-independent integrin clustering can result from enhanced lateral mobility of integrins in the membrane through release from cytoskeletal restraints (42–45). This clustering mode is usually reflected in induction of integrin patches on the cell membrane (8, 46, 47). However, we did not detect any increased levels of VLA-4 patches in resting or PMA-stimulated CD81-expressing cells (Fig. 3C). In addition, low doses of cytochalasin D shown to increase integrin mobility in various cell types (42, 43, 46) strongly decreased the adhesion strengthening of VLA-4 on both VCAM-1 and immobilized VLA-4 binding mAbs. Furthermore, PKC-mediated phosphorylation of the cytoskeletal remodeling effector MacMarcks and its related Marcks (48) has been shown to increase LFA-1 mobility and avidity to ICAM-1 (44). However, phorbol ester stimulation of LFA-1 avidity in U937 cells required the presence of functional MacMarcks (49). We found that both CD81-expressing and -null U937 clones expressed negligible amounts of MacMarcks or the related Marcks (48) has been shown to increase LFA-1 mobility and avidity to ICAM-1 (44). However, phorbol ester stimulation of LFA-1 avidity in U937 cells required the presence of functional MacMarcks (49). We found that both CD81-expressing and -null U937 clones expressed negligible amounts of MacMarcks or the related Marcks (48). Notably, MacMarcks is also not expressed in murine splenic B cells (50), where CD81 facilitates VLA-4 avidity to VCAM-1 (Fig. 1). Nevertheless, we cannot exclude a role for an alternative PKC-dependent mod-

---

**Fig. 7.** Impaired PKC-dependent stimulation of VLA-4 avidity despite normal PKC function. Frequency of VLA-4-mediated tethers of control or PMA-stimulated (10 ng/ml, 2 min) CD81-null or CD81-expressing U937 cells interacting with VCAM-1 (0.25 $\mu$g/ml) or immobilized $\alpha_e$-specific mAb, HP1/2 (1 $\mu$g/ml). The different tether categories were determined in two fields at 1 dyn/cm$^2$ and results are an average of each tether category. All PMA-induced tethers were inhibited by the PKC blocker, bisindolylmaleimide I. Similar expression of the three major DAG-dependent PKC isoforms in CD81-null and -expressing U937 cells determined with $\alpha$-PKC isoform-specific antibodies. Actin expression is shown as control. D, similar PKC signaling to ERK in CD81-expressing and -null U937 cells. Cells were left untreated or stimulated with PMA (10 ng/ml, 2 min) at 37 °C and immunoblotted as described in the legend to Fig. 5A. E, MHC-I but not CD81 is a scaffold for activated PKC$_{\beta II}$. PMA-activated cells were immunoprecipitated with $\alpha$-CD81 or $\alpha$-MHC-I antibodies, and then immunoblotted for PKC$_{\beta II}$ (top panel), CD81 (middle panel), or VLA-4 (lower panel).
ulator of VLA-4 avidity regulated by CD81, because this kinase may modify several regulatory cytoskeletal partners of β1 integrins, like talin (51). Notably, a restriction of VLA-4 mobility has been correlated with normal tethering but reduced adhesion strengthening (5, 43, 52). It is therefore still possible that the enhanced PKC triggered VLA-4 avidity observed in CD81-expressing cells but not in CD81-null cells, is, at least partially because of enhanced integrin mobility. We thus conclude that the CD81-stimulated VLA-4 avidity is likely to involve a novel post-ligand occupancy process by which integrins strengthen their multivalent contacts, possibly by enhanced mobility and additional outside-in rearrangements, the full nature of which remains to be explored.

CD81 is a ubiquitous tetraspanin that associates with other tetraspanins in specialized webs (16, 17) and acts as a scaffold for co-stimulatory molecules, including CD19, MHC-II, MHC-I, CD4, CD8, and cytoplasmic signaling kinases (16). U937 monocyte cells express multiple tetraspanins, including CD82 and CD151 which, like CD81, were shown in other cells to associate with activated DAG-dependent PKCs (19). Within this large group of tetraspanins, CD81 and CD9 comprise a subgroup of tetraspanins, which target endothelial and extracellular sites into proadhesive in-

ACKNOWLEDGMENTS—We thank Drs. R. Lobb, D. Mochny-Rosen, E. Rubinstein, C. Boucheix, and M. Hemler for gifts of reagents and cell lines. We thank Dr. S. Schwarzbaum for editorial assistance and Dr. C. Brodie (Bar-Ilan University, Israel) for helpful discussions.

REFERENCES

1. Springer, T. A. (1994) Cell 76, 301–314

2. Alon, R., and Feigelson, S. (2002) Semin. Immunol. 14, 93–104

3. Berlin, C., Bargatze, R. F., Campbell, J. J., von Andrian, U. H., Szabo, M. C., Hassel, S. R., Nelson, R. D., Berg, E. L., Erlandsen, S. L., and Butcher, E. C. (1995) Cell 80, 413–422

4. Henderson, R. B., Lim, L. H., Tessier, P. A., Gavins, F. N., Mathies, M., Ferretti, M., and Hogg, N. (2001) J. Exp. Med. 194, 219–226

5. Alon, R., Kassner, P. D., Carr, M. W., Finger, E. R., Hemler, M. E., and Springer, T. A. (1995) J. Cell Biol. 128, 1243–1253

6. Sigal, A., Bleiss, D. A., Grabovsky, V., van Vliet, S. J., Dwir, O., Figdor, C. G., van Kooyk, Y., and Alon, R. (2000) J. Exp. Med. 192, 495–505

7. Berditchevski, F., Zutter, M. M., and Hemler, M. E. (1996) Trends Cell Biol. 6, 390–396

8. Maecker, H. T., Talbot, S. C., and Levy, S. (1997) FASEB J. 11, 428–442

9. Hemler, M. E. (1998) Curr. Opin. Cell Biol. 10, 578–585

10. Grabovsky, V., Feigelson, S., Winter, E., Chen, L. L., Pepsnyk, R. B., Yednock, T., Yablonka, D., Lobb, R., and Alon, R. (2001) J. Biol. Chem. 276, 12891–12901

11. Grabovsky, V., Feigelson, S., Chen, C., Bleiss, R., Peled, A., Cinnamon, G., Baleux, F., Arzenaus-Sieder, F., Lapidot, T., van Kooyk, Y., Lobb, R., and Alon, R. (2000) J. Exp. Med. 192, 495–505

12. Berditchevski, F., Tolias, K. F., Wong, K., Carpenter, C. L., and Hemler, M. E. (1999) J. Biol. Chem. 274, 2598–2604

13. Hemler, M. E. (1998) J. Biol. Chem. 273, 2139–2145

14. Brodie, F., Rubinstein, C., Boucheix, and M. Hemler for gifts of reagents and cell lines. We thank Dr. S. Schwarzbaum for editorial assistance and Dr. C. Brodie (Bar-Ilan University, Israel) for helpful discussions.

15. Berditchevski, F., Zutter, M. M., and Hemler, M. E. (1996) Trends Cell Biol. 6, 390–396

16. Maecker, H. T., Talbot, S. C., and Levy, S. (1997) FASEB J. 11, 428–442

17. Hemler, M. E. (1998) Curr. Opin. Cell Biol. 10, 578–585

18. Rubinstein, E. (1999) Biochem. J. 341, 271–277

19. Zhang, X. A., Bontrager, A. L., and Hemler, M. E. (2001) J. Biol. Chem. 276, 25005–25015

20. Berh, S., and Schriever, F. (1995) J. Exp. Med. 182, 1191–1199

21. Fenclz, C. A., Sethi, T., Ramos, J. W., Hughes, P. E., and Ginsberg, M. H. (1997) Nature 390, 81–85

22. Shibagaki, N., Hamada, K., Yamashita, H., Shimada, S., and Hamada, H. (1999) Eur. J. Immunol. 29, 4081–4091

23. Lammerding, J., Kazarov, A. R., Huang, L., Lee, R. T., and Hemler, M. E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7616–7621

24. Torcini, M., Raimondi, V., Lamy, L., Wijdenes, J., Lindberg, F. P., Brown, E. J., and Bernard, A. (2001) FASEB J. 15, 341–350

25. Jirovsky, M. R., Gillig, J. B., Gonzalez, C. M., Heath, W. F., McDonald, J. H., and Heath, W. F. (1995) J. Cell Biol. 130, 171–181

26. Baker, B. W., Boettiger, D., Spooner, E., and Norton, J. D. (1992) Nucleic Acids Res. 20, 5234

27. Maecker, H. T., and Levy, S. (1997) J. Exp. Med. 185, 1505–1510

28. Dwir, O., Kanssa, G. S., and Alon, R. (2000) J. Biol. Chem. 275, 18682–18691

29. Dwir, O., Yeung, Y. P., Tsetoura, D., DeKruyff, R. H., Umesato, D. T., and Levy, S. (2000) J. Immunol. 165, 5054–5061

30. Lin, K., Ateoq, H. S., Hsiung, S. H., Chong, L. T., Zimmerman, C. N., Castro, A. L., McCord, C. E., Kalkunte, S., Chen, L. L., Pepsnyk, R. B., Leon, D. R., Sprague, A. G., Abraham, W. M., Gill, A., Lobb, R. B., and Adams, S. P. (1999) J. Med. Chem. 42, 920–934

31. Todd, S. C., Lippe, S. G., Crusca, L., Salomon, D. B., and Tsukada, S. U. (1996) J. Exp. Med. 184, 2055–2060

32. Takagi, J., Okada, M., Matsumoto, J., and Shinkuma, M. (1995) J. Biol. Chem. 270, 4243–4248

33. Sierra, V., Vazquez, F., Billard, M., Azorza, D. O., Lanza, F., Boucheix, C., and Rubinstein, E. (1999) Biochem. J. 340, 103–111

34. Ganju, R. K., Brubaker, S. A., Meyer, J. D., Dutt, P., Yang, Y., Qin, S., Newman, W., and Groupman, J. E. (1998) J. Biol. Chem. 273, 23169–23175

35. Ramata, T., Puzon, W., and Takada, Y. (1999) Biochem. J. 345, 945–951

36. Brodie, F., Liu, L., and Mochly-Rosen, D. (1995) J. Biol. Chem. 270, 24170–24176

37. Besson, A., Wilson, T. L., and Yong, W. V. (2002) J. Biol. Chem. 277, 22073–22084

38. Kuric, D. F., Dustin, M. L., Miller, J. M., and Brown, E. J. (1996) J. Clin. Invest. 97, 2139–2144

39. Auerh, R. L., Felsenfeld, D. P., Kraeft, S. K., Chen, L. B., Sheetz, P. M., and Hemler, M. E. (1997) J. Exp. Med. 186, 1–9

40. CD81-regulated Integrin Avidity in Leukocytes
