A novel form of integrin dysfunction involving β1, β2, and β3 integrins

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Alison McDowall,[1] David Inwald,[2] Birgit Leitinger,[1] Alison Jones,[3] Ri Liesner,[4] Nigel Klein,[2] and Nancy Hogg[1]

[1]Leukocyte Adhesion Laboratory, Cancer Research UK London Research Institute, Lincoln's Inn Fields Laboratories, London, United Kingdom
[2]Immunobiology Unit, Institute of Child Health, London, United Kingdom
[3]Department of Clinical Immunology, and
[4]Department of Haematology, Great Ormond Street Hospital for Children NHS Trust, Great Ormond Street, London, United Kingdom

The adhesion receptors known as integrins perform key functions for hematopoietic cells. The platelet integrin αIIbβ3 is critical in hemostasis, and the β1 and β2 integrins on leukocytes have many roles in cell-mediated immunity. Mutations in the β2 subunit lead to integrin nonexpression and to an immune deficiency, leukocyte adhesion deficiency-1. Mutations in either the α or β subunit of αIIbβ3 usually lead to integrin nonexpression and a bleeding tendency termed Glanzmann thrombasthenia. Here we describe a unique patient with clinical features of both Glanzmann thrombasthenia and leukocyte adhesion deficiency-1. The patient has normal expression of β1, β2, and β3 integrins, but all are dysfunctional. The key findings are that "inside-out" signaling pathways leading to integrin activation are defective and that this is associated with abnormal integrin clustering. The integrins themselves are intact and capable of function following extracellular stimulation. T cell motility is normal, as are the expression levels and electrophoretic characteristics of all cytoskeletal and signaling proteins tested, except PKC-α, which has enhanced expression in the patient's cells. To our knowledge, this is the first description of a dysfunction affecting three classes of integrins. We propose that it is caused by a lesion in an intracellular factor or signaling pathway essential for integrin activation in hematopoietic cells and results in lack of regulation of clustering, an essential component of integrin-mediated adhesion.

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Introduction

The integrins are a widely expressed family of noncovalently linked α and β subunits that mediate cell-cell and cell-extracellular matrix interactions. The integrins on circulating leukocytes and platelets bind minimally to their ligands, but their adhesive capacity can be increased by stimulation of intracellular signaling pathways, for example by chemokines and bacterial peptides, triggering of the T cell receptor complex, or artificially, with phorbol esters. This mechanism of integrin activation has been termed inside-out signaling. It is also possible to activate integrins with divalent cations such as Mg^{2+} and Mn^{2+} or with special anti-integrin mAb's. These agonists act directly on the integrin ectodomain and stimulate adhesion to ligand (reviewed in ref. 1).

Inside-out signaling induces two major forms of alteration to integrins that enable efficient ligand binding: integrins can undergo conformational change leading to higher affinity receptors and can diffuse laterally in the membrane to form higher avidity clusters. In leukocytes, signals leading to clustering of integrins seem to predominate, and the clustering depends on cytoskeletal reorganization and the protease calpain (2, 3). In platelets, inside-out signaling primarily leads to affinity alteration of integrin αIIbβ3, but clustering is also part of the activation process (4, 5). Once activated (and ligand bound), the integrin signals back into the cell, and this is termed outside-in signaling.

Active β1 and β2 integrins play a crucial role in the extravasation of leukocytes from the circulation to sites of injury or infection and in the homing of lymphocytes to tissues, particularly the secondary lymphoid organs. Specifically, integrins α4β1 and LFA-1 mediate firm adhesion of the leukocytes and transendothelial migration. Neutrophils and monocytes are the first cells to be

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Address correspondence to: Nancy Hogg, Leukocyte Adhesion Laboratory, Cancer Research UK London Research Institute, Lincoln's Inn Fields Laboratories, London WC2A 3PX, United Kingdom. Phone: 44-207-269-3255; Fax: 44-207-269-3093; E-mail: nancy.hogg@cancer.org.uk.
Birgit Leitinger's present address is: Sackler Institute for Muscular Skeletal Research, Department of Medicine, University College London, London, United Kingdom.

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Nonstandard abbreviations used: leukocyte adhesion deficiency-1 (LAD-1); Glanzmann thrombasthenia (GT); phorbol-12,13-dibutyrate (PdBu); platelet-rich plasma (PRP); thrombin receptor agonist peptide (TRAP); platelet-poor plasma (PPP); integrin-linked kinase (ILK).

recruited to inflammatory sites where, making use of β2 integrins (CD11/CD18), LFA-1, Mac-1, and p150,95, they phagocytose and undergo respiratory burst in response to bacterial infection. The platelet integrin αIIbβ3 is stimulated to bind fibrinogen by agonists, such as thrombin, ADP, and thrombospondin, released at sites of vascular injury. This event is pivotal to the aggregation of platelets that is required for formation of the platelet plug and clotting of blood.

In humans, two inherited autosomal recessive diseases result from germline mutations in the genes encoding integrins specific to cells of hematopoietic origin. These disorders are leukocyte adhesion deficiency-1 (LAD-1) and Glanzmann thrombasthenia (GT) (6, 7). The hallmark of these disorders is lack of expression of the affected integrin. LAD-1 is caused by mutations in the β2 subunit of the leukocyte integrins. The lack of β2 integrin function results in elevated numbers of circulating neutrophils because these cells fail to adhere to or migrate across the endothelium. LAD-1 patients are susceptible to recurring, life-threatening, bacterial infections, which are typically evident in soft tissue. Severely affected people often die of infection in childhood or early adulthood unless bone marrow transplantation is successfully accomplished. The bleeding disorder GT is usually caused by mutations in either the α or β subunit of the platelet integrin αIIbβ3 (CD41/CD61). The platelets are unable to bind to fibrinogen and thus fail to aggregate and form a primary hemostatic plug in response to agonists. GT patients suffer easy bruising, mucocutaneous bleeding, and, occasionally, gastrointestinal and intracranial bleeding.

Here we describe an unusual patient with clinical features of both LAD-1 and GT. The results suggest that the patient has a novel form of integrin dysfunction in which the β1, β2, and β3 integrins are expressed on the cell surface at normal levels but cannot be stimulated to bind ligand by intracellular signaling pathways.

Methods

The following Ab's were gifts: KIM 185 (β2 activating) from M. Robinson (Celltech Group PLC, Slough, United Kingdom); HUTS21 (β1 activation reporter) from C. Cabañas (CSIC-Universidad Complutense, Madrid, Spain); IB4 (β2 function blocking) from S.K. Law (University of Oxford, Oxford, United Kingdom); 2E7 (β2 blotting) from C. Gahmberg (University of Helsinki, Helsinki, Finland); LIBS6 (αIIbβ3 activating) from M. Ginsberg (The Scripps Research Institute, La Jolla, California, USA); anti-Vav from V. Tybulewicz (National Institute for Medical Research, London, United Kingdom); anti–PKC-ζ from P. Parker (Cancer Research UK, London, United Kingdom). The following mAb's were produced at Cancer Research UK: 38 (αL function blocking and blotting); ICRF44 (αM); 3.9 (αX); 24 (β2 activation reporter); 7.2R (α4 blotting); UCHT-1 (CD3). The hybridoma cell lines producing TS1/18 (β2), P5D2 (β1), and TS2/16 (β1 activating) were obtained from American Type Culture Collection (Rockville, Maryland, USA). HP2/1 (α4 function blocking) and PM6/13 (β3 blotting) were purchased from Serotec Ltd. (Oxford, United Kingdom); SAM-1 (α5 function blocking) was purchased from Eurogenetics UK Ltd. (Hampton, United Kingdom); SZ22 (αIIb blotting) was purchased from Immunotech (Marseilles, France); anti-fibrinogen, 5B12 (αIIb), and Y2/51 (β3) were purchased from DAKO Ltd., (Ely, United Kingdom) in FITC-conjugated form; G25.2 (αL nonfunction blocking), FITC-conjugated PAC-1 (αIIb activation reporter), and Abs against Rac-1, Cdc42, Rap-1, SLAP-130, ILK, and PKC-α, -β, -δ, and -θ were purchased from Becton Dickinson UK Ltd. (Oxford, United Kingdom); B3B11 (β1 blotting) and anti-filamin were purchased from Chemicon International Ltd. (Harrow, United Kingdom). Ab's against talin, α-actinin, vinculin, ezrin, paxillin, and actin were purchased from Sigma-Aldrich (Poole, United Kingdom). Ab's against RhoA and Rac-2 were obtained from Autogen Bioclear UK Ltd. (Calne, United Kingdom. The reagents FMLP, phorbol-12,13-dibutyrate (PdBu), ionomycin, thapsigargin, and 2′,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) were obtained from CN Biosciences UK Ltd. (Nottingham, United Kingdom). ICAM-1Fc and ICAM-3Fc were produced as described previously (8), VCAM-1Fc was a gift from M. Robinson (Celltech Group PLC). ADP, fibronectin, and fibrinogen were purchased from Sigma-Aldrich and thrombin receptor agonist peptide (TRAP) was purchased from Peninsula Laboratories (St. Helen's, United Kingdom).

Preparation of platelets, neutrophils, T cells and Epstein-Barr virus–transformed B cells. Blood samples were collected following approval by the Great Ormond Street/Institute of Child Health Ethics Committee and the parents' written informed consent. Platelet-rich plasma (PRP) was prepared by collecting blood into 0.38% sodium citrate followed by centrifugation at 150 *g* for 10 minutes at room temperature. To prepare washed platelets, PRP was spun at 800 *g* for 5 minutes, and the pellet was washed with HBSS without cations and resuspended in RPMI-1640. All steps were performed in the presence of 30 ng/ml prostacyclin (Glaxo-SmithKline, Uxbridge, United Kingdom) to prevent platelet activation.

Neutrophils and T cells were purified from EDTA- or heparin-anticoagulated venous blood. Neutrophils and PBMCs were fractionated, and T cells were cultured as described previously (8).

Epstein-Barr virus–transformed (EBV-transformed) B lymphoblastoid cells were derived from patient FM and a control donor by the Research Cell Services, Cancer Research UK, using standard procedures. The cells were maintained in RPMI-1640 with 10% FCS.

Flow cytometry. Leukocytes (5×10^5) were incubated for 20 minutes on ice in 50 μl of PBS/0.2% BSA containing primary mAb's. For detection of activation epitopes, the mAb's were diluted in HEPES buffer (20 mM HEPES, 140 mM NaCl, 2 mg/ml glucose, pH 7.4) containing 0.5 mM $MnCl_2$, and the incubation performed

at 37°C. Bound mAb was detected by incubation with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) for 20 minutes on ice.

For platelets, 5 μl of PRP was added to 50 μl of platelet buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, pH7.4) (9) containing fluorochrome conjugated mAb and agonists and incubated at 37°C for 20 minutes in an adaptation of a previously described method (10). Experiments looking at PAC-1 and fibrinogen binding were also performed in the presence of 20 μg/ml eptifibatide (integrilin) (Schering-Plough Ltd., Welwyn Garden City, United Kingdom), an αIIbβ3 antagonist.

For soluble ligand binding, ICAM-1Fc and VCAM-1Fc at 300 and 2 μg/ml, respectively (saturating levels), were incubated with T cells using methodology as described previously (11).

Aggregometry. For aggregometry, the residual blood following PRP collection was centrifuged at 1,200 *g* for 15 minutes at room temperature to obtain platelet-poor plasma (PPP). PRP with a final platelet count of 250×10^9/l was prepared by diluting PRP with PPP. Aggregometry was performed at 37°C with a stir speed of 900 rpm. Platelet agonists were added to the cuvettes and light transmission recorded.

SDS-PAGE and Western blotting of detergent soluble cell extracts. T cells were suspended at 5×10^7/ml and platelets at 10^9/ml in ice-cold lysis buffer (50 mM Tris pH 8 containing 150 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 μg/ml aprotonin, 20 μg/ml PMSF, and 1% Triton X-100), and lysed for 20 minutes on ice. The lysate was microfuged for 15 minutes to remove insoluble material. Proteins were separated by SDS-PAGE. After transfer to nitrocellulose membrane and incubation with primary Ab's, the bound Ab was detected with HRP-conjugated sheep anti-mouse Ig (Amersham Biosciences UK Ltd., Chalfont St. Giles, United Kingdom) or HRP-conjugated goat anti-rabbit Ig (DAKO Ltd.) and enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences UK Ltd.). Quantification of bands at subsaturating levels was performed using NIH Image 1.60 Software.

Adhesion assays. T cell adhesion to immobilized ICAM-1Fc, ICAM-3Fc, VCAM-1Fc (all at 5 μg/ml) or fibronectin (20 μg/ml), and EBV-transformed B cell binding to immobilized ICAM-1 (5 μg/ml) or fibronectin (20 μg/ml) were performed using similar methods, as described previously (8). Neutrophil adhesion to immobilized fibrinogen (0.5 mg/ml) was performed as described previously (8).

Microscopy. For video microscopy, 35 mm glass-bottom microwell dishes (Mattek Corp, Ashland, Massachusetts, USA) were coated overnight with 10 μg/ml ICAM-1Fc, then blocked with 2.5% BSA/PBS. One milliliter of T cells (4×10^5/ml in HEPES buffer) was allowed to settle for 4 minutes before addition of 500 μl of HEPES buffer containing 15 mM $MgCl_2$/3 mM EGTA. Images were taken at 5 second intervals for 20 minutes using a Nikon Diaphot 300 microscope and AQM[2001] Kinetic Acquisition Manager (Kinetic Imaging Ltd., Bromborough, United Kingdom). Cells were tracked using Motion Analysis software (Kinetic Imaging Ltd.) and the data analyzed using a Mathematica notebook (Wolfram Research Europe Ltd., Long Hanborough, United Kingdom) developed by Daniel Zicha (Cancer Research UK).

Samples were prepared for confocal microscopy as described previously (12), except that 2×10^6 cells were used.

Results

Patient FM was born at term by elective Caesarean section to nonconsanguineous Maltese parents. She had two female siblings, of whom one is 7 years old and well, whereas the other died within hours of birth with widespread bruising and bleeding. Despite her atraumatic delivery, patient FM was noted to have extensive bruising and petechiae within hours of birth. She had an antenatal intraventricular hemorrhage and later required insertion of a ventriculo-peritoneal shunt for posthemorrhagic hydrocephalus. A platelet count and routine clotting screen performed at this point were in the normal range. The umbilical cord separated normally at 1 week of age. At three months of age she was referred for investigation of prolonged bleeding following minor trauma.

The platelet count, thrombin time, activated partial thromboplastin time, prothrombin time, and fibrinogen levels were all within the normal range. However, platelet aggregation was absent in response to ADP, collagen, and arachidonic acid. In contrast, platelets from both parents aggregated normally in response to these agonists. Flow cytometry revealed that the patient's platelets had normal expression of GPIb and αIIbβ3. These results suggested a diagnosis of type 2 GT with dysfunctional, rather than absent, platelet αIIbβ3. She has been managed with tranexamic acid and platelet transfusions as required.

From 5 months of age the patient developed recurrent bacterial infections and at 11 months she developed leg ulcers and was commenced on prophylactic antibiotics. She was found to have leukocytosis (38.4×10^9/l; normal range 5×10^9–15×10^9/l), suggestive of a leukocyte adhesion defect, but had normal expression of the leukocyte integrin αL, αM, αX, and β2 subunits and the selectin ligand sialyl Lewis x. She had normal humoral immune responses to tetanus toxoid and *Haemophilus influenza*, neutrophil phagocytosis of *Staphylococcus aureus,* and oxidative burst. Her lymphocyte count, however, was high (15.7×10^9/l; normal range of 1.5×10^9–4×10^9/l). Her T cell mitogenic activity to phytohemagglutinin was reduced by 50% compared with healthy age-matched controls. The patient is now 3 years old and has undergone a successful bone marrow transplant. There is no family history of either LAD-1– or GT-type disorders.

Integrin expression on the patient's platelets, neutrophils, and T cells. Because the patient displayed symptoms indicative of both leukocyte and platelet integrin dysfunction,

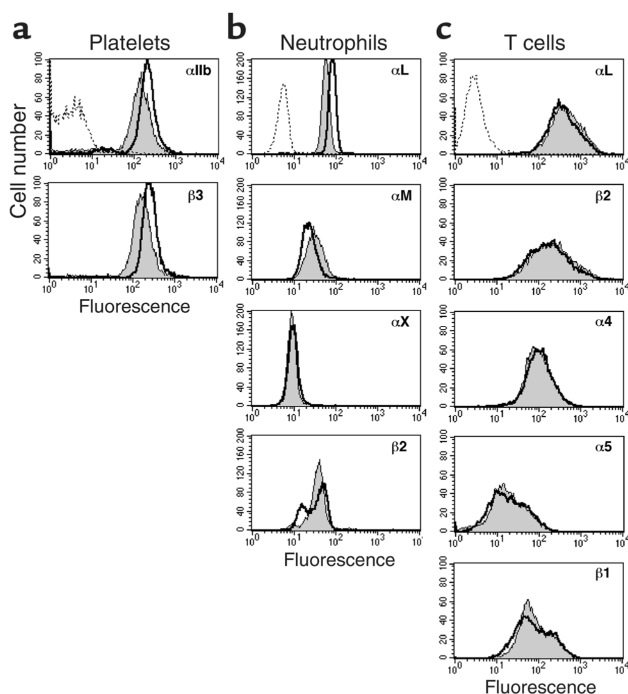

Figure 1
Comparison of integrin expression on platelets, T cells, and neutrophils from patient and control. Expression of (**a**) αIIb and β3 subunits on platelets (n = 2). (**b**) αL, αM, αX, and β2 subunits on neutrophils. (**c**) αL, β2, α4, α5, and β1 subunits on T cells from control (black line) and patient (gray region). Background binding is indicated (dotted line) and is identical for control and patient. Representative histograms (n = 3) are shown.

the cell surface expression of the major integrins on platelets, neutrophils and T cells was analyzed by flow cytometry. The overlapping profiles in Figure 1 show that the expression of the αIIb and β3 subunits on platelets, the αL, αM, αX, and β2 subunits on neutrophils, and the αL, β2, α4, α5, and β1 subunits on T cells was similar for the patient and a control donor.

To determine whether the patient's integrins were abnormally processed or posttranslationally modified, cell lysates were subjected to SDS-PAGE and Western blotting for the relevant integrin subunits. No differences were detected in the electrophoretic characteristics of the αIIb or β3 integrin subunits of platelet lysates (Figure 2a) or the αL, β2, α4, or β1 subunits of T cell lysates (Figure 2b) prepared from the patient and a control donor. Therefore, the patient's integrins resembled normal controls in both expression and biochemical characteristics.

Functional analysis of αIIbβ3 on the patient's platelets. Although the patient had normal cell surface expression of the three classes of integrins tested, it was possible that her symptoms were due to the inability of these integrins to function normally. The function of the platelet integrin αIIbβ3 was assessed by aggregometry. Although control platelets responded to 5 μM ADP as expected, the patient's platelets did not aggregate (Figure 3a). We next assessed the ability of two standard platelet agonists, ADP and TRAP, which signal through two distinct platelet receptors, to cause platelets to bind soluble fibrinogen (Figure 3b). Both stimuli induced binding of control platelets to fibrinogen, and this was inhibited by the αIIbβ3 antagonist, eptifibatide; however, the patient's platelets failed to bind soluble fibrinogen under any circumstances of inside-out stimulation. These agonists induced upregulation of α-granule contents, such as P-selectin, indicating platelet activation was normal (data not shown). Another way to activate integrins is to use mAb's, such as LIBS-6, which stimulate αIIbβ3 by direct activation of the ectodomain (termed outside-in signaling) (13). LIBS-6 induces expression of the αIIbβ3 activation epitope recognized by the mAb PAC-1 (14). Here LIBS-6 induces the PAC-1 epitope on both patient and control platelets (Figure 3c).

Functional analysis of Mac-1 on the patient's neutrophils. The function of the β2 integrin Mac-1 was examined by inducing neutrophil binding to immobilized fibrinogen. A variety of activating stimuli were used, which tested both inside-out and outside-in signaling to integrins. All treatments caused control neutrophils to bind to fibrinogen in a β2 integrin-dependent manner (Figure 4). In contrast, the patient's neutrophils failed to bind in response to either FMLP or the phorbol ester PdBu (inside-out signaling), but did bind to fibrinogen following exposure to the β2 activating mAb KIM 185. Therefore, the ability of Mac-1 on the patient's neutrophils to bind fibrinogen was impaired in response to typical stimulants of inside-out signal transduction.

However, the patient's neutrophils were able to mobilize intracellular Ca^{2+} in response to FMLP and the Ca^{2+} mobilizing agent thapsigargin, and FMLP induced similar levels of L-selectin shedding and Mac-1 upregulation in patient and control neutrophils (data not shown). Therefore, the patient's neutrophils are responsive to FMLP in non-integrin–dependent ways.

Functional analysis of LFA-1 on the patient's T cells. The function of the β2 integrin LFA-1 was examined on T cells by inducing adhesion to immobilized ligands. PdBu, the Ca^{2+} mobilizer ionomycin, or CD3 mAb

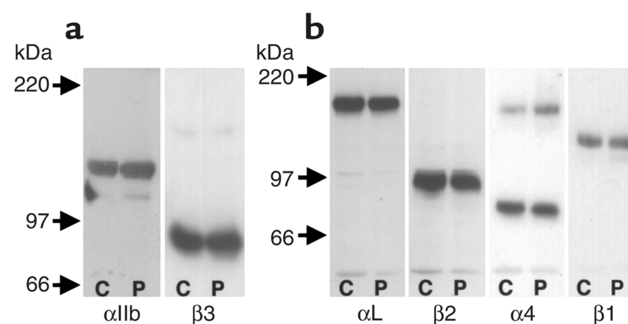

Figure 2
Electrophoretic characteristics of integrin subunits from patient and control platelets and T cells. (**a**) Platelet lysates from a control donor (C) and the patient (P) blotted for αIIb and β3 subunits. (**b**) T cell lysates from a control donor (C) and the patient (P) blotted for αL, β2, α4, and β1 subunits. Representative blots (n = 2) are shown.

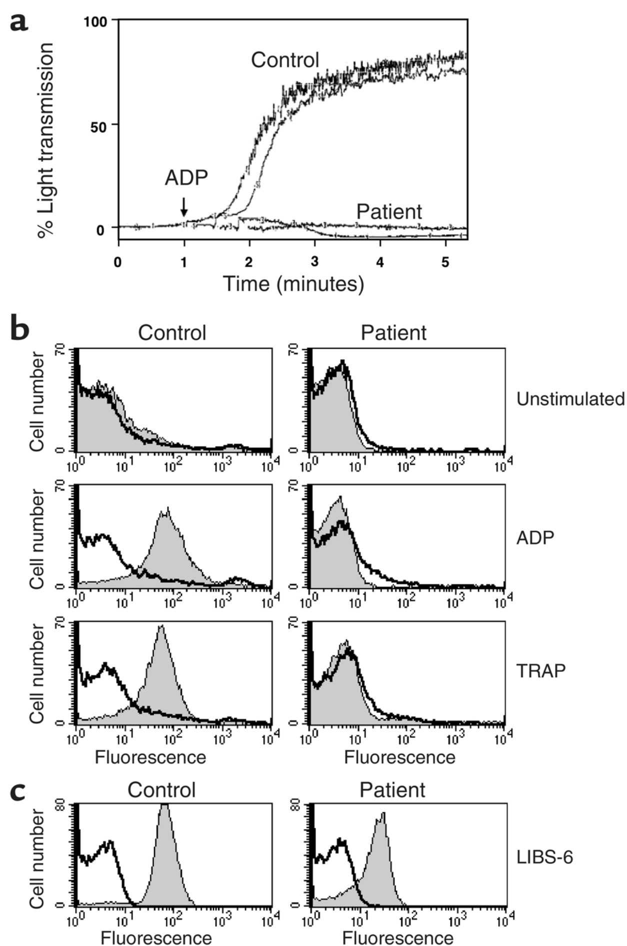

Figure 3
Comparison of integrin αIIbβ3 function in patient and control platelets. (**a**) Platelet aggregation in response to 5 μM ADP (single experiment performed in duplicate). (**b**) Binding of FITC-conjugated antifibrinogen to platelets in the presence (black line) or absence (gray region) of 20 μg/ml eptifibatide. Platelets were either unstimulated or stimulated for 20 minutes with 10 μM ADP or 1 μM TRAP. Data are representative of four separate experiments. (**c**) Binding of mAb PAC-1 to platelets stimulated with 10 μg/ml β3 integrin mAb LIBS-6 in the presence (black line) and absence (gray region) of eptifibatide. Data are representative of two separate experiments.

UCHT-1, which cross links the T cell receptor/CD3 complex, were used to test LFA-1 activation by inside-out signaling, whereas the β2-activating mAb KIM 185, Mg^{2+}/EGTA, or Mn^{2+} were used to directly activate LFA-1. All the stimuli induced LFA-1–mediated binding of control T cells to both ICAM-1 and ICAM-3 (Figure 5, a and b). However, only KIM 185, Mg^{2+}/EGTA, and Mn^{2+} induced adhesion of the patient's T cells. None of the stimuli that act through intracellular signaling pathways induced LFA-1–mediated adhesion of the patient's T cells to either ligand (Figure 5, a and b).

Functional analysis of α4β1 and α5β1 on the patient's T cells. T cells express β1 as well as β2 integrins, with α4β1 and α5β1 being involved in many immune processes in association with the β2 integrins. When adhesion to the α4β1 ligand VCAM-1 (Figure 5c) or the α4β1/α5β1 ligand fibronectin (Figure 5d) was assessed, all the stimuli tested induced adhesion of control T cells, whereas the patient's T cells only adhered when stimulated by Mg^{2+}/EGTA, Mn^{2+}, or the β1-activating mAb TS2/16. Thus β1 and β2 integrins on the patient's T cells were able to bind their ligands when stimulated directly through the ectodomain, but failed to bind when inside-out stimuli were used. These results suggested a possible lesion in an intracellular signaling pathway.

Functional analysis of β1 and β2 integrins on the patient's B cells. To assess whether the defect in inside-out stimulation of integrin-mediated adhesion also affected B cells, we used EBV-transformed B lymphoblastoid cells derived from the patient's blood and a control donor's blood. Both these cell lines were able to adhere to ICAM-1 (Figure 6a) or fibronectin (Figure 6b) when stimulated with Mn^{2+}, but the patient's cells failed to adhere when stimulated with PdBu.

The state of integrin affinity and avidity on the patient's T cells. We next investigated the activation state of the integrins on the T cells. The β2 integrin LFA-1, when in higher affinity form, is recognized by mAb 24 (15) and binds soluble ICAM-1 with increased affinity (11). Exposure to Mn^{2+} of control and patient T cells induced equivalent levels of both mAb 24 and soluble ICAM-1 binding (Figure 7a), indicating that the capacity for LFA-1 to adopt a higher-affinity form was intact when stimulated from outside the cell. Similarly, β1 integrins from patient and control T cells could be induced to express the β1 activation epitope, recognized by mAb HUTS 21 (16), and to bind soluble VCAM-1 (α4β1 only) (Figure 7a). Therefore, the lack of β1 and β2 integrin function on the patient's T cells could not be explained by an inability to assume a higher affinity conformation.

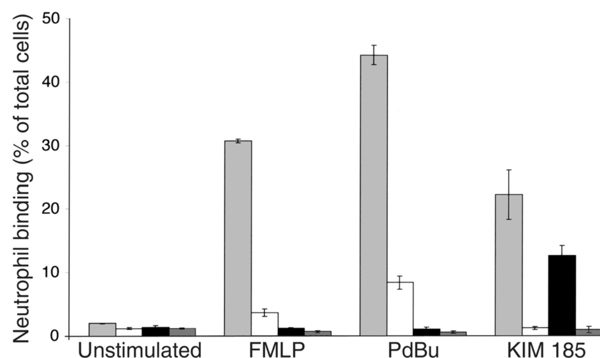

Figure 4
Comparison of Mac-1–mediated adhesion of patient and control neutrophils. The binding of control (light gray bars) and patient (black bars) neutrophils to fibrinogen-coated plates when stimulated with 100 nM FMLP, 50 nM PdBu, or 10 μg/ml KIM 185 for 30 minutes. The presence of β2 mAb IB4 at 10 μg/ml inhibits adhesion of control (white bars) and patient (dark gray bars) cells. Data (mean of triplicates ± SD) from one representative experiment (n = 2) are shown.

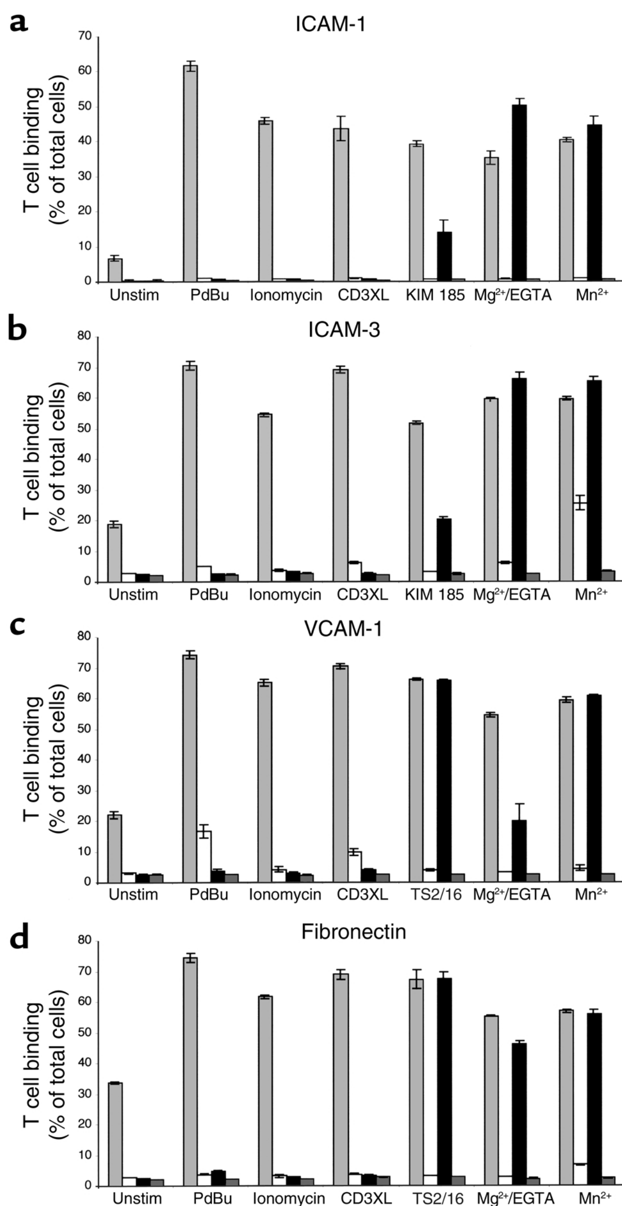

Figure 5
Adhesion of patient and control T cells to LFA-1 ligands ICAM-1 and ICAM-3, to $\alpha 4\beta 1$ ligand VCAM-1, and to $\alpha 4\beta 1/\alpha 5\beta 1$ ligand fibronectin. The binding of control (light gray bars) and patient (black bars) T cells to plates coated with (**a**) ICAM-1, (**b**) ICAM-3, (**c**) VCAM-1, and (**d**) fibronectin when stimulated with 50 nM PdBu, 1 μM ionomycin, 10 μg/ml UCHT-1, 10 μg/ml KIM 185 or TS2/16, 5 mM $MgCl_2$/1 mM EGTA, or 0.5 mM $MnCl_2$. The presence of αL mAb 38 at 10 μg/ml in **a** and **b**, α4 mAb HP2/1 at 10 μg/ml in **c**, and α4 mAb HP2/1 plus α5 mAb SAM-1 both at 10 μg/ml in **d** inhibits adhesion of control (white bars) and patient (dark gray bars) cells. Data (mean of triplicates ± SD) from one representative experiment (n = 3) are shown. Unstim, unstimulated.

When β2 integrins on leukocytes are triggered through intracellular pathways, they become laterally mobile and cluster (2, 17). T cell adhesion to ICAM-1 is dependent on this clustered form of LFA-1 (2, 3). When viewed by confocal microscopy, control T cells exhibited increased LFA-1 clustering following exposure to PdBu or the Ca^{2+} mobilizer thapsigargin, but not when exposed to Mg^{2+}/EGTA (Figure 7b), as reported previously (2). In contrast, LFA-1 was already in a clustered state on the patient's T cells and additional stimulation with PdBu and thapsigargin caused no further increase (Figure 7b). Preliminary evidence indicated parallel findings for β1 integrins on control and patient T cells using both α4 and β1 mAbs (n = 2; data not shown). Other abundant cell surface membrane proteins such as CD2, CD4, CD8, CD55, and MHC class I were not clustered on the patient's cells (data not shown). These findings suggest a disruption of signaling pathways causing dysregulation of integrin clustering or lateral mobility on the patient's T cells.

Analysis of the cytoskeleton. For leukocytes and platelets, the link between integrins and the cytoskeleton is critical for their function, and the cytoskeleton is also involved in the process of integrin clustering. To address whether associations with the cytoskeleton are defective in the patient's T cells, lysates were blotted for some of the most commonly reported integrin-associated cytoskeletal proteins. Filamin, talin, α-actinin, vinculin, ezrin, paxillin (not shown), and actin (Figure 8a) were all present at equivalent levels in the control and patient's T cells and migrated as expected on SDS-PAGE. The findings indicate that none of these cytoskeletal proteins in the patient's cells had been cleaved or subjected to altered posttranslational modification.

When T cells are stimulated through LFA-1 they polarize and migrate on immobilized ICAM-1 (18), suggesting that activated LFA-1 can signal remodeling of the cytoskeleton. Therefore, to determine whether a component of the patient's T cell cytoskeletal network was dysfunctional, Mg^{2+}/EGTA-treated T cells were adhered to ICAM-1 and their ability to polarize and migrate assessed. Both the patient and control T cells polarized (Figure 8b) and migrated (Figure 8c) on ICAM-1 in a comparable manner. The average speed of control T cells was calculated to be 12.7 ± 6.3 μm/min

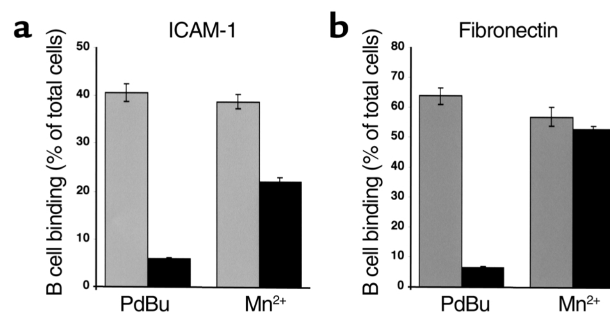

Figure 6
Adhesion of patient and control B lymphoblastoid cells to fibronectin and ICAM-1. The binding of control (gray bars) and patient (black bars) EBV-transformed B cells to plates coated with (**a**) ICAM-1 and (**b**) fibronectin when stimulated with 50 nM PdBu or 0.5 mM $MnCl_2$ for 30 minutes. Data (mean of triplicates ± SD) from one representative experiment (n = 3) are shown.

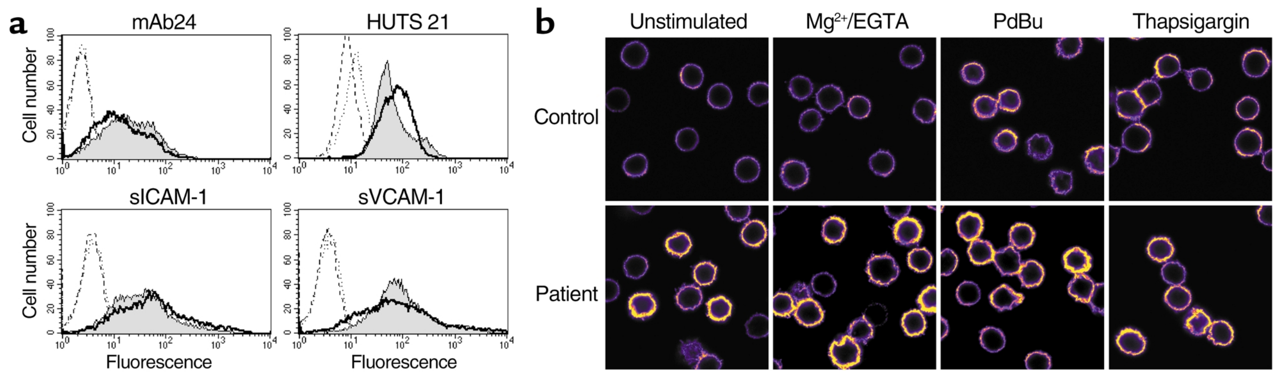

Figure 7
Comparison of the affinity and avidity state of integrins. (**a**) Control (black line) and patient (gray region) T cells incubated with mAbs 24 (β2 integrin activation reporter) or HUTS 21 (β1 integrin activation reporter) at 25 μg/ml, or ICAM-1Fc (300 μg/ml) or VCAM-1Fc (2 μg/ml) for 20 minutes at 37°C in the presence of 0.5 mM $MnCl_2$; control (dotted line) and patient (dashed line) T cells incubated with mAbs or soluble ligand as above for 20 minutes at 4°C in the presence of 1 mM EDTA. Data are from one representative experiment (*n* = 3). (**b**) T cells were either unstimulated or treated with 5 mM Mg^{2+}/1 mM EGTA, 50 nM PdBu, or 5 μM thapsigargin then labeled with LFA-1 mAb G25.2 and analyzed by confocal microscopy. A false color scheme is employed, which depicts the intensity of the fluorescent signal from blue (low) to yellow (high) (2). One optical section is shown at midheight of the cells. Data are representative of four experiments. The total fluorescent signal was quantified and averaged over four experiments as follows: no treatment, control 61.5 ± 5.0, patient 98.8 ± 4.9; Mg^{2+}/EGTA, control 59.5 ± 3.5, patient 105.5 ± 6.8; PdBu, control 78.8 ± 2.9, patient 101.0 ± 1.8; thapsigargin, control 81.0 ± 3.4, patient 96.5 ± 6.6.

and of patient T cells was 12.4 ± 5.9 μm/min. These data provide evidence that both the cytoskeleton and the adapter proteins linking the cytoskeleton and integrins function normally in the patient's cells.

Expression of GTPases, PKCs, and other adhesion-related molecules. Inside-out signaling pathways leading to integrin activation are poorly characterized. However, in an attempt to discover the nature of the signaling lesion giving rise to the lack of integrin function in the patient, we decided to assess the expression of various signaling molecules that have been associated with adhesion of leukocytes. The adaptor protein SLAP-130 (19, 20), the guanine nucleotide exchange factor Vav-1 (21), and the GTPase Rap-1 (22) have been shown recently to have a role in LFA-1 clustering and adhesion. The GTPases RhoA, Rac-1, and Cdc42 are involved in integrin-mediated cell migration (23) and mutations in Rac-2 give rise to neutrophil defects similar to the patient's abnormalities (24). PKCs have been implicated in several aspects of leukocyte adhesion (18, 25), and the patient's leukocytes do not adhere in response to activation of PKC with phorbol ester. Finally, the integrin-linked kinase (ILK) is involved in adhesion of several classes of integrin (26). The expression and electrophoretic characteristics of 12 of the 13 proteins tested were identical in patient and control T cells (Figure 9). Expression of PKC-α, however, was elevated 2.5-fold in the patient's T cells.

Discussion

We report a patient with a novel form of inherited integrin dysfunction in which the β1 and β2 integrins are expressed on the leukocyte cell surface at normal levels, but cannot be stimulated to bind ligand by intracellular signaling pathways. The defect also affects the integrin αIIbβ3, because the patient's platelets cannot be induced to bind soluble fibrinogen or to aggregate by

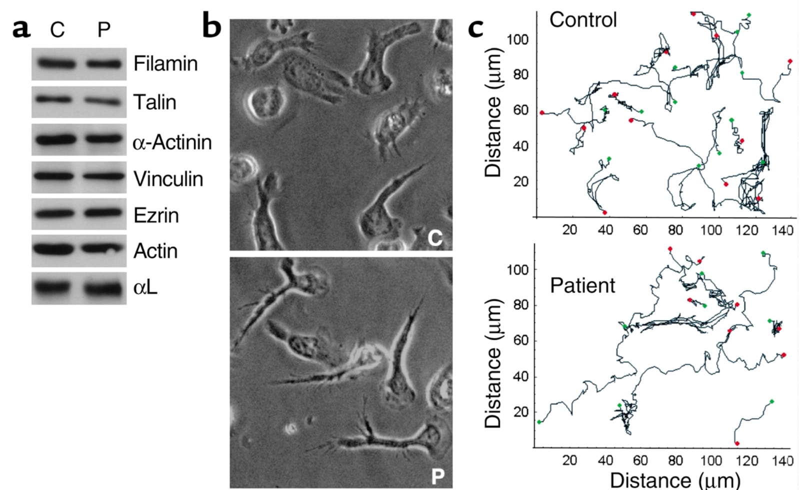

Figure 8
Western blotting of cytoskeletal proteins and morphology of migrating T cells from patient and control. (**a**) Control (C) and patient (P) T cell lysates were titrated and blotted for the indicated cytoskeletal proteins and also for the αL subunit of LFA-1. Representative blots are shown (*n* = 3). (**b**) Video microscopy pictures of control (C) and patient (P) T cells migrating on ICAM-1–coated coverslips following stimulation with 5 mM $MgCl_2$/1mM EGTA. (**c**) Cell tracks of randomly migrating T cells treated as in **b**.

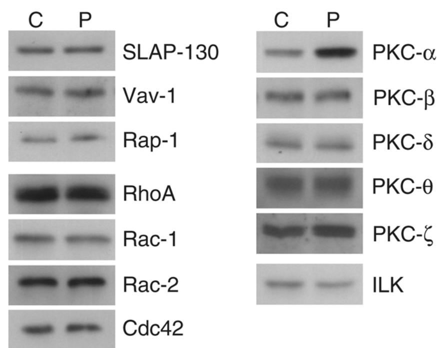

Figure 9
Western blotting of Rho family GTPases, PKC isoforms, and other adhesion-related molecules. Control (C) and patient (P) T cell lysates were blotted for the indicated proteins as for Figure 8. Representative blots are shown (*n* = 3).

platelet agonists. However, all three classes of integrins can be activated directly either with divalent cations Mg^{2+}/Mn^{2+} or activating mAbs.

Characteristically LAD-1 patients lacking β2 integrins have elevated numbers of circulating neutrophils and fail to clear bacterial infections because these cells have restricted ability to traffic into infected tissue. The patient described here also has an abnormally elevated number of circulating lymphocytes, a feature that is not evident in classical LAD-1. This is probably due to the lack of function of both the β1 and β2 families of integrins, resulting in impaired responses of both myeloid cells and lymphocytes to inflammatory signals. In experiments with knockout mice, lack of β2 integrin LFA-1 can be partially compensated for by the β1 integrins, but blocking function of both classes of integrins further increases the numbers of circulating neutrophils and lymphocytes and substantially impairs inflammatory responses (27).

To date, four patients with nonclassical forms of LAD-1 have been described. The leukocytes in two cases expressed approximately 60% of the normal levels of β2 integrins, but had no β2 integrin function (8, 28). These LAD-1 variant cases are related to classical LAD-1, having a mutation in one allele that prevents expression and a mutation in the other allele that allows expression but not ligand binding. Similar GT patients have been described who have αIIbβ3 expression but no function (29).

There are two reported LAD-1 variant cases in which no mutations in the integrin β subunit genes have been detected. The first case had the clinical indicators of LAD-1 and the β2 integrins were expressed but nonfunctional (30). After some years, defects in β3 integrin function also became apparent; but, in contrast to our patient, β1 integrins were not affected. For the second patient, there were problems with β2 and β1 integrin function, and a bleeding problem then developed that was not explored experimentally (31). A speculation is that the genetic lesions in these two patients and patient FM are individual, but may be related and potentially highlight a specific common pathway dedicated to integrin activation.

Because the activities of at least three integrin families are affected in patient FM and the integrins can function when activated directly from outside the cell, it is unlikely that the dysfunction is due to mutations in the integrin subunits themselves. It is more probable that the faulty gene encodes a protein that is critical for integrin function. Moreover, the expression of the affected gene product is predicted to be confined to hematopoietic cells or have redundant function in other cell types. The fact that murine β1 integrin knockouts are embryonic lethal (32) implies that the patient would not have survived if β1 integrin functioning was universally affected. The results demonstrating that other leukocyte and platelet functions are relatively normal provide evidence that the lesion is confined to a component of a key pathway dedicated to integrin activation.

Further insight into the nature of the patient's lesion has come from confocal microscopy, which revealed that LFA-1 and the β1 integrins on the patient's T cells are constitutively clustered and that the state of clustering does not change when the cells are activated. Integrin clustering is believed to be dynamic, but much is unknown about the sequence of events regulating this process. It has been proposed that integrins on resting cells are tethered to the cytoskeleton in an unclustered form and that activation of the cell removes the cytoskeletal constraint allowing the integrin to move and form clusters in the membrane, mediate firm ligand binding, and potentially reassociate with the cytoskeleton (2, 3, 17). The observation that the patient has constitutively clustered integrins, yet these integrins do not function, implies that dynamic clustering is required for inside-out stimulated adhesion and that, in the patient's cells, integrin mobility may be restricted. Although exposure to low concentrations of cytochalasin D promotes adhesion of naive T cells by removing the cytoskeletal tethering of the integrins (3), this procedure did not alter the adhesion capabilities of the patient's T cells (data not shown). It is interesting that outside-in signaling through LFA-1 was sufficient for T cell adhesion, polarization, and migration, suggesting that the state of clustering is irrelevant for these adhesion-dependent activities, or, alternatively, that it can be altered by signaling directly through LFA-1.

Although the platelet integrin αIIbβ3 is activated primarily through signaling that leads to a change in affinity (reviewed in ref. 5), avidity regulation also plays a role in the activation of this integrin (4). In the patient's platelets, inside-out agonists, such as ADP and thrombin, failed to induce binding of soluble fibrinogen, suggesting that this pathway is not functioning. The relationship between affinity and avidity regulation remains to be resolved, but the evidence gathered from the unusual patient described here suggests that, at least in platelets, both integrin activation pathways may be defective or, alternatively, that they are interdependent.

As far as LFA-1 clustering is concerned, the adapter protein SLAP-130 (also known as Fyb and ADAP) and the Rac-1 guanine nucleotide exchange factor,Vav-1, recently have been reported to be involved (19–21). Deletion of either protein in murine T cells prevents LFA-1 clustering and adhesion to ICAM-1 and the β1 integrin ligand fibronectin in response to CD3 engagement. These cells, however, do adhere when stimulated with phorbol ester, which is not the case for the patient's T cells. Caution is necessary when extrapolating results obtained with mouse cells to human studies, but the above findings, as well as the fact that the expression of SLAP-130 and Vav-1 in the patient's T cells is normal, suggests that it is unlikely that these proteins are defective in this patient. Additionally, SLAP-130 is not expressed in B cells, whereas adhesion of the patient's EBV-transformed B lymphoblastoid cells is defective. Expression of an active form of the GTPase Rap-1 in thymocytes also leads to constitutive clustering of LFA-1 (22), and Rap-1 is reported to lie in the signaling pathway leading to LFA-1, Mac-1, and αIIbβ3 activation (reviewed in ref. 33). Rap-1, however, is not hematopoietic cell specific, is expressed at normal levels in the patient, and, as with SLAP-130 and Vav-1, the effects on clustering and adhesion differ from the patient's problem, which consists of constitutively clustered nonfunctioning integrins.

Other Rho family GTPases, RhoA, Rac, and Cdc42, have been directly implicated in adhesion and are involved in changes in the F-actin cytoskeleton that are important for cell migration (23). Of interest is another patient with clinical features of LAD-1, where the lesion was found to be a mutation in the gene encoding the hematopoietic cell-specific small GTPase Rac-2, preventing GTP binding (24). However, the patient described here has elevated numbers of lymphocytes and an intact superoxide burst, distinguishing her from the Rac-2 defective patient for whom the described dysfunctions are restricted to neutrophils. We found no difference in the expression or electrophoretic characteristics of Rac-1, Rac-2, RhoA, or Cdc42, or with T cell migration, suggesting that our patient's problem does not lie with these proteins.

PKC isozymes have been implicated in adhesion processes (34). In our study, phorbol ester was unable to activate β1 or β2 integrin–mediated adhesion, suggesting that the lesion could be either in a PKC subtype or downstream of such a kinase. Consistent with this phenotype, phorbol ester–sensitive Jurkat cells mutant in ERK-1 have been generated, in which β1 and β2 integrin function is lacking (25). The patient's cells, however, have normal ERK and p38 MAP kinase expression (data not shown). PKC-β1 has been suggested to have a role in LFA-1–mediated adhesion stimulated from outside (18), a pathway that is functional in the patient's cells.

We found T cells to express normal amounts of PKC-β, -δ, -θ, and -ζ, but 2.5-fold increased levels of PKC-α, and it is of interest to speculate about the meaning of this increase. The use of the Ca^{2+} mobilizer, thapsigargin, is one of the inside-out signaling protocols that failed to induce adhesion of the patient's cells. As this form of adhesion is not sensitive to the broadly based PKC inhibitor Ro 31-8220 (2), it seems that the patient's adhesion lesion is evident in at least one model of β1 and β2-induced adhesion that is not PKC dependent. It is therefore unlikely that the increased PKC-α level is the cause of the lesion, but is probably a consequence of it. PKC-α has been implicated in various adhesion phenomena. It is physically associated with β1 integrins and involved in membrane trafficking by controlling integrin internalization (35). Overexpression of a number of PKC isozymes, including PKC-α, cause adhesion of Jurkat T cells (36). Taken together, these results suggest that the overexpression of PKC-α observed in the patient's T cells may well be a downstream effect of the clustered state of the integrins. In any event, this observation provides a valuable clue to the aberrant molecular events in the patient's leukocytes and will require further investigation.

Various other factors associated with integrin activity seemed unlikely to explain the patient's lesion. A number of cytoskeletal proteins, reported to associate with integrins (reviewed in ref. 37), were present at normal levels and with normal electrophoretic characteristics in the patient's T cells. Moreover, T cell polarization and migration following triggering of LFA-1 were normal, suggesting a functioning cytoskeleton. Therefore, the dysfunction is probably not due to lack of expression of one of the cytoskeletal proteins involved in migration. Other factors that associate more directly with integrins and the cytoskeleton, such as cytohesin-1, which binds to LFA-1, or β3-endonexin, which binds αIIbβ3, are integrin-type specific, diminishing the likelihood of their involvement in this integrin dysfunction (see ref. 37). In this context, the kinase ILK is also of interest because it links several types of integrins to the cytoskeleton (26). ILK, however, is expressed at normal levels in the patient and is, moreover, best characterized for its role in β1 integrin-matrix interactions that are key for epithelial cells, not hematopoietic cells, where the problems lie in this patient. We have proposed previously that inside-out signaling in T cells leads to clustering of LFA-1, which is dependent upon cytoskeletal rearrangement and activity of the cysteine protease, calpain (2). Calpain is also activated following FMLP stimulation of neutrophils (38). However, in platelets, calpain activation is reported to lie downstream of αIIbβ3 ligation, because calpain inhibitors have no effect on platelet aggregation but do affect fibrin clot retraction (39). Because the patient in this study has no platelet aggregation, it is unlikely that the defect affects calpain.

PI3K is reported to have a role in chemokine induction of LFA-1 affinity increase in murine T cells (40). In contrast, we have failed to find a role for PI3K in LFA-1–mediated adhesion of human T cells stimulated by TCR-CD3 or phorbol esters, as assessed by the lack of effect of inhibitor LY294002 (B. Leitinger, unpublished data). Therefore, it is unlikely that the

patient's adhesion defect lies in the PI3K pathway. In addition, the p110δ subunit of PI3K is predominantly expressed in leukocytes, and recent characterization of mice expressing an inactive form of p110δ show CD3-stimulated α4β1/α5β1- and LFA-1–mediated adhesion to be normal (41).

In summary, a defect in the activation of three classes of integrin on leukocytes and platelets has been described. A key observation is that the β2 and β1 leukocyte integrins are constitutively clustered, and it is speculated that the lack of regulation of this clustering leads to a defect in the ability of the integrins to function correctly and, in turn, to the LAD-1- and GT-like symptoms of this unique patient. It is hoped that study of this patient will yield further key insights into the common features that lead to integrin activity on leukocytes.

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