A LAD-III syndrome is associated with defective expression of the Rap-1 activator CalDAG-GEFI in lymphocytes, neutrophils, and platelets

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Leukocyte and platelet integrins rapidly alter their affinity and adhesiveness in response to various activation (inside-out) signals. A rare leukocyte adhesion deficiency (LAD), LAD-III, is associated with severe defects in leukocyte and platelet integrin activation. We report two new LAD cases in which lymphocytes, neutrophils, and platelets share severe defects in β1, β2, and β3 integrin activation. Patients were both homozygous for a splice junction mutation in their CalDAG-GEFI gene, which is a key Rap-1/2 guanine exchange factor (GEF). Both mRNA and protein levels of the GEF were diminished in LAD lymphocytes, neutrophils, and platelets. Consequently, LAD-III platelets failed to aggregate because of an impaired αIIbβ3 activation by key agonists. β2 integrins on LAD-III neutrophils were unable to mediate leukocyte arrest on TNFα-stimulated endothelium, despite normal selectin-mediated rolling. In situ subsecond activation of neutrophil β2 integrin adhesiveness by surface-bound chemoattractants and of primary T lymphocyte LFA-1 by the CXCL12 chemokine was abolished. Chemokine inside-out signals also failed to stimulate lymphocyte LFA-1 extension and high affinity epitopes. Chemokine-triggered VLA-4 adhesiveness in T lymphocytes was partially defective as well. These studies identify CalDAG-GEFI as a critical regulator of inside-out integrin activation in human T lymphocytes, neutrophils, and platelets.

Leukocyte arrest at target endothelial sites is nearly exclusively mediated by integrin receptors (1). As circulating leukocytes maintain their integrins in a generally nonadhesive state, a key checkpoint in leukocyte arrest is the rapid modulation of integrin affinity and avidity to endothelial ligands (2) by various agonists, predominately chemoattractants or chemokines, presented on the endothelium (3, 4). Likewise, platelets maintain their major fibrinogen receptor, the integrin αIIbβ3, in an inactive conformation, which is converted by multiple agonists, predominately ligands to G protein–coupled receptors (GPCRs), into an activated receptor with high affinity to multiple ligands (5).

We recently described a human genetic deficiency of leukocyte adhesion to endothelium leukocyte adhesion deficiency (LAD) III that is

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distinct from LAD-I. Whereas LAD-I is a genetic defect in β3 integrin expression or function (14), LAD-III leukocytes express intact integrins with an impaired ability to generate high avidity to their endothelial ligands at vascular endothelial contacts in response to rapid endothelial chemoattractant signals (15, 16). Patient leukocytes, however, express functionally intact GPCRs. A role for Rap-1 malfunction in the LAD-III syndrome was inferred by our finding that LAD-III lymphoblasts express normal levels of Rap-1, which fail to undergo activation in response to the prototypic chemokine CXCL12. Although Rap-1 is also implicated in the survival and function of many nonhematopoietic tissues (17–19), no severe developmental disorders or abnormalities in nonhematopoietic tissues were reported in the LAD-III patients (14). Thus, we suggested that LAD-III and related integrin activation defects in hematopoietic systems are the result of a loss in a key Rap-1 guanine exchange factor (GEF) that is essential for the transduction of leukocyte and platelet GPCR signals into Rap-1 and integrin activation. In this study, we report two similar LAD-III cases in which neutrophil and lymphocyte integrins cannot acquire adhesiveness upon rapid activation by GPCR agonists under shear flow conditions. Chemokine-induced activation of two LFA-1 conformations associated with integrin extension and high affinity states is also largely impaired in LAD-III T lymphocytes. In addition, platelets derived from the two patients fail to aggregate in response to prototypic inside-out signals, and their key integrin, αIIbβ3, does not acquire a high affinity state essential for ligand binding and aggregation. These LAD cases share a homozygous mutation in the acceptor splice junction at the beginning of exon 16 of the Rap-1 GEF, CalDAG-GEFI (RasGRP2). Furthermore, LAD-derived total blood levels of CalDAG-GEFI mRNA and the protein expression in LAD platelets, neutrophils, and lymphocytes are diminished. These results are the first example of a human inherited disease caused by a Rap GEF deficiency that is linked to profound defects in both leukocyte and platelet integrin activation and adhesive functions in the vasculature. RESULTS Patients The two patients, a 1-yr-old male (patient K) and a 2-yr-old female (patient A) of Turkish origin were each born to consanguineous parents. The two families, although not related, originally lived in the close villages in the eastern region of Turkey, suggesting a common ancestor. Both sets of parents are asymptomatic. Both patients shared a similar clinical presentation consisting of petechia from birth caused by a severe bleeding tendency and requiring repeated blood transfusions. They suffered recurrent and severe bacterial infections associated with marked leukocytosis (25–70,000/mm3, 60% neutrophils and 30% lymphocytes), but normal platelet counts. More than 95% of patient neutrophils expressed CD18, CD11a, and CD11b integrins, ruling out a LAD-I syndrome. Lymphocyte subsets and immunoglobulin levels were within normal range. Patient A suffered from recurrent severe pneumonias, necessitating constant antibiotic treatment. Patient K suffered from recurrent severe pneumonias and sepsis. He had surgery to correct a bowel intususception when he was 14 mo old, resulting in a large, nonhealing wound after the operation. Blood culture yielded B-hemolytic Pseudomonas aeruginosa. Patient K died at 2 yr of age from sepsis and pulmonary bleeding. Laboratory data showed hemoglobin around 8 gr%. Failure to thrive was consistent in both patients from early life, and both children were below the fifth percentile for both height and weight. Patient A’s sister died at age 15 mo from pneumonia and sepsis and had suffered from anemia and bleeding tendency from early life. Patient A has only one healthy brother aged 6 yr. Patient K does not have siblings. Neither of the patients displayed overt neurological disorders. Platelets derived from LAD patients fail to aggregate because of the inability of agonists to trigger high affinity αIIbβ3 As both patients displayed major bleeding disorders in addition to their severe leukocytosis, we first analyzed their agonist-stimulated platelet aggregation. Platelet aggregation triggered by all GPCR agonists tested, i.e., ADP, epinephrine, arachidonic acid, and thrombin, was completely absent in patient-derived platelets (Fig. 1 A and not depicted), despite only moderate reduction in surface αIIbβ3 (GPIIbβ3) expression (Fig. 1 B, inset). As this integrin is the main fibrinogen receptor on platelets (5), these results suggested a major defect in GPCR-mediated inside-out activation of patient αIIbβ3 (Fig. 1 A). An alternative GPCR-independent inside-out activation pathway that stimulates αIIbβ3-mediated platelet aggregation involves the binding of collagen to platelet receptors such as GPVI and the αIIbβ3 integrin (20). Patient-derived platelets were also unable to aggregate in response to collagen signals (Fig. 1 A). However, these multiple defects were not caused by a global platelet aggregation defect because when GPCR signaling to αIIbβ3 was bypassed with the vWF agonist ristocetin, patient platelets underwent robust aggregation, although at a twofold lower magnitude (Fig. 1 A). Defective platelet aggregation could result from a failure of αIIbβ3 to undergo activation or from cytoskeletal defects that impair postligand occupancy of this integrin (5). Direct activation by agonists of the αIIbβ3 integrin, from an inactive state to a fully active integrin with high affinity to ligands, can be probed by binding of the ligand mimetic activation reporter antibody, PAC-1 (21). PAC-1–specific staining of activated platelets derived from the LAD patients was slightly reduced in response to platelet GPCR agonists, whereas PAC-1 staining on control platelets was dramatically increased (Fig. 1 B). In contrast, P-selectin was normally activated by the same agonists in LAD-derived platelets (unpublished data). These results collectively indicate that affinity up-regulation of αIIbβ3 by inside-out signals is severely impaired in patient platelets.
Absence of CalDAG-GEFI transcripts and protein in LAD patient-derived platelets and neutrophils

The Rap-1 GEF CalDAG-GEFI has been implicated as a key regulator of agonist-induced αIIbβ3 activation and platelet aggregation (22). Therefore, we analyzed the level of CalDAG-GEFI by RT-PCR in total blood. Strikingly, several CalDAG-GEFI transcripts tested were nearly completely absent from blood obtained from both patients (Fig. 2 A and not depicted), whereas normal levels of this transcript were found in all four parents (Fig. 2 A). In contrast, levels of a second Rap-1 GEF, CalDAG-GEFIII, were completely normal in both patients compared with parents and age-matched control (Fig. 2 A). Quantitative PCR analysis revealed that CalDAG-GEFI was expressed at >30-fold lower levels in LAD patient A compared with healthy age-matched control, whereas the expression level of CalDAG-GEFIII was comparable (Fig. 2 B). In agreement with the loss of CalDAG-GEFI in LAD patients, Western blot analysis with two mAbs against CalDAG-GEFI revealed a complete loss of the protein in lysates derived from platelets and neutrophils purified from patient A (Fig. 2 C and not depicted). Consistent with the RT-PCR results, both of patient A’s parents expressed normal levels of the CalDAG-GEFI protein in both neutrophil and platelet lysates (unpublished data).

Both LAD patients share an identical homozygous mutation in an intronic acceptor splice junction in CalDAG-GEFI

We next assessed whether the two patients were homozygous at the CalDAG-GEFI chromosomal locus 11q13.1. We identified a single microsatellite marker, located at chr11:64,428,757–64,429,006 (National Center for Biotechnology Information build 36), which is 159,253 bp away from the gene (chr11:64,250,960–64,269,504), and thus the closest published polymorphic marker to CalDAG-GEFI. We initially used this marker to analyze DNA of the two patients, their parents, and the healthy sibling of patient A (Fig. 3 A). In family A, two alleles, 252 and 260 bp long, were detected in the samples derived from the parents (Fig. 2 A). In contrast, levels of a second Rap-1 GEF, CalDAG-GEFIII, were completely normal in both patients compared with parents and age-matched control (Fig. 2 A). Quantitative PCR analysis revealed that CalDAG-GEFI was expressed at >30-fold lower levels in LAD patient A compared with healthy age-matched control, whereas the expression level of CalDAG-GEFIII was comparable (Fig. 2 B). In agreement with the loss of CalDAG-GEFI in LAD patients, Western blot analysis with two mAbs against CalDAG-GEFI revealed a complete loss of the protein in lysates derived from platelets and neutrophils purified from patient A (Fig. 2 C and not depicted). Consistent with the RT-PCR results, both of patient A’s parents expressed normal levels of the CalDAG-GEFI protein in both neutrophil and platelet lysates (unpublished data).

We next sequenced the genomic region of CalDAG-GEFI from 2,000 bp upstream to 500 bp downstream of the cDNA sequence (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070058/DC1). 20 deviations from the reference genome were found, comprising 17 known single-nucleotide polymorphisms (SNPs) and 3 novel SNPs (Fig. S2). The PCR products containing novel SNPs were generated and sequenced in LAD patient A’s healthy brother. Only one novel SNP, IVS15nt718c>a, was unique in LAD patient A (Fig. 3 B and Fig. S3). The same mutation was found in LAD patient K (Fig. 3 B and Fig. S3). Importantly, this mutation was absent in all 116 control chromosomes tested from Turkish donors (unpublished data). The mutation occurs 3 bp upstream of the beginning of exon 16, disrupting the splice junction present at that location. An automated splice site
analysis program based on information theory (23) was used to predict the possible effects of this mutation on splicing. The program predicts that this mutation creates a putatively active cryptic splice site located 1 bp before the actual site, which is concomitantly weakened (Fig. S4). If this cryptic site is preferred by the transcription machinery, the products of such a splice would have a shifted reading frame and a premature stop codon, resulting in nonsense-mediated decay (NMD) (24). Collectively, the LAD patients share a homozygous mutation in a critical acceptor splice region of CalDAG-GEFI that predicts the loss of message, which was indeed observed (Fig. 2, A and B).

CalDAG-GEFI–null neutrophils fail to arrest on inflamed endothelial cells and on ICAM-1 in spite of normal selectin-mediated capture and rolling

Neutrophils from the initial LAD-III patient characterized by us (15) expressed normal levels of the two major \( \beta_2 \) integrins implicated in neutrophil arrest on vascular endothelium, LFA-1 and Mac-1. Neutrophils derived from the two new LAD cases also expressed normal levels of LFA-1 and Mac-1 (Fig. 4 A and not depicted). The LAD-derived CalDAG-GEFI–null neutrophils were next perfused over a monolayer of TNF-stimulated human umbilical vein endothelial cells (HUVECs) under physiological shear flow; these cells express high levels of E-selectin and multiple \( \beta_2 \) integrin ligands (25, 26). Patient and control neutrophils were captured by the cytokine-activated HUVEC at comparable rates and initiated normal rolling on the endothelial monolayer (Fig. 4 B). Shortly after capture on the endothelial surface, normal neutrophils spontaneously arrest on the activated endothelium, in a \( \beta_2 \) integrin–dependent manner (26) (unpublished data), whereas CalDAG-GEFI–null neutrophils failed to arrest and continued to roll over the cytokine-activated EC monolayer (Fig. 4 B), as was previously observed for LAD-III neutrophils (15). Inhibition of phospholipase C (PLC) in control neutrophils completely eliminated their ability to arrest in a \( \beta_2 \) integrin–dependent manner on the activated HUVECs (unpublished data), which is consistent with a role for PLC, an upstream regulator of CalDAG-GEFI (22) in CalDAG-GEFI–mediated \( \beta_2 \) integrin activation in normal neutrophils. Furthermore, whereas both normal and LAD neutrophils were efficiently captured by
E-selectin coimmobilized with ICAM-1 (Fig. 4 C), nearly all normal neutrophils either immediately arrested on the E-selectin/ICAM-1 surface or rolled for several seconds before arrest, but none of the LAD neutrophils were able to arrest (Fig. 4 C). Exclusion of Mg\(^{2+}\) from the binding medium resulted in integrin inactivation and loss of arrest on the E-selectin/ICAM-1 surface only in control neutrophils (Fig. 4 C). As expected, this integrin inactivation did not affect the capture and rolling adhesions initiated by either control or LAD neutrophils. These results collectively indicate intact rolling capacity, but loss of adhesiveness of both LFA-1 and Mac-1 integrins in LAD neutrophils interacting with inflamed endothelial cells, as well as with purified ICAM-1 under shear flow conditions.

**Impaired spontaneous β2 integrin adhesiveness to ICAM-1 in CalDAG-GEFI-null neutrophils is associated with reduced levels of high affinity integrin conformations**

The inability of both LFA-1 and Mac-1 expressed by LAD neutrophils to develop firm adhesions could result from improper inside-out activation through E-selectin (27, 28). We therefore next compared β2-mediated attachments of normal and LAD neutrophils to isolated ICAM-1 coated at high density in the absence of E-selectin. Control blood-derived neutrophils retained moderate levels of spontaneous β2 integrin adhesiveness to high density ICAM-1, manifested by the ability of these leukocytes to generate both transient and firm tethers on the ligand under low shear flow (Fig. 5 A). Notably, β2 integrins on LAD-derived CalDAG-GEFI-null neutrophils failed to interact with ICAM-1 even under these permissive flow conditions (Fig. 5 A). Furthermore, prolonged association with ICAM-1 under static conditions allowed β2 integrins on normal, but not on LAD, neutrophils to generate high shear–resistant adhesions (Fig. 5 B). Thus, a considerable fraction of normal neutrophils settled on ICAM-1 for 1 min developed resistance to detachment by high shear stress, whereas only a negligible fraction of LAD neutrophils developed shear resistance on ICAM-1 after prolonged association with the ligand under static conditions (Fig. 5 B). These results suggested that LAD β2 integrins are inherently defective in their acquisition of the intermediate and/or high affinity states necessary for rapid ligand recognition and contact-dependent adhesion strengthening on ligands (29, 30). Comparing the levels of these β2 conformational states probed by specific reporter mAbs, we found, however, identical levels of the β2 integrin extension reporter KIM127 on both normal and LAD neutrophils (Fig. 5 C, top). Nevertheless, the appearance of the high affinity 327C β2 I domain epitope on LAD β2 integrins was reduced by fivefold (Fig. 5 C, bottom), which is consistent with defective acquisition of high affinity states by LAD β2 integrins.

**Mac-1 in CalDAG-GEFI-null leukocytes fails to undergo subsecond activation of adhesiveness by chemoattractants**

In a previous study on LAD-III neutrophils, we found that the neutrophil Mac-1 could undergo normal conformational activation by chemoattractants, but failed to respond to in situ inside-out stimulation by the same surface-bound chemoattractants under shear flow (15). To directly assess in the present LAD syndrome the ability of Mac-1 to undergo inside-out activation by prototypic chemoattractants, we next assessed whether platelet-activating factor (PAF) can alter the integrin activation state in patient neutrophils. CBMRM1/5 is a ligand mimetic mAb that detects high affinity Mac-1 subsets (31).
In contrast to platelets, PAF, as well as IL-8, effectively triggered the CBRM1/5 Mac-1 neoepitope in patient CalDAG-GEFI–null neutrophils, which is consistent with normal PAF and IL-8 signaling in these cells (Fig. 6 A and not depicted). Nevertheless CalDAG-GEFI–null neutrophils failed to mount any PAF- or IL-8–dependent in situ activation of their Mac-1 when perfused over fibrinogen coimmobilized with these chemokines (Fig. 6 B). The ability of IL-8 to trigger LAD neutrophil attachments to ICAM-1, which is a shared ligand of Mac-1 and LFA-1 (32), was also abrogated (Fig. 6 C). Notably, neutrophils from the patient’s mother, although confirmed to be a heterozygote carrier of the mutated gene (Fig. 3 and Fig. S3), were indistinguishable from healthy neutrophils in all adhesion assays tested (not depicted). Collectively, these results suggest that β2 integrins in LAD-derived, CalDAG-GEFI–null neutrophils are completely defective in their ability to undergo rapid, chemoattractant–triggered activation under shear stress conditions, despite retained chemoattractant–triggered conformational activation of their Mac-1 β2 integrin.

Abolished chemokine activation of LFA-1 and of VLA-4–mediated arrest in CalDAG-GEFI–deficient LAD lymphocytes

Murine splenocytes express negligible levels of CalDAG-GEFI (Fig. 7 A) (22). Strikingly, high levels of the GEF were found in primary freshly isolated T lymphocytes from healthy donors (Fig. 7 A). As CalDAG-GEFI was completely lost in primary freshly isolated T lymphocytes (Fig. 7 A), we next assessed how the GEF deficiency affects the function of the two major lymphocyte integrins, the β2 integrin LFA-1 and the β1 integrin VLA-4. LAD lymphocytes expressed both integrins, as well as CXCR4, which is the main receptor for the prototypic lymphocyte chemokine CXCL12 (SDF-1α) at normal levels (Fig. 7 B). Nevertheless, CXCL12–mediated activation of LFA-1, assessed by the induction of the integrin extension epitope KIM127, as well as the high affinity integrin conformation, probed by the 327C mAb (33), was greatly reduced in LAD-derived, CalDAG-GEFI–deficient T lymphocytes (Fig. 7 C). In agreement with defective chemokine–induced inside-out activation of LFA-1 in CalDAG-GEFI–deficient lymphocytes, CXCL12 failed to trigger any LFA-1 adhesions to ICAM-1 under shear flow (Fig. 7 D). Thus, CalDAG-GEFI is critical for CXCL12–mediated inside-out activation of LFA-1 in primary human T cells. We next assessed the role of CalDAG-GEFI in chemokine–mediated activation of VLA-4 in T cells interacting with VCAM-1 and coimmobilized CXCL12 under shear flow. In healthy T lymphocytes, this prototypic chemokine triggers robust VLA-4–dependent lymphocyte tethering, rolling, and arrest on VCAM-1 under shear flow (25). Notably, VLA-4 of patient CalDAG-GEFI–null T lymphocytes could still undergo in situ activation by CXCL12 (Fig. 7 E), but CXCL12–triggered VLA-4 failed to arrest patient CalDAG-GEFI–null T lymphocytes on VCAM-1. These data suggest that CalDAG-GEFI is only partially required for inside-out activation of VLA-4. As VLA-4 activation epitopes are not induced in T lymphocytes by soluble CXCL12 (25), and there are no available VLA-4 ligand mimetic mAbs, we could not confirm an inside-out defect in chemokine–triggered VLA-4 conformation or affinity state based on activation epitope analysis, as we could for LFA-1 (Fig. 7 C). We could rule out a defect in outside-in activation of VLA-4 by VCAM-1 because CalDAG-GEFI–null
Neutrophils derived from patient’s mother and analyzed.

- Neutrophils were perfused over substrates at a shear stress of 0.5 dyn/cm².
- Presence of immobilized 1P4 or 2 μg/ml IL-8 coated alone or in the presence of low density fibrinogen (coated at 0.5 μg/ml) coated alone or in the presence of immobilized 100 nM PAF or 2 μg/ml IL-8.

Analysis of neutrophils derived from patient’s mother and analyzed in LAD neutrophils at subsecond contacts.

- Normal PAF-triggered induction of the activation Mac-1 neoepitope CBRM1/5 on LAD (patient A) neutrophils. Control or LAD neutrophils were either left intact or stimulated with 100 nM PAF. Background antibody stainings had fluorescence intensity values of <5.
- Capture and arrest of neutrophils on low density fibrinogen (coated at 0.5 μg/ml) coated alone or in the presence of immobilized 100 nM PAF or 2 μg/ml IL-8.

Conditions relevant for leukocyte interactions with blood vessels.

Our genetic analysis has identified a homozygous mutation in the acceptor splice junction at the beginning of exon 16 of the CalDAG-GEFI gene in both LAD patients. Familial segregation analysis also indicates that the patients’ parents are heterozygous for this mutation, yet they are healthy and their leukocytes express normal levels of the GEF. Thus, we establish for the first time an autosomal recessive mutation in a LAD-III syndrome. Furthermore, this is a first implication for the first time an autosomal recessive mutation in a LAD-III syndrome. Furthermore, this is a first implication of a single Rap-1 GEF as a critical inside-out regulator of integrin activation in two major types of human leukocytes and platelets.

The role of CalDAG-GEFI in lymphocyte integrin activation has been obscure because this GEF was not detected in the white pulp of the murine spleen or in the thymus. We confirmed these results, but found considerable expression of the protein in healthy human peripheral blood T cells. Because primary LAD T cells lacked this GEF, we were prompted to dissect the contribution of CalDAG-GEFI to rapid chemokine-induced integrin activation in T lymphocytes. Chemokine activation of lymphocyte integrins involves simultaneous bidirectional activation by both inside-out and outside-in rearrangements in integrin headpieces and tails. Choosing CXCL12 as a prototypic chemokine for lymphocyte integrin activation, we assessed the ability of both LFA-1 and VLA-4 in CalDAG-GEFI–null LAD T lymphocytes to undergo in situ stimulation of adhesiveness under physiological conditions of shear flow. Because integrin tethers form within leukocyte contacts with integrin ligands in the range of 0.04–0.1 s, any immediate adhesive tethers in situ stimulated by the surface-bound chemokine involves the contact of the integrin and the GPCR with their cognate ligands in this short time frame. Our results therefore indicate an indispensable role for CalDAG-GEFI in the earliest stages (i.e., subsecond-lived contacts)
of chemokine-induced LFA-1 firm adhesiveness. Correspondingly, we also find this GEF to be essential for the triggering in T cells of two conformational states of LFA-1 associated with integrin extension and high affinity to ligand (30). Furthermore, VLA-4 in CalDAG-GEFI–null T cells also fails to develop high avidity binding to its major endothelial ligand VCAM-1 in response to in situ activation signals from CXCL12, although a significant fraction of VLA-4 on CalDAG-GEFI–null lymphocytes could still undergo normal in situ activation by CXCL12 at subsecond contacts. Importantly, α4 integrin conformations can be regulated by shear forces without noticeable changes in integrin affinity to ligand under shear-free conditions (40, 41). Interestingly, the subset of firm arrest-mediating VLA-4–VCAM-1 tethers developed by healthy, but not by GEF-deficient, T cells is sensitive to inhibition by low levels of soluble VLA-4 ligands known to selectively block high affinity VLA-4 (25). Although VLA-4–mediated adhesions require proper integrin anchorage to the cytoskeleton, we have ruled out a VLA-4 anchorage defect in LAD T cells because these cells normally tethered and arrested on high density VCAM-1 (unpublished data). Nevertheless, we cannot exclude the possibility that defective VLA-4–mediated T cell arrest in LAD T cells is caused by a failure of chemokine-activated VLA-4 to anchor to the cytoskeleton and develop optimal shear resistance in CalDAG-GEFI–deficient T cells. The GEF is dispensable, however, for chemokine-triggered VLA-4–mediated transient and rolling adhesions, which are generally mediated by low or intermediate affinity VLA-4 subsets (64). We thus conclude that T cells lacking CalDAG-GEFI undergo incomplete chemokine–triggered activation of VLA-4, suggesting that unlike LFA-1, VLA-4 regulation by chemokines involves additional and redundant CalDAG-GEFI–independent signaling pathways.

A final step in integrin activation is the binding of the cytoskeletal adaptor talin to integrin tails (42). A novel integrin activation pathway, which links inside-out signals to αIIbβ3 affinity modulation via a Rap-1–RIAM–talin signaling complex was recently identified (43). It is likely that CalDAG-GEFI acts upstream of this complex not only in platelets (20, 22) but also in neutrophils and lymphocytes. Neutrophil Rap-1 is strongly activated by cytosolic calcium and by...
phorbol ester DAG analogues (44), implicating these two secondary messengers and their key regulatory enzyme, PLC, in Rap-1 activation of neutrophil integrins. Indeed, β2-mediated neutrophil arrest on inflamed endothelium is highly sensitive to inhibition of PLC (unpublished data). Rap-1 is found in multiple membranal pools in various cell types (45), but its activation by GPCRs occurs mainly in the plasma membrane (46, 47). Because these signals are transmitted within a fraction of a second at leukocyte–endothelial contacts (48), at least on circulating cells, CalDAG-GEFI and its immediate targets are expected to preexist near the plasma membrane in proximity to their target integrins. CalDAG-GEFI may also activate integrins by triggering the small GTPase R–Ras, which was previously implicated in affinity modulation of the β3 integrin VLA-5 (49). It is noteworthy that, apart from the loss of CalDAG-GEFI, we did not find any expression defect in either Rap-1 or talin in patient platelets and leukocytes (unpublished data). The involvement of CalDAG-GEFI in platelet, neutrophil, and lymphocyte integrin activation suggests that some of the previously published clinical studies of integrin activation defects in leukocytes and platelets (50–53) may involve deficiency in this key GEF. With the exception of RAPL, none of the aforementioned Rap-1 effectors are specific to hematopoietic cells; thus, a genetic defect in any of these effectors would not be restricted to the hematopoietic lineage, in contrast to the LAD-III defect described here. Indeed, the loss of CalDAG-GEFI in LAD patient blood did not appear to impair any Rap-1-related functions in nonhematopoietic cellular environments; although the two patients exhibited dense bone in x-ray scans, this is apparently caused by impaired migration of bone remodeling precursors into their skull tissues (54). Similar to the phenotype of our patients, CalDAG-GEFI–null mice do not display any severe nonhematopoietic–associated disorder (22).

It is quite surprising that the loss of CalDAG-GEFI in platelets and leukocytes could not be compensated by any other Rap-1 activating GEF. Leukocytes and platelets express multiple Rap-1 GEFs in addition to CalDAG-GEFI, including CalDAG-GEFIII, the receptor tyrosine kinase–stimulated GEF C3G, and the cAMP-triggered GEF EPAC (17, 55). EPAC is expressed at very low levels in neutrophils (53), and thus cannot serve as a major Rap1 GEF in these cells. Importantly, we detected comparable levels of the two other Rap1 GEFs, CalDAG-GEFIII and C3G (Fig. 2 B and not depicted), in LAD neutrophils and lymphocytes, which suggest that these Rap-1 GEFs cannot functionally compensate for a loss of CalDAG-GEFI. Future studies will be required to address this point. Another open question is whether deficiency in CalDAG-GEFI in murine neutrophils results in a similarly dramatic loss of integrin inside–out activation under the experimental conditions studied in the present work. Lastly, although this Rap-1 GEF is missing in murine lymphocytes, Rap-1 activation is considered as important in murine integrin regulation as it is in human integrin regulation (56). If so, one should expect to find in murine lymphocytes an alternative Rap-1 GEF that is critical for lymphocyte integrin activation.

In addition to regulating inside-out integrin activation in platelets, neutrophils, and lymphocytes, Rap-1 and CalDAG-GEFI may also control critical outside–in activation steps, imposed by ligand-induced rearrangements (2, 57). Indeed, in the present work, as well as in a study on neutrophils from a previous LAD-III patient (15), the Mac-1 integrin could undergo normal inside-out activation in the presence of the prototypic chemoattractant PAF, but still failed to generate adhesiveness in response to rapid PAF signals under shear stress conditions, suggesting a defect in outside–in integrin activation of this integrin. Recent studies suggest that without proper anchorage to the cytoskeleton and a series of rearrangements of the β2 integrin LFA-1 by its ligand during subsecond contacts, this integrin, and possibly other integrins operating at leukocyte–endothelial contacts, may fail to generate shear-resistant adhesiveness (30). Rearrangement of integrins by their ligands transduces specific cytoplasmic changes in integrin tails (58), which may be stabilized by in situ GPCR–mediated Rap-1 activation (16). Indeed, inhibition of Rap-1 by overexpression of its GAP, SPA-1, can interfere with integrin adhesiveness, even when the integrin ectodomain is artificially stabilized at a high affinity state by Mn2+ or by activating mAbs (59), which is consistent with a major role of Rap-1, and potentially of CalDAG-GEFI, in outside-in integrin activation. Rap-1 is also implicated in diverse signaling pathways that link shear stress signals to integrin activation in various cell types (12, 60). Future studies will need to address these multiple potential roles of CalDAG-GEFI as an integrator of both inside-out and outside-in integrin activation events in different neutrophil and lymphocyte subsets.

**MATERIALS AND METHODS**

**Reagents and mAbs.** Recombinant ICAM-1-IgG1 and E-selectin-IgG1 fusion protein, as well as human SDF-1α (CXCL12) and CXCL8 (IL-8), were purchased from R&D Systems. Recombinant soluble seven-domain human VCAM-1, VCAM-1 (61), was a gift from R. Lobb (Biogen, Cambridge, MA). BSA (fraction V), Ca2++, and Mg2+-free HBSS, EGTA, Hepes, fibrinogen, and Ficol-Hypaque 1077 were obtained from Sigma-Aldrich. Human serum albumin (fraction V) was purchased from Calbiochem. The anti-β2-integrin subunit mAb TS1.18, the anti–LFA-1 TS2.4, and the anti–Mac-1 integrin mAbs CBRM1/2 and CBRM1/5 (62) were gifts from T. Springer (Harvard University, Boston, MA). The Alexa Fluor 488–conjugated anti–β2-integrin neoepitope 327C mAb (33) was a gift from D. Staunton (ICOS Corpora–

**Platelet isolation and aggregation studies.** Informed consent was obtained from each individual studied. This study was approved by the Institutional Review Board of the Rambam Medical Center, which is consistent with the provisions of the Declaration of Helsinki. Citrated blood was centrifuged at 150 g for 10 min at RT, and platelet-rich plasma was isolated (63). Platelets were adjusted to a concentration of 3 × 10^6/ml with 1/10 volume of 3.2% buffered sodium citrate. Aggregation was initiated by adding 5 μM ADP, 10 μM epinephrine, 10 μg/ml arachidonic acid, 2 μg/ml collagen, or 1.25 mg/ml ristocetin. Aggregation was monitored by light transmission using a two–channel aggregometer (Chrono-Log Corp.).
Isolation and culture of leukocytes. Human peripheral blood neutrophils and T lymphocytes were isolated from citrate-anticoagulated whole blood, as previously described (25, 64). Murine T lymphocytes were purified from C57BL/6 splenocytes, as previously described (65). Murine platelets were isolated and purified from wild-type and CalDAG-GEFI knockout mice, as previously described (22). Leukocytes were stored in cation-free HBSS containing 10 mM Hepes, pH 7.4, and 2 mg/ml BSA at room temperature for up to 2 h before experimentation.

RT-PCR analysis. RNA prepared from EDTA-anticoagulated peripheral blood was extracted using the RNeasy mini kit (QIAGEN). cDNA was synthesized using random hexamer primers (Promega) with Superscript II R-Nase H-negative reverse transcriptase (Invitrogen). cDNA was amplified by PCR using specific primers, which are listed in Fig. S1. QRT-PCR was performed as previously described (66), using a LightCycler (Roche) according to the manufacturer’s instructions. PCR reactions were performed in duplicate. PCR amplification consisted of 35–50 cycles of denaturation, annealing, and extension. Denaturation was performed for 15 s at 95°C, annealing was performed at 60°C, and the extension was performed at 72°C for 20 s, with fluorescence detection at 72°C after each cycle. After the final cycle, melting point analyses of all samples were performed within the range of 62–99°C. Expression levels of GusB and of HPRT1 were used for sample normalization. A standard curve was obtained with serial dilutions of a reference cDNA sample amplified concomitantly with the tested samples. CalDAG-GEFI and CalDAG-GEFIII mRNA levels were determined by comparing experimental levels to the standard curves and are expressed as arbitrary units. The primers used are listed in Fig. S1.

Microsatellite analysis. Blood was drawn and DNA was isolated by standard methods. PCR amplification of one microsatellite marker with a maximal length of 260 bp, which contained 11.75 TATC perfect repeats and was located 159,253 bp upstream of CALDAG-GEFI on the minus strand on chromosome 11q13.1, was performed in singleplex reaction by touchdown PCR (MJ Research). Two primers, flanking the microsatellite marker, were used in the reaction, one of which was fluorescently labeled: forward PCR primer, Fam-CCGCGAGACATATAAACCC; reverse PCR primer, ACTTGAGATCTGGAGGGCC. PCR products were separated on a 16-capillary automated genetic analyzer (AB 3100; Applied Biosystems). Results were analyzed using the GeneScan analysis software (AB 3100).

DNA sequencing. The full CalDAG-GEFI gene, including the 2,000-bp region upstream of the first exon and the 500-bp region downstream of the last exon (chr11:6,240,405-6,271,447), were PCR amplified, and 90% of these segments were successfully sequenced in LAD patient A as previously described (67). The primers used for each segment are listed in Fig. S1. Primers of fragment 32 (Fig. S1) were used to amplify the region containing the IVS15nt718c→g mutation, and thus were subsequently used to screen the genomic DNA of LAD patient K, the healthy brother of LAD patient A, the 4 parents of both patients, and 58 Turkish control donors. Sequence analysis of each 5-s interval, the number of cells that remained bound was expressed as a percentage of the total number of cells. The base of the mutation is highlighted. Shown in Fig. S3 is the sequence of the genomic DNA surrounding the putative mutation. The genomic DNA was amplified using primer set 32 (Fig. S1), and analyzed as described in Fig. 3. The base of the mutation is illustrated. Shown are the base alignment (left) and the trace alignment (right). Fig. S4 shows splice overlay overnight at 4°C with the indicated concentrations of ICAM-1-Fc. sVCAM-1 was coimmobilized with CXCL12 (both at 2 μg/ml), as previously described (25). E-selectin/ICAM-1 substrates were prepared by coating 0.2 μg/ml E-selectin-Fc on high density protein A, followed by ICAM-1-Fc coating (5 ng/ml). Neutrophil arrests on this substrate were totally blocked by pretreatment with the β2 integrin-blocking mAb TS1.18. Fibronogen was coated directly at 0.5 μg/ml and washed, and PAF (Sigma-Aldrich) was overlayed at 100 nM for an additional hour. PAF was verified to potently activate neutrophil integrins via their PAF receptors (26). For adhesion experiments on resting or TNFα-activated endothelial cells, primary HUVECs (passage 2 or 3) were left intact or stimulated for 18 h with heparin-free culture media supplemented with TNFα (2 ng/ml, 100 U/ml; R&D Systems) (70).

Analysis of leukocyte attachments and resistance to detachment developed during short, static contacts. All shear flow experiments were performed at 37°C. Neutrophils and T lymphocytes were suspended in binding medium (cation-free HBSS, containing 10 mM Hepes, pH 7.4, and 2 mg/ml BSA supplemented with Ca2+ and Mg2+ at 1 mM each) and immediately perfused through the chamber at controlled flow rates, as previously described (25). All cellular interactions with the adhesive substrates were determined by a MatLab-based computerized tracking of individual cell motion within at least two fields of view (each 0.17 mm² in area). “Transient” tethers were defined as cells attached briefly (<2 s) to the substrate; “rolling tethers” were attached cells that persistently rolled for at least 2 s over the substrate; “rolling then arrested” were cells that stopped for at least 3 s after a rolling period; “arrest (firm) tethers” were defined as tethered cells that immediately stopped for at least 3 s (30). For analysis of integrin-mediated adhesion strengthening at short stationary contacts, leukocytes were perfused into the flow chamber and allowed to settle onto the substrate for 1 min. Flow was then initiated and increased step-wise every 5 s through a programmed set of flow rates. At the end of each 5-s interval, the number of cells that remained bound was expressed relative to the number of cells originally settled on the substrate.

Online supplemental material. Fig. S1 shows the primers used for PCR amplification and sequencing of CalDAG-GEFI. Fig. S2 shows the polymorphisms found in the genomic sequencing. Note that all alleles refer to the minus strand. Fig. S3 depicts the sequence of the genomic DNA surrounding the putative mutation. The genomic DNA was amplified using primer set 32 (Fig. S1), and analyzed as described in Fig. 3. The base of the mutation is illustrated. Shown are the base alignment (left) and the trace alignment (right). Fig. S4 shows splice site analysis using information theory (23). The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070058/DC1.

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Note added in proof. A paper that describes multiple integrin activation in neutrophils derived from CalDAG-GEFI−/− mice associated with loss of firm adherence to inflamed vessels was published online on May 10, 2007 in the Journal of Clinical Investigation (Bergmeier, W., T. George, H.-W. Wang, J.R. Crittenden, A.C.W. Baldwin, S.M. Cifuni, D.E. Housman, A.M. Graybiel, and D.D. Wagner. J. Clin. Invest. 117:1699–1797).

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