Stromal Cell-Derived Factor-1-Induced LFA-1 Activation During In Vivo Migration of T Cell Hybridoma Cells Requires Gq/11, RhoA, and Myosin, as well as Gq and Cdc42

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Ron D. M. Soede, Ingrid S. Zeelenberg, Yvonne M. Wijnands, Marga Kamp, and Ed Roos

Dissemination of T cell hybridomas in mice, a model for in vivo migration of memory T cells and for T lymphoma metastasis, depends on the chemokine stromal cell-derived factor-1 (SDF-1) and the integrin LFA-1 and correlates well with invasion into fibroblast cultures. In addition to the known role of the pertussis toxin-sensitive GTPase Gq, we show that also the pertussis toxin-insensitive GTPase Gq/11 is required for dissemination and invasion. Furthermore, we show that the small GTPases, Cdc42 and RhoA, are involved, and that invasion is blocked by inhibitors of actinomyosin contraction. Gq/11, RhoA, and contraction are specifically required for LFA-1 activation, since 1) they are essential for LFA-1-dependent migration toward low SDF-1 concentrations through ICAM-1-coated filters, but not for migration toward high SDF-1 levels, which is LFA-1 independent; 2) G protein (AIF4)-induced adhesion to ICAM-1 requires RhoA and contraction; 3) constitutively active Gq induces aggregation, mediated by LFA-1. We previously reported that binding of this activated LFA-1 to ICAM-1 triggers a signal, transduced by the ζ-associated protein 70 tyrosine kinase, that activates additional LFA-1 molecules. This amplification of LFA-1 activation is essential for invasion. We show here that ζ-associated protein 70-induced LFA-1 activation requires neither Cdc42 and RhoA nor contraction and is thus quite different from that induced by SDF-1. We conclude that two modes of LFA-1 activation, with distinct underlying mechanisms, are required for the in vivo migration of T cell hybridomas. The Journal of Immunology, 2001, 166: 4293–4301.

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Dissemination of T cell hybridomas in mice, a model for in vivo migration of memory T cells and for T lymphoma metastasis, depends on the chemokine stromal cell-derived factor-1 (SDF-1) and the integrin LFA-1 and correlates well with invasion into fibroblast cultures. In addition to the known role of the pertussis toxin-sensitive GTPase Gq, we show that also the pertussis toxin-insensitive GTPase Gq/11 is required for dissemination and invasion. Furthermore, we show that the small GTPases, Cdc42 and RhoA, are involved, and that invasion is blocked by inhibitors of actinomyosin contraction. Gq/11, RhoA, and contraction are specifically required for LFA-1 activation, since 1) they are essential for LFA-1-dependent migration toward low SDF-1 concentrations through ICAM-1-coated filters, but not for migration toward high SDF-1 levels, which is LFA-1 independent; 2) G protein (AIF4)-induced adhesion to ICAM-1 requires RhoA and contraction; 3) constitutively active Gq induces aggregation, mediated by LFA-1. We previously reported that binding of this activated LFA-1 to ICAM-1 triggers a signal, transduced by the ζ-associated protein 70 tyrosine kinase, that activates additional LFA-1 molecules. This amplification of LFA-1 activation is essential for invasion. We show here that ζ-associated protein 70-induced LFA-1 activation requires neither Cdc42 and RhoA nor contraction and is thus quite different from that induced by SDF-1. We conclude that two modes of LFA-1 activation, with distinct underlying mechanisms, are required for the in vivo migration of T cell hybridomas.
latter are specifically blocked by reagents that affect LFA-1 function, which have no effect at high SDF-1 levels. For instance, only the migration toward low SDF-1 levels involves the tyrosine kinase ζ-associated protein 7 (ZAP-70) (12). We showed that ZAP-70 acts downstream of LFA-1 and is essential because it activates additional LFA-1 molecules on the same cell (14), thus amplifying the SDF-1 signal, and this amplification is essential for invasion to occur. We proposed that invasion occurs in two steps: 1) initiation by limiting amounts of SDF-1 on the fibroblasts or in tissues in vivo, causing polarization and motility and activation of some LFA-1 molecules; and 2) this activated LFA-1 binds ICAM-1 or ICAM-2, which triggers ZAP-70 that causes activation of additional LFA-1 molecules, thus amplifying and propagating the signal. According to this model, invasion involves two modes of LFA-1 activation, induced by chemokine and integrin-to-integrin signals, respectively.

Rho-like small GTPases control activities of the actin cytoskeleton (15). Hence, they play a pivotal role in cellular shape changes that are essential for cell migration. For lymphocytes, their specific roles in the migration process have been only partially defined. Activation of the integrin α4β1 by the chemotactic peptide fMLP was reported to activate RhoA (2), and chemotaxis of T cells induced by SDF-1 was reported to depend on Cdc42 (16). LFA-1-mediated adhesion can be induced or enhanced by constitutively active V12Rac1 (17, 18), but, independently, also by active protein kinase C and the Ras-like GTPase Rap1 (18, 19). In noninvasive BW5147 lymphoma cells, invasiveness could be induced by Tiam1, an exchange factor for Rac1 (20), as well as by active V12Rac1 (21), but, independently, also by active V12Cdc42 (22). It is not clear, however, which of these integrin activation pathways are actually used by cells as they migrate through tissues in vivo.

Chemokine receptors, like all heptahelical receptors that can induce chemotaxis (23), are coupled to heterotrimeric GTPases of the Gq/11 subfamily. However, chemokine receptors also couple to other types of G proteins, and there is some evidence suggesting that these other G proteins play a role in migration (24). We recently found that a dominant-negative mutant of Gq/11 blocked dissemination of a myeloid leukemia to bone marrow, spleen, and liver (25). Remarkably, the Gi protein inhibitor, pertussis toxin, blocked only the dissemination to spleen and liver, not that to bone marrow, so that for influx into bone marrow Gi/11 is required, whereas Gq is not. For that cell line, however, we have not yet identified the factor(s) that triggers Gi and the process in which Gi/11 is involved.

In the present study we have investigated the roles of the small GTPases Cdc42 and RhoA and the heterotrimeric GTPase Gq/11 in the two steps of the T cell hybridoma invasion process. We show that Cdc42, RhoA, and Gq/11 are essential for T cell hybridoma dissemination to all tissues. Furthermore, we conclude that step 1 of the invasion process, SDF-1-induced LFA-1 activation, involves Gq/11, and requires both Cdc42 and RhoA activity as well as actinomyosin contraction. In contrast, the mechanism underlying step 2, LFA-1-induced LFA-1 activation, is quite distinct, since it requires neither contraction nor RhoA or Cdc42. Finally, we show that the LFA-1-independent migration toward high SDF-1 concentrations does not require RhoA and, remarkably, does not involve myosin activity.

Materials and Methods

Cells and culture conditions

TAM2D2 T cell hybridoma cells (9) were cultured in hybridoma medium (9), an enriched RPMI 1640 medium. Phoenix cells (26), provided by Dr. G. P. Nolan, and rat embryo fibroblasts were cultured in DMEM, supplemented with 10% FCS and 1% 1-glutamine.

Generation and transduction of DNA constructs

The G208A mutant of G11 (27), provided by Dr. C. D. Tsoukas (San Diego State University, San Diego, CA), was cloned into plZRS-ires-Hygro-EFP, made by replacing the Zeocin resistance protein-encoding cDNA in the LZRS-encoding vector (28) with a cDNA encoding a fusion protein of the hygromycin resistance protein and the enhanced green-fluorescent protein (GFP). The internal ribosome entry site (IRES) causes correlated expression of the G11 mutant and GFP and thus allows for selection of high expressors by FACS sorting. The construct was transfected by calcium phosphate precipitation into Phoenix cells (29). The supernatant was collected 72 h after transfection and centrifuged at 1200 rpm for 5 min to remove cell debris, and 1 ml was mixed with 10 μl of DOTAP (Roche, Mannheim, Germany) and added to 107 TAM2D2 cells. After 24 h the TAM2D2 cells were transferred to fresh medium and another 24 h to medium containing 1 mg/ml hygromycin B (Calbiochem, La Jolla, CA). GFP fluorescence of the G208A-G11-transduced cell population was measured by FACS, and clones with high expression levels were isolated by single-cell sorting. We selected the clones with stable and homogeneous GFP levels for further analysis. The cDNA encoding the constitutively active Q209L mutant of G11 (30), provided by Dr. J. S. Gutkind, was cloned into the retroviral vector pMFG-ires-geo, which contains an IRES- and a cDNA-encoding geo, a fusion of the neomycin resistance and β-galactosidase (lacZ) proteins, 3′ of the inserted cdna (12). Transfection into Phoenix cells and infection of TAM2D2 cells were performed similarly to that described above. From the Gq/11-resistant-transduced cells, clones with high β-galactosidase activity were selected by lacZ staining.

LZRS-IRES-Zeo vectors containing Myc epitope-tagged N17Cdc42 and N19RhoA (28) were provided by Dr. J. G. Collard. Zeocin (200 μg/ml) was added to the medium 72 h after transduction of these vectors. Populations of transduced cells were used rather than clones. The LFA-1 levels of all the above transfected clones were checked by FACS analysis and found to be identical with those of the nontransduced cells.

Expression and activity assays

G208A-G11 and Myc-tagged N17Cdc42 and N19RhoA were detected by Western blotting. SDS-PAGE-separated cell lysates were blotted to nitrocellulose, which was then blocked with 3% fat-free dried milk and 1% BSA. The blots were incubated for 1 h at 20°C with the rabbit anti-human G11 polyclonal antisierum QL (31), provided by Dr. S. Hermouet, or mouse mAbs against the Myc epitope, Cdc42 or RhoA (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with donkey anti-rabbit or sheep anti-mouse Abs coupled to HRP, and detection with the ECL kit (Amersham). Cdc42 and RhoA activity assays were performed as previously described (32, 33). In brief, 2 × 105 cells were lysed on ice in lysis buffer (50 mM Tris·HCl (pH 7.4), 1% Nonidet P-40, 100 mM NaCl, 10% glycerol, 5 mM MgCl2, and protease inhibitors). Cleared lysates were incubated for 30 min at 4°C with GST-PAK (p21-activated kinase) or GST-rhotekin, bound to glutathione-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) to precipitate GTP-bound Cdc42 and Rho, respectively. Precipitated complexes were washed three times in lysis buffer and boiled in sample buffer. Total lysates and precipitates were analyzed on Western blot using the mAbs against Cdc42 and RhoA.

Invasion and migration assays

Invasion assays were performed as previously described (9). Briefly, TAM2D2 cells or transfectants were added to confluent rat embryo fibroblasts in serum-free medium. After 1 h at 37°C and 5% CO2, the monolayers were extensively washed and then paraformaldehyde-fixed. The invaded cells were counted using phase contrast microscopy, and the percentage of invaded cells was calculated. Chemotactic migration was assayed in Transwells, with 8-μm pore size filters, that were either or not coated with soluble ICAM-1, as previously described (12). Briefly, the lower chamber was filled with 250 μl of RPMI containing 0.1% OVA and 0.1 or 100 ng/ml SDF-1. The Transwell, placed on top, and 150 μl of medium with 105 cells was inserted into the upper chamber. The data presented are the percentages of added cells that have been collected from the lower chamber after 2 h at 37°C and 5% CO2.

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Adhesion assays

Cells secreting murine sICAM-1 were a gift from Dr. F. Takei. Purification of sICAM-1 was conducted as described by Welder et al. (34). For adhesion assays with TAM2D2 cells, microtiter plates were coated overnight at 4°C with 100 μl of sICAM-1/well (2 μg/ml in PBS). Unbound sites were subsequently blocked with 0.5% OVA in 20 mM Tris buffer, pH 7.2, for 2 h at room temperature. TAM2D2 cells or transfectants were incubated for 15 min in 20 mM Tris buffer, pH 7.2, containing 150 mM NaCl, 6 mM KCl, 5 mM D-glucose, 1 mM CaCl₂, and 1 mM MgCl₂; and supplemented, or not, with AIF₄⁻ (10 mM NaF and 40 μM AlCl₃) or 100 ng/ml PMA and then transferred in that medium to the ICAM-1-coated wells. AIF₄⁻ and PMA remained present during the adhesion assay. Cells were centrifuged onto the plate for 1 min at 1200 rpm to synchronize adhesion. After incubation for 30 min at 5% CO₂ and 37°C, nonadherent cells were washed off, and the number of adherent cells was determined by assaying hexosaminidase activity, using known numbers of cells as standard (35).

Dissemination

Cells (5 × 10⁵) in 200 μl of PBS supplemented with 1 mM CaCl₂ and 1 mM MgCl₂ were injected into a lateral tail vein of 2- to 3-mo-old syngeneic AKR mice. All animals were sacrificed when mice injected with control cells became moribund or after 100 days. All mice were examined for the presence of macroscopically visible tumor in different organs. To test tumorigenicity, 10⁶ cells were injected i.p. After 2 wk, the peritoneal cavity was flushed with PBS, and the cells were counted.

Aggregation assay

Aggregation of the Q209L Gq transfectants was assayed using 10⁶ cells from a dense culture (≈10⁷ cells/ml) in 0.5 ml of HBSS, pH 7.0, supplemented with 20 mM HEPES, 0.35 g/l NaHCO₃, 1 mM CaCl₂, and 1 mM MgCl₂ in a 10-ml tube. The cells were incubated in a water bath at 37°C for 2 h in an upright position and shaken at low speed. By mild agitation with a wide-bore pipette, the suspensions were then dispersed, and 100-μl samples transferred to a flat-bottom dish and photographed using an inverted microscope. Aggregation induced by subsaturating concentrations of the M17/4 mAb against LFA-1 was assessed similarly as described previously (14).

Inhibitors

In some experiments we used 30 μM 1-(5-chloronaphtalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine-HCl (ML-7; Biomol, Plymouth Meeting, PA), an inhibitor of myosin light chain kinase, or 20 mM 2,3-butanedione-2-monoxime (BDM; Sigma, St. Louis, MO), a myosin inhibitor. The cells were preincubated for 30 min with the inhibitors in the buffers or media appropriate for the assays and remained present during the assay.

Results

Cdc42 and RhoA are involved in dissemination of T cell hybridoma cells

Both N17Cdc42 and N19RhoA blocked invasion of the T cell hybridoma cells into fibroblast monolayers (Fig. 3A), a process that is dependent on SDF-1 produced by the fibroblasts and LFA-1 present on the T cell hybridoma cells and that correlates well with dissemination capacity (see Introduction). N17Cdc42 inhibited invasion almost completely and N19RhoA by about 90% despite the relatively low expression level of N19RhoA. N17Cdc42 also blocked migration toward both high and low SDF-1 concentrations (Fig. 3B). In striking contrast, N19RhoA did not affect the migration induced by 100 ng/ml SDF-1, which is independent of LFA-1. However, it did block migration at 1 ng/ml, which only occurs when filters are coated with the LFA-1 ligand ICAM-1 and which filter.

FIGURE 2. Dissemination of N17Cdc42 and N19RhoA transfectants to the liver. Shown is the liver weight 4 wk after injection of 5 × 10⁶ cells into a tail vein. Also shown is the number of mice with macroscopically visible tumors (since some livers within the normal weight range still contained detectable tumors). The dashed line indicates the upper limit of normal weight range. Untr., untransfected cells; Empt.v., empty vector-transduced control cells; Cdc/1, Cdc/2 and Rho/3, Rho/6, two independent Zeocin-resistant populations transduced with N17Cdc42 and N19RhoA, respectively.

FIGURE 1. Expression of N17Cdc42 and N19 RhoA in transfectants. Upper panel, Myc tag Ab detecting only the transfected proteins. Lower panels, Abs against Cdc42 (left) and RhoA (right) detecting both the transfected and the endogenous proteins. Untr., untransfected cells; Cdc/1, Cdc/2 and Rho/3, Rho/6, two independent Zeocin-resistant populations transduced with N17Cdc42 and N19RhoA, respectively.
requires LFA-1 (12). Thus, RhoA is essential only when LFA-1 is involved. RhoA is required for G protein-induced, but not PMA-induced, LFA-1 activation

The requirement of RhoA for LFA-1 function indicated that RhoA is involved in SDF-1-induced LFA-1 activation. To study this, we attempted to assess SDF-1-induced adhesion to ICAM-1 as has been described by others (3), but this adhesion was very weak and poorly reproducible. However, LFA-1 activation by G proteins can be mimicked with AlF$_4^-$ (36), which activates heterotrimeric G proteins. As shown in Fig. 3C, both N17Cdc42 and N19RhoA inhibited AlF$_4^-$-induced adhesion to ICAM-1, whereas adhesion induced by PMA was not affected. We thus conclude that both Cdc42 and RhoA are specifically involved in G protein-induced LFA-1 activation.

Both Cdc42 and RhoA are activated in the T cell hybridoma cells

The above results suggested that both Cdc42 and RhoA are activated by SDF-1. To study this, we performed Cdc42 and RhoA activity assays (32, 33). As shown in Fig. 4, active Cdc42 and active RhoA were present in the cells in the absence of added stimuli, i.e., also in serum-free medium. These assays were performed several months after the experiments described above. During that period the levels of N17Cdc42 and especially N19RhoA had decreased substantially in the transduced cells, as is evident when comparing the endogenous with the larger tagged transfected proteins in Figs. 1 and 4. Nevertheless, the amount of active Cdc42 and RhoA was still substantially reduced in the N17Cdc42 and N19RhoA transfectants, respectively. We did not observe an increase in activation by SDF-1 at any concentration or at any time point up to 30 min after addition to the cells, at least not to an extent large enough to be detected on top of the GTPase activity already present. However, upon stimulation with AlF$_4^-$ we did observe an increase in Cdc42 activity (Fig. 4), but not in RhoA (not shown).

Role of heterotrimeric GTPase $G_q/11$ in dissemination, invasion, and migration

AlF$_4^-$ activates all heterotrimeric G proteins. Furthermore, the SDF-1 receptor couples to other G proteins in addition to the pertussis toxin-sensitive members of the $G_i/G_o$ subfamily that are required for chemotactic responses to SDF-1 in these T cell hybridoma cells (12). $G_q$, in particular, is involved in certain responses to SDF-1, such as calcium mobilization and activation of phospholipase C (PLC) (24). Furthermore, $G_q$ appears to activate RhoA in certain cell types (37). Together, this suggested a role for $G_q$ proteins in SDF-1-induced migration. To study this, we transduced the...
G208A mutant of G11. G11 is highly homologous to Gq and is coexpressed with Gq in most cells, and the two have largely redundant functions (38). The G208A mutation is analogous to that in Gq and Ga mutants, which were shown to prevent GTP-induced activation and to specifically inhibit Gq and Ga functions in a dominant-negative fashion (39–41). Also, the G208A-G11 mutant has been reported to inhibit Gq/11 signaling specifically (27).

The G208A-G11 mutant was coexpressed with GFP. The transfectant populations showed a wide range of GFP expression by FACS analysis. From these populations, clones with high, homogeneous, and stable expression were isolated by FACS sorting. In Fig. 5A, GFP expression levels are shown of the two clones that were tested. As shown in Fig. 5B, expression of the G208A-G11 mutant in these cells was substantially higher than that of endogenous Gq/11, which is only visible after prolonged exposure (not shown). As a control for in vivo experiments, we also sorted a population of empty vector transfectants with comparable expression (Fig. 5A).

The G208A-G11 mutant strongly reduced dissemination of the TAM2D2 cells. After 4 wk all mice injected with either the parental cells or the empty vector transfectant population had died (Fig. 6), whereas mice injected with the G208A-G11 transfectants survived for a prolonged period, and most survived for 100 days and contained no macroscopically detectable tumor upon autopsy. The mutant also substantially reduced the (SDF-1- and LFA-1-dependent) invasion into fibroblast monolayers (Fig. 7A) and LFA-1-dependent migration toward 1 ng/ml SDF-1 (Fig. 7B). In contrast, the LFA-1-independent migration toward 100 ng/ml SDF-1 was not affected (Fig. 7B), indicating that Gq/11 is specifically involved in the activation of LFA-1.

Myosin activity is required for G protein-induced LFA-1 activation, but not for SDF-1-induced migration

The above results indicate that both RhoA and Gq/11 are involved in the SDF-1-induced activation of LFA-1. RhoA is known to trigger contraction by influencing myosin light chain phosphorylation,
Constitutively active \( G_q \) activates LFA-1, and this requires myosin activity

Activation of LFA-1 by \( G_{q/11} \) cannot be demonstrated by an effect of the G208A-G11 mutant on AlF\(_4^-\)-induced adhesion, since the mutant acts by competing for receptor binding and does not affect the direct activation of \( G_{q/11} \) by AlF\(_4^-\). In an attempt to demonstrate this effect of \( G_q \), we transduced the constitutively active Q209L mutant of \( G_q \) (30). Strikingly, this mutant induced extensive aggregation (Fig. 9). The transfected mutant was not tagged, and it was barely detectable as an increase in the endogenous \( G_q/11 \) levels (not shown), indicating that it has a strong effect at low levels and may be toxic at higher levels. Aggregation was already seen in the culture flasks, but was enhanced by mild agitation. Blocking mAbs against LFA-1 and ICAM-2 inhibited aggregation completely (not shown). BDM and ML-7 also blocked completely, as shown for BDM in Fig. 9. RhoA activity was not increased in this transfectant (not shown). In line with this, we saw no effect of dominant-negative G208A-G11 on RhoA activity in these cells, indicating that this activity is not due to \( G_{q/11} \) activation. Although active \( G_q \) apparently activated LFA-1, it actually inhibited invasion completely. This may be due to the fact that LFA-1 is activated on the entire surface, which may interfere with the sequential local activation required during invasion. Alternatively, part of the relevant signaling required may have been down-regulated by the constant \( G_q \) signal.

LFA-1-induced LFA-1 activation, required for invasion, does not involve RhoA or myosin

We showed previously that the tyrosine kinase ZAP-70 is required for invasion and that it is specifically involved in LFA-1-dependent processes, since dominant-negative ZAP-70 blocked migration induced by 1, but not 100, ng/ml SDF-1 (12). Furthermore, we demonstrated that ZAP-70 is activated downstream of LFA-1 (12) and is involved in activation of additional LFA-1 molecules (14), thus amplifying and propagating the initial signal. As an assay for this LFA-1 to LFA-1 signal we use the aggregation of the cells induced by subsaturating concentrations of blocking LFA-1 mAbs (14). Cross-linking of the occupied LFA-1 molecules causes a signal that activates the free LFA-1 molecules, which then bind to

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<th>Inhibition by</th>
<th>G-Protein (AlF(_4^-))−Induced Adhesion to ICAM-1</th>
<th>Aggregation Induced by Subsaturating LFA-1 mAb</th>
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<tr>
<td>Piceatannol</td>
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<tr>
<td>Dominant-negative ZAP-70</td>
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<td>N17Cdc42</td>
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*Strong or complete inhibition is indicated by +. Aggregation induced by subsaturating concentrations of LFA-1 mAb, an assay for LFA-1-induced LFA-1 activation was described previously (14), as were the cells expressing dominant-negative ZAP-70 and the effects of the ZAP-70 inhibitor piceatannol, the PLC inhibitor U73122 and the calpain inhibitor calpeptin (12, 14).
ICAM-2 on adjacent cells. This causes aggregation that is blocked by dominant-negative ZAP-70 and the ZAP-70 inhibitor piceatannol. Here, we have tested the effect of inhibitors on this LFA-1-induced as well as G protein-induced LFA-1 activation. The results are listed in Table I. Inhibitors of PLC and calpain blocked both processes. Dominant-negative ZAP-70 and piceatannol did not affect the G protein-induced adhesion, confirming that ZAP-70 acts downstream of LFA-1. G208A-G11 did not affect LFA-1 mAb-induced aggregation, as expected, since it acts on signals of G protein-coupled receptors. Remarkably, however, N17Cdc42, N19RhoA, BDM, and ML-7 also had no effect, showing that actinomyosin contraction is clearly not required, and the roles of small GTPases in the two processes are different. An alternative way to assess the LFA-1 to LFA-1 signal is to assay the adhesion of cells to a substrate coated with the blocking Abs (14). The results of this assay confirmed those of the aggregation assay (not shown). Apparently the mechanisms underlying the two modes of LFA-1 activation, by G protein and by LFA-1, are quite distinct.

Discussion

Our present and previous results show that during T cell migration in vivo as well as invasion into fibroblast monolayers in vitro, the integrin LFA-1 is activated by two distinct mechanisms. The first activation is induced by the chemokine SDF-1, involves $G_{i}$ and $G_{q}$, and depends on Cdc42, RhoA, and myosin activity. The second occurs upon binding of thus activated LFA-1 to ICAM-1 or -2, causes activation of additional LFA-1 molecules on the same cell, and does not depend on Cdc42, RhoA and myosin. This idea is illustrated in Fig. 10.

Our conclusion that the $G_{q11}$ subfamily of heterotrimeric G proteins is involved in LFA-1 activation by SDF-1 is based on the effect of the dominant-negative G208A-G11 mutant. This mutant is analogous to $G_{i}$ and $G_{q}$ mutants with demonstrated specific dominant-negative activity, and evidence has been provided that this is also true for the G11 mutant (27). The mutant did not inhibit migration toward a high concentration of SDF-1, indicating that $G_{i}$ is not required. It cannot be formally excluded that the mutant is less effective when the $G_{q11}$ signal is strong, but this seems unlikely since we saw no inhibition at all. As shown previously (12), invasion is better mimicked by the LFA-1-dependent migration toward low SDF-1 levels, when the signal is much weaker. In fact, invasion and migration toward 1 ng/ml SDF-1 were both strongly inhibited by the mutant, and the same was true for migration in vivo, as assessed by metastasis formation in mice. This indicates that $G_{i}$ is specifically involved in the SDF-1-induced LFA-1 activation. This idea is supported by our finding that constitutively active $G_{q}$ induces LFA-1-dependent aggregation. We have recently shown that the G208A-G11 mutant blocked dissemination of myeloid leukemia cells to the liver, spleen, and bone marrow (25). Remarkably, whereas the colonization of liver and spleen was also dependent on $G_{i}$ proteins, this was not true for the bone marrow. For these myeloid cells we had no information on the relevant chemokines or other activating factors and on the integrins involved. Our present results suggest that also in myeloid cells $G_{q11}$ is required for integrin activation by factors in tissues such as the bone marrow.

$G_{q}$ is a strong activator of members of the PLC-β subfamily (44). PLC is likely to be involved, since the PLC inhibitor U73122 blocks invasion (14). PLC hydrolyzes PIP2 to generate diacylglycerol and inositol trisphosphate that activate protein kinase C and release intracellular $Ca^{2+}$, respectively. Protein kinase C inhibitors do not influence invasion of these cells (14), but $Ca^{2+}$ is probably required, since an inhibitor of the calcium-dependent protease calpain blocks invasion (14) (see Table I) and also the $G_{q}$-induced aggregation (not shown). In fact, a rise in intracellular $Ca^{2+}$ has been shown to activate LFA-1, and this involves calpain (45). However, we found that calpain is also involved in migration toward high SDF-1 levels (14), which is not dependent on LFA-1 and, as shown here, is not inhibited by dominant-negative $G_{q11}$. More importantly, we show that activation of LFA-1 by $G_{q}$ requires actinomyosin contraction, induced by MLCK, in contrast to migration toward high SDF-1 levels. Contraction and LFA-1 activation are probably caused by Ca/calmodulin-dependent MLCK (46), activated by the $Ca^{2+}$ that is released by the inositol trisphosphate generated by PLC. At high SDF-1 levels PLC is also required (14), apparently not to trigger MLCK but to activate calpain and possibly yet other effectors. This PLC activation may be induced by $ββ$ dimers, which can also activate PLC-β. Although this activation is much weaker than that induced by $G_{q}$ (47), it may be sufficient when the signal is strong, so that the SDF-1-induced $G_{q}$ activity may not be required at such high SDF-1 levels. We propose that at the low SDF-1 concentrations, which are apparently more relevant in vivo, $G_{q}$ is required for a sufficiently strong PLC response to the weak chemokine signal.

RhoA activity is also required for chemokine-induced and, more generally, G protein-induced LFA-1 activation, but its role is not clear. RhoA can cause contraction upon activation of Rho kinase, which phosphorylates myosin light chain directly (48) or has an indirect effect by phosphorylation of myosin phosphatase (49). However, the Rho kinase inhibitor Y-27362 did not inhibit LFA-1 activation by $G_{q}$ or AlF$_4^-$ (not shown). Furthermore, although $G_{q}$ has been claimed to activate RhoA (37), we have seen no such activation by $G_{q}$ or AlF$_4^-$. In fact, active RhoA was already present, even in suspended cells in serum-free medium, and further
activation may not be required. This activity is not triggered downstream of G_{q/11}, since it was not reduced in the cells expressing the dominant-negative G_{208A-G11}. In noninvasive BW5147 lymphoma cells, invasiveness can be induced by transfected active Rac1 (21). It is noteworthy that this invasion only occurs in the presence of (serum-derived) LPA (lyosphosphatidic acid), in part because it activates RhoA (22). The T cell hybridomas used here invade massively in the absence of serum or LPA, possibly because RhoA is already active. Thus, it seems clear that RhoA activity is an important permissive factor for invasion, but it remains to be determined how this RhoA is activated and which of the RhoA effector pathways are involved.

An obvious possible role for actinomyosin contraction in chemokine-induced LFA-1 activation is the clustering of the LFA-1 molecules (43), causing enhanced avidity for ligand. We have, however, found little evidence for this explanation. First, we showed previously with immunogold labeling and electron microscopy that LFA-1 is already aggregated in these cells, even in suspension, in multimolecular clusters of approximately 50 nm (50), indicating that the contraction is not required for clustering at a molecular scale. Alternatively, contraction may lead to the formation of large aggregates of clusters. In this study we have not observed such large clusters by confocal microscopy in cells induced to adhere by AlF_{4}^{-} i.e., by G protein signals (not shown), even though such adhesion was blocked by MLCK and myosin inhibitors and thus depended on contraction. It is, however, quite likely that contraction occurs only locally and transiently during G protein-induced adhesion and is therefore not readily detected. Another possibility is that contraction has more subtle effects on the conformation or arrangement of individual LFA-1 molecules within the clusters.

Our results with dominant-negative Cdc42 confirm findings by others that Cdc42 activity is required for chemotaxis (51, 52) and, in particular, chemotaxis induced by SDF-1 (16). Indeed, we found that Cdc42 activity is enhanced in cells treated with AlF_{4}^{-}. This suggests that Cdc42 acts as an effector of G_{i} proteins, but the connection is as yet not defined in mammalian cells, whereas in yeast there is evidence for the participation of G protein βγ subunits and Cdc42 in the same pathway (53). The role of Cdc42 is likely to trigger polarization of the cells (54) and to induce actin polymerization (55). Remarkably, the LFA-1-independent migration toward high SDF-1 concentrations required no myosin activity and, therefore, no contraction of the actin cytoskeleton. This indicates that the actin polymerization-driven continuous extension of pseudopods is sufficient to propel cells forward, without contraction. This underscores the specificity of the myosin activity for G protein-induced integrin activation, since it is clearly not required for motility per se. A novel finding was that Cdc42 is also required for G protein-induced adhesion, possibly because actin polymerization is essential to allow the cells to spread on the ICAM-1-coated substrate. This role for Cdc42 (and also for RhoA) is specific for G proteins, since neither Cdc42 nor RhoA is required for adhesion induced by PMA. The latter does require actinomyosin contraction, but this is apparently not dependent on RhoA. The PMA effect may also depend on actin polymerization that is triggered by PMA-activated protein kinase C (56), but apparently Cdc42 is not involved.

We showed previously that invasion and dissemination of the T cell hybridomas are effectively inhibited by a dominant-negative mutant of the tyrosine kinase ZAP-70 (12). We concluded that ZAP-70 acts downstream of LFA-1 and is involved in activation of additional LFA-1 molecules on the same cell, a process that amplifies and propagates the initial LFA-1 activation signal of the chemokine (14). This amplification is essential when the chemokine signal is too weak to trigger enough LFA-1 activation for chemotaxis to occur. This interplay of chemokine and integrin signals allows for the precise regulation of lymphocyte trafficking in vivo by adjustment of levels and gradients of chemokines and integrins as well as chemokine receptors and integrin ligands. The idea that ZAP-70 acts downstream of LFA-1 is confirmed by the lack of effect of the dominant-negative ZAP-70 mutant on G protein-induced adhesion. Conversely, neither dominant-negative Cdc42 and RhoA nor contraction inhibitors had any effect on the LFA-1-induced LFA-1 activation, as assessed in an Ab-induced aggregation assay and an adhesion assay on Ab-coated substrate, as described previously (14). ZAP-70 activates Vav, an exchange factor for Rac1, which can activate LFA-1 (18) as well as other integrins (17), and Rac1 is thus a likely candidate for involvement in the LFA-1 to LFA-1 signal. Unfortunately, however, we did not obtain transfectants expressing dominant-negative Rac1, probably because it impairs the survival and/or proliferation of these cells, and are thus unable to study this possibility by this approach. We conclude that two completely different modes of LFA-1 activation are relevant for in vivo migration of T cells and, remarkably, that both are required simultaneously.

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References


