

Invasion of T-lymphoma cells: cooperation between Rho family GTPases and lysophospholipid receptor signaling

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Rho-like GTPases orchestrate distinct cytoskeletal changes in response to receptor stimulation. Invasion of T-lymphoma cells into a fibroblast monolayer is induced by Tiam1, an activator of the Rho-like GTPase Rac, and by constitutively active V12Rac1. Here we show that activated V12Cdc42 can also induce invasion of T-lymphoma cells. Activated RhoA potentiates invasion, but fails by itself to mimic Rac and Cdc42. However, invasion is inhibited by the Rho-inactivating C3 transferase. Thus, RhoA is required but not sufficient for invasion. Invasion of T-lymphoma cells is critically dependent on the presence of serum. Serum can be replaced by the serum-borne lipids lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) (10^{-7} – 10^{-6} M), which act on distinct G protein-linked receptors to activate RhoA and phospholipase C (PLC)-Ca²⁺ signaling. LPA- and S1P-induced invasion is preceded by Rho-dependent F-actin redistribution and pseudopodia formation. However, expression of both V14RhoA and V12Rac1 does not bypass the LPA/S1P requirement for invasion, indicating involvement of an additional signaling pathway independent of RhoA. The PLC inhibitor U-73122, but not the inactive analog U-73343, abolishes invasion. Our results indicate that T-lymphoma invasion is driven by Tiam1/Rac or Cdc42 activation, and is dependent on LPA/S1P receptor-mediated RhoA and PLC signaling pathways which lead to pseudopod formation and enhanced infiltration.
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Introduction

Rho-like GTPases, including RhoA, Rac1 and Cdc42, have been implicated in the control of a wide range of biological processes, such as the regulation of cytoskeletal structures, adhesion, motility, transcriptional activation and cell cycle progression (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Each of the Rho-like GTPases regulates distinct actin cytoskeletal rearrangements in response to receptor stimulation. In fibroblasts, Cdc42 controls the formation of microspikes (Kozma *et al.*, 1995; Nobes and Hall, 1995), whereas Rac regulates the formation of lamellipodia and membrane ruffles (Ridley *et al.*, 1992). Activation of RhoA leads to the induction

of stress fibers (Ridley and Hall, 1992). Moreover, all three GTPases induce the formation of integrin adhesion complexes in Swiss 3T3 fibroblasts (Hall, 1998), and stimulate transcriptional activation and DNA replication (Olson *et al.*, 1995; Vojtek and Cooper, 1995; Perona *et al.*, 1997).

In search of genes involved in the process of tumor cell invasion and metastasis, we previously have identified the *Tiam1* gene by retroviral insertional mutagenesis in combination with *in vitro* selection of invasive T-lymphoma variants (Habets *et al.*, 1994). The *Tiam1* protein consists of 1591 amino acids and contains several conserved protein motifs, including a Dbl homology (DH) domain. The DH domain is the catalytic domain in many guanine nucleotide exchange factors (GEFs), which activate members of the Rho subfamily of small GTPases (Collard, 1996; Whitehead *et al.*, 1997). GEFs promote the exchange of bound GDP for GTP on these GTPases, which results in their activation. Whereas *Tiam1* displays GEF activity towards all three Rho-like GTPases Rac1, Cdc42 and RhoA *in vitro*, *Tiam1* specifically activates Rac *in vivo* (Michiels *et al.*, 1995). In fibroblasts, *Tiam1* induces the formation of membrane ruffles and stimulates the activation of the Jun N-terminal kinase (JNK), similarly to activated V12Rac1 (Michiels *et al.*, 1997). Furthermore, V12Rac1 induces invasion of T-lymphoma cells, suggesting that *Tiam1*-induced invasiveness of T-lymphoma cells is caused by activation of Rac (Michiels *et al.*, 1995).

Rac1 regulates the organization of the cortical actin cytoskeleton, and in fibroblasts it induces the formation of lamellipodia and membrane ruffling, which are believed to play an important role in cell motility (Stossel, 1993). The role of RhoA in cell adhesion and motility has been studied extensively by using C3 transferase, a toxin that specifically inhibits Rho activity (Sekine *et al.*, 1989). Lysophosphatidic acid (LPA), a serum-borne lipid mitogen (Eichholtz *et al.*, 1993), activates its cognate G protein receptor to stimulate multiple signaling pathways including RhoA signaling (Moolenaar *et al.*, 1997). LPA induces RhoA-dependent stress fiber formation in fibroblasts (Ridley and Hall, 1992) as well as neurite retraction in neuroblastoma cells (Jalink *et al.*, 1994). Both processes are inhibited by C3 transferase. A related platelet-derived serum lysophospholipid, sphingosine-1-phosphate (S1P), has been implicated in the control of cell proliferation, invasion and apoptosis (Cuvillier *et al.*, 1996; Spiegel and Merrill, 1996; Yatomi *et al.*, 1997). Like LPA, S1P activates its own G protein-coupled receptor (Bunemann *et al.*, 1996; Okajima *et al.*, 1996; Postma *et al.*, 1996; Meyer zu Heringdorf *et al.*, 1997; Zondag *et al.*, 1998), although S1P has also been proposed to have an intracellular messenger function (Spiegel and Merrill, 1996). In N1E-115 neuroblastoma cells, LPA and S1P activate their respective receptors to trigger RhoA-dependent neurite

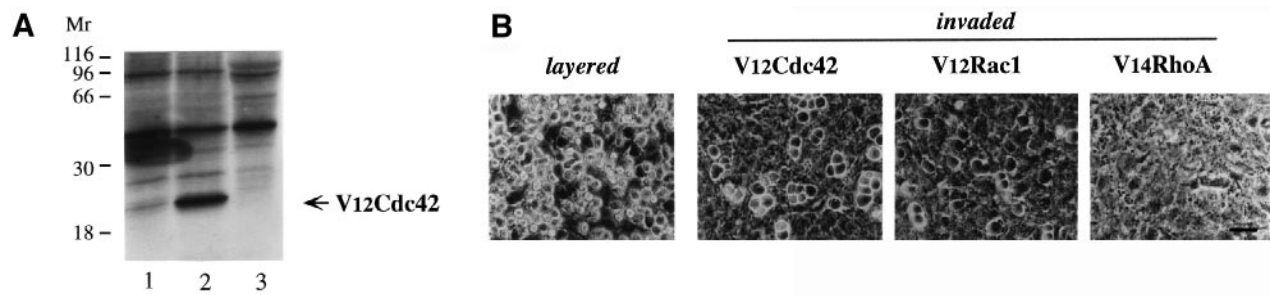


Fig. 1. Constitutively active Cdc42 induces invasion of T-lymphoma cells. (A) Western blot analysis of total lysates of T-lymphoma cells expressing V12Cdc42. T-lymphoma cells were retrovirally transduced with an empty vector (control, lane 3) or with Myc-tagged V12Cdc42 (lanes 1 and 2). The latter were subjected to invasion selection. Lane 1, expression of V12Cdc42 prior to selection; lane 2, expression of V12Cdc42 after three selection rounds. Selection for invasion yielded invasive cells expressing high levels of V12Cdc42. (B) Phase-contrast images of *in vitro* invasion of T-lymphoma cells into monolayers of REF cells. Layered: T-lymphoma cells layered on a monolayer of fibroblasts. Invaded: fibroblast monolayers with infiltrated cells expressing V12Cdc42 or V12Rac1. The non-infiltrated cells were washed away after 4 h incubation. Note that none of the V14RhoA-expressing cells infiltrated the monolayer. Bar indicates 30 μ m.

retraction (Jalink *et al.*, 1994; Postma *et al.*, 1996). Furthermore, LPA induces RhoA-dependent invasion of hepatoma cells (Imamura *et al.*, 1993; Yoshioka *et al.*, 1995), while invasiveness of T-cell hybrids also depends on RhoA (Verschuere *et al.*, 1997). In contrast, S1P exerts inhibitory effects on the motility of smooth muscle and melanoma cells (Sadahira *et al.*, 1992; Bornfeldt *et al.*, 1995; Yamamura *et al.*, 1997).

In this study, we have investigated the role of Cdc42, Rac1 and RhoA in T-lymphoma invasion into a fibroblast monolayer and explored the nature of the serum components required for invasion. Our results indicate that T-lymphoma invasion is driven by Tiam1/Rac or Cdc42 activation. We find that LPA and S1P can fully replace serum in promoting T-lymphoma invasion. Invasion appears to depend on LPA/S1P receptor-mediated RhoA and phospholipase C (PLC) signaling pathways, leading to pseudopod formation and subsequent infiltration into fibroblast monolayers.

Results

Constitutively active V12Cdc42 induces invasion of T-lymphoma cells similarly to V12Rac1

Previously, we have shown that constitutively active V12Rac1 induces invasiveness of T-lymphoma cells (Michiels *et al.*, 1995). To determine the effect of Cdc42 on invasion of T-lymphoma cells, we introduced constitutively active V12Cdc42 into non-invasive BW5147 cells by retroviral transduction. Pools of transduced cells were subjected to three rounds of invasion selection (see Materials and methods). The invasive cells thus selected highly expressed V12Cdc42 (Figure 1A, lanes 1 and 2). Control cells transduced with the empty vector or a vector encoding V14RhoA did not become invasive, as shown previously (Michiels *et al.*, 1995). The efficiency of invasion was assayed by layering the lymphoma cells on a monolayer of rat embryo fibroblasts (REFs) for 4 h, after which non-invaded cells were washed away (Figure 1B). Similar to V12Rac1-transduced cells (Michiels *et al.*, 1995), 10–20% of selected V12Cdc42-expressing cells infiltrated within 4 h into the fibroblast monolayer. The infiltrated cells showed high levels of V12Cdc42 expression, but rapidly lost expression as well as invasive capacity after propagation, due to V12Cdc42-induced

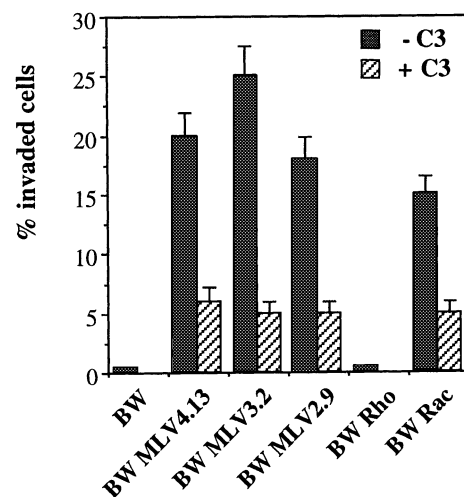


Fig. 2. C3 transferase pre-treatment inhibits invasion. Different T-lymphoma cell lines were pre-treated with or without 30 μ g/ml C3 transferase for 24 h, and tested for invasiveness on a REF monolayer as described in Materials and methods and as illustrated in Figure 1B. The number of cells invaded after 4 h is expressed as a percentage of the number of cells added. Data are presented as mean \pm SEM.

polyploidization and growth arrest. From these studies, we conclude that similarly to V12Rac, overexpression of V12Cdc42 leads to an invasive phenotype in T-lymphoma cells. Since V12Cdc42-expressing cells were less stable than V12Rac1-expressing cells, they were not included in further studies.

RhoA activity is required but not sufficient for Tiam1- and Rac1-induced invasion

Although expression of V14RhoA was not sufficient to induce invasiveness, endogenous RhoA could still be involved in T-lymphoma invasion. To test this, T-lymphoma cells, which had acquired invasiveness either by proviral insertion in the *Tiam1* gene (Habets *et al.*, 1994) or by expression of exogenous V12Rac1 (Michiels *et al.*, 1995), were pre-incubated with C3 transferase for 24 h. C3 transferase inactivates RhoA by ADP ribosylation in its putative effector domain (Sekine *et al.*, 1989). As shown in Figure 2, pre-treatment of the cells by C3 transferase resulted in an inhibition of invasive capacity for all invasive cell lines tested. This indicates that invasion

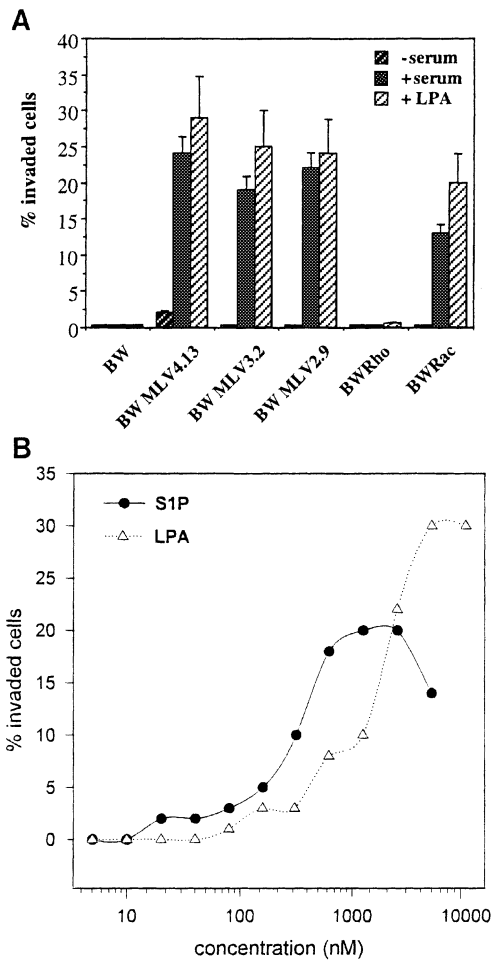


Fig. 3. LPA and S1P can substitute for serum to stimulate invasion. (A) Different invasive T-lymphoma cell lines (see Figure 2) were tested for invasive capacity in medium containing 10% FCS, in serum-free medium, and in serum-free medium containing 5 μ M LPA. Data are presented as mean \pm SEM. (B) Dose-response relationship of LPA- and S1P-stimulated invasion. The invasive capacity of the cell line BW MLV2.9 was tested in serum-free medium with BSA and the indicated concentrations of LPA and S1P. Other cell lines showed similar responses.

of these T-lymphoma cells requires not only an activated Tiam1/Rac signaling pathway, but also RhoA activity.

Serum is required for invasion and can be substituted for by LPA and S1P

T-lymphoma invasion was critically dependent on the presence of serum (Figure 3A): in serum-containing media, ~15–25% of the various T-lymphoma variant cells invaded into the fibroblast monolayer within 4 h. Hardly any invasion occurred under serum-free conditions. Since LPA is a serum factor known to activate RhoA, whilst T-lymphoma invasion is RhoA-dependent, we tested whether LPA could substitute for serum. Indeed, we found that LPA can completely replace the serum requirement during invasion (Figure 3A). LPA (5 μ M) promoted invasion more efficiently than serum (10%). Other receptor ligands such as bombesin (10 nM), platelet-derived growth factor (PDGF; 30 ng/ml), epidermal growth factor (EGF; 100 ng/ml), bradykinin (10 ng/ml) or insulin (1 μ g/ml) had no effect on invasion (not shown). These results

Table I. Stimulation and inhibition of invasion of T-lymphoma cells

Addition ^a	Concentration	Invasion ^b	
		A	B
None	–	<2%	4–7%
Fetal calf serum	10%	15–25%	20–30%
LPA	5 μ M	20–30%	25–35%
Phosphatidic acid	5; 50 μ M	<2%	4–7%
S1P	1 μ M	15–20%	15–25%
Sphingosine phosphatidylcholine	10 μ M	<2%	4–7%
Brain ceramide	10 μ M	<2%	4–7%
C8-ceramide	10 μ M	<2%	4–7%
Ionomycin	0.3; 0.5; 0.7 μ M	ND	6–9%
Thapsigargin	1; 3 μ M	ND	6–9%
PMA (10 min pre-treatment)	1 μ M	ND	<2%

Inhibitor ^c			
None	–	20–30%	25–35%
Suramin ^d	1 mg/ml	<2%	4–7%
U-73122	1 μ M	<2%	2–4%
U-73343	1 μ M	20–30%	25–35%
Butan-1-ol ^d	20 mM	20–30%	25–35%
Pertussis toxin	200 ng/ml	20–30%	20–30%
C3 transferase	30 μ g/ml	5–7%	ND
Cytochalasin B	1 μ M	<2%	<2%
KT5926	5 μ M	<2%	<2%
PMA (24 h pre-treatment)	1 μ M	ND	20–30%
Ro-31-8220	5 μ M	ND	20–35%
PD98059 ^d	5 μ M	20–30%	20–30%
BAPTA-AM ^d	20 μ M	3–7%	6–10%

^aInvasion assay performed in serum-free medium with the additions as indicated.

^bInvasion was scored after 4 h incubation on an REF monolayer. Less than 2% invasion is considered as background; ND, not determined. (A) Range of invasive capacity of different BWMLV and BWRac cell lines, as determined after at least two assays per cell line. (B) Invasion of different BWRacRho cell lines with high V14RhoA expression, as determined after at least two assays per cell line.

^cCells were pre-treated as described in Materials and methods.

Invasion was performed in serum-free medium with LPA (5 μ M).

Invasion in serum-free medium with S1P (1 μ M) gave similar results.

^dPresent during the invasion assay.

suggest that LPA is the active serum ingredient responsible for invasion.

Another serum-borne lysolipid, S1P (Yatomi *et al.*, 1997), reproduces many effects of LPA but acts on a distinct receptor (Postma *et al.*, 1996; Meyer zu Heringdorf *et al.*, 1997). S1P acted similarly to LPA and stimulated T-lymphoma invasion under serum-free conditions. The stimulation of invasion by S1P and LPA was dose-dependent, with LPA having an EC₅₀ of 2 μ M, with maximal invasion being reached at 5 μ M. S1P stimulated invasion with an EC₅₀ of ~350 nM and maximal invasion at ~1 μ M (Figure 3B). Other related lipids such as sphingosyl-phosphorylcholine, phosphatidic acid and cell-permeable C8-ceramide had no effect (Table I). Suramin (1 mg/ml), a known inhibitor of the interaction between LPA/S1P and their respective receptors (Jalink *et al.*, 1995; Postma *et al.*, 1996), abolished the stimulation of invasion by both LPA and S1P (Table I). Furthermore, both LPA and S1P evoke a transient Ca²⁺ signal in T-lymphoma cells (see below; Figure 6), indicating the presence of functional LPA/S1P receptors on the lymphoma cell surface. From these results, we conclude

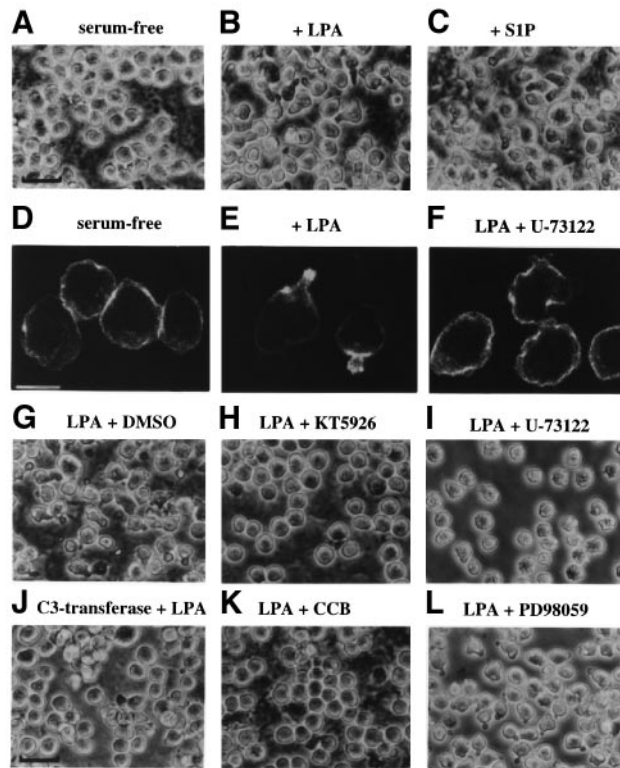


Fig. 4. LPA and S1P induce shape changes in T-lymphoma cells. (A–C) BWRac cells were washed twice in PBS, resuspended in RPMI medium containing 0.5 mg/ml BSA and layered on a REF monolayer. Subsequently, LPA (5 μ M) or S1P (1 μ M) was added, and 1 h later phase-contrast images were made. Both LPA and S1P induce shape changes within 1 h. Bar represents 25 μ m. (D–F) Confocal laser scanning micrographs showing F-actin reorganization induced by LPA (5 μ M) (compare D and E). F-actin was stained with TRITC-labeled phalloidin. The cellular extrusions, pseudopodia, induced by LPA, are clearly enriched in F-actin which accumulates at the bases of the pseudopodia. U-73122 inhibits LPA-induced shape changes (compare E and F). Bar represents 10 μ m. (G–L) Phase-contrast images of shape changes induced by LPA (5 μ M) in the presence of DMSO (negative control) or inhibitors, as indicated. Note that when shape changes are inhibited, invasion also is inhibited (Table I). Bar represents 25 μ m.

that LPA and S1P stimulate invasion of T-lymphoma cells through activation of their cognate G protein-coupled receptors.

Effects of LPA and S1P on the actin cytoskeleton

In fibroblasts and neuroblastoma cells, LPA and S1P induce rapid RhoA-mediated rearrangements of the actin cytoskeleton. We investigated cytoskeletal rearrangements in T-lymphoma cells treated with LPA or S1P. Figure 4A and D shows that T-lymphoma cells are rounded under serum-free conditions. Addition of LPA (5 μ M) or S1P (1 μ M) induced marked morphological changes within 20–60 min, notably a transition from a rounded to a pluriform shape with extending pseudopodia (Figure 4B, C and E). This response was observed consistently in all T-lymphoma cell lines tested. Fluorescence staining of F-actin showed that a layer of cortical actin is present underneath the plasma membrane. Following LPA or S1P treatment, F-actin relocalized to the sites of newly formed pseudopodia (Figure 4D and E). Cytochalasin B (CCB; 1 μ M) abolished the morphological responses to LPA and

S1P (Figure 4K) as well as invasion (Table I), confirming that these events require an intact actin cytoskeleton.

RhoA stimulates myosin light chain (MLC) phosphorylation leading to enhanced actomyosin contractility (Chrzanowska-Wodnicka and Burridge, 1996; Kimura *et al.*, 1996). Both the Rho-inactivating C3 transferase (30 μ g/ml) and the myosin light chain kinase (MLCK) inhibitor KT5926 (5 μ M) blocked the induction of pseudopods by LPA (Figure 4J and H) and inhibited invasion (Table I). This supports the notion that RhoA regulation of actomyosin contractility is required for invasion. Thus, in addition to forming stress fibers in fibroblasts and triggering neurite retraction in neuroblastoma cells, LPA and S1P induce actin-mediated pseudopodial extensions in T-lymphoma cells.

Constitutively active RhoA enhances invasion induced by activated Rac1, but invasion is still largely dependent on serum

Since LPA and S1P activate RhoA, we reasoned that V14RhoA might bypass the LPA/S1P (or serum) dependence of invasion. To test this notion, we generated invasive T-lymphoma cells expressing both V12Rac1 and V14RhoA. Using retroviral transduction, V14RhoA was introduced into invasive V12Rac1-transduced (BWRac) cells and V12Rac1 was introduced into non-invasive V14RhoA-transduced (BWRho) cells. The BWRacRho bulk populations, obtained by both methods, were also subjected to single cell cloning in order to prevent selective loss of putative serum-independent, but perhaps more slowly growing invasive clones. Figure 5A shows the expression levels of V12Rac1 and V14RhoA in BWRac-, BWRho- and BWRacRho-transduced cells, as determined by Western blot analysis. Expression levels of V14RhoA in BWRacRho bulk populations and subclones were variable, whereas V12Rac1 levels (detected through the same Myc-tag) were relatively high in all invasive cell lines (Figure 5A, lanes 3–9).

Transduction of V14RhoA into the already invasive V12Rac1-expressing cells increased the invasive potential of these cells. High expression levels of V14RhoA in the BWRacRho cell lines correlated with increased invasive capacity in the presence of serum (Figure 5B). These cells also showed some increased invasive capacity (up to 7%) in the absence of serum, suggesting that V14RhoA can bypass the serum requirement to a limited extent. However, invasion of BWRacRho cells still appeared to be largely dependent on serum (Figure 5B). Similar results were obtained when serum was replaced by either LPA or S1P. From these results, we conclude that LPA- and S1P-induced invasion depends on both RhoA-dependent and RhoA-independent signaling pathways.

Possible involvement of PLC

To gain insight into the RhoA-independent signaling pathway(s) required for invasion of T-lymphoma cells, we analyzed the involvement of known effectors that are activated by LPA and S1P. In fibroblasts, the LPA receptor couples to G proteins of the G_i , G_q and $G_{12/13}$ subclass (Moolenaar *et al.*, 1997). G_i mediates activation of the mitogenic Ras/MAPK pathway and inhibition of adenylate cyclase. $G_{12/13}$ couples to RhoA activation, whilst G_q activates phospholipase C- β (PLC β). Pre-treatment of the

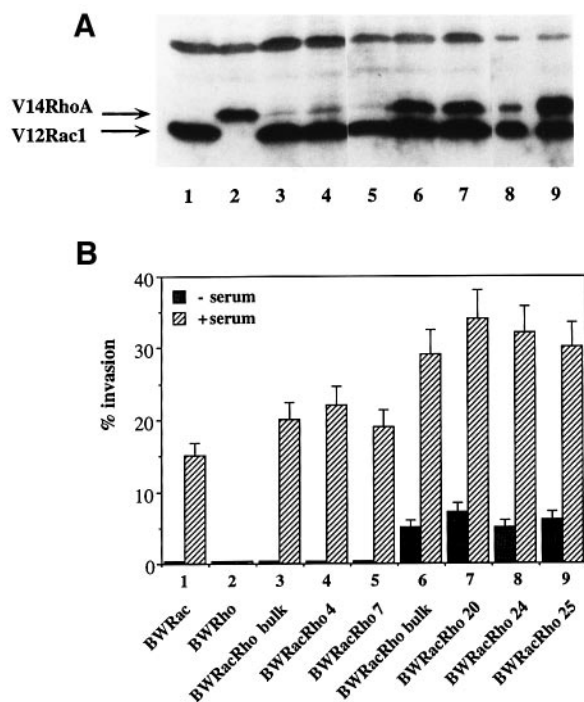


Fig. 5. Expression of constitutively active V14RhoA and V12Rac1 and their effect on invasiveness. (A) Western blot analysis of total protein lysates (100 µg protein/lane) of the indicated bulk populations and single cell lines. Representative examples of ~50 analyzed BWRacRho cell lines are shown. Blots were immunoprobed with α Myc antibody to determine the expression of Myc-tagged V14RhoA and V12Rac1 proteins. (B) Serum dependence of invasion of the different cell lines as analyzed in (A). Invasion was assayed in the presence or absence of serum, and is expressed as a percentage of the number of cells added.

cells with pertussis toxin (PT), which inactivates G_i , did not inhibit invasion of the BWRac and BWMLV cells (Table I), ruling out a critical role for G_i . Unlike the situation in fibroblasts, addition of LPA (2–10 min) to serum-deprived T-lymphoma cells (BWRac and BW5147) induced only a slight increase in phosphorylation of ERK, which was inhibited by the MEKK inhibitor PD98059 at 5 µM concentration (not shown). However, at this concentration, PD98059 did not inhibit LPA-induced invasion (Table I) or pseudopod formation (Figure 4L). These results argue against a role for the G_i -Ras-ERK pathway in LPA-induced invasion of T-lymphoma cells.

LPA and SIP receptors also couple to activation of PLC and PLD (Desai *et al.*, 1992; Van der Bend *et al.*, 1992; Cross *et al.*, 1996; Okajima *et al.*, 1996). PLC stimulation results in the generation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate which activate protein kinase C (PKC) and mobilize Ca^{2+} , respectively. Addition of LPA to T-lymphoma cells evoked a rapid but transient rise in intracellular Ca^{2+} (Figure 6), indicative of PLC activation. Due to homologous desensitization, a second addition of LPA was ineffective; however, it did not interfere with a subsequent SIP-induced Ca^{2+} transient (Figure 6). This indicates that LPA and SIP activate distinct Ca^{2+} -mobilizing receptors in T-lymphoma cells.

The PLC inhibitor U-73122 (1 µM) inhibited invasion by >90%, whereas the inactive congener U-73343 had no effect (Table I). Furthermore, addition of butan-1-ol, to prevent PLD-induced formation of phosphatidic acid

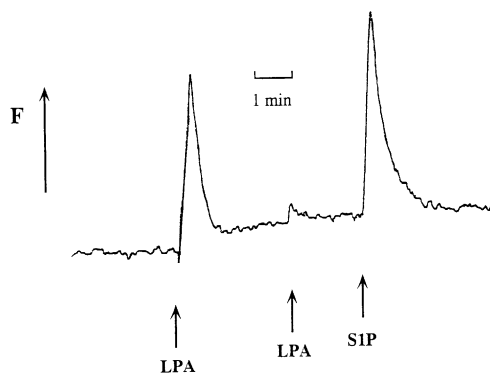


Fig. 6. LPA and SIP induce calcium mobilization in T-lymphoma cells. Time course of changes in cytoplasmic free calcium in BWRac cells, induced by LPA and SIP. F denotes Ca^{2+} -dependent indo-1-AM fluorescence (see Materials and methods). Other T-lymphoma variants gave similar responses. Shown is a representative example of five experiments, which gave similar results.

(PA), did not affect invasion (Table I). These results suggest a role for PLC, but not PLD, in LPA/SIP-induced invasion. Inhibition of PLC by U-73343 also inhibited induction of shape changes by LPA (Figure 4F and I). Phase-contrast microscopy showed cells with an uneven, rough surface (Figure 4I), and the cortical F-actin layer seemed more fragmented than in control cells (Figure 4D and F). These results suggest that LPA/SIP-induced PLC activation is required for both invasion and pseudopodia formation.

To gain insight into the PLC-directed downstream pathways involved in invasion, we pre-treated invasive cells with phorbol 12-myristate 13-acetate (PMA) for 24 h to downregulate the level of classical PKC isoforms. Downregulation of PKC by PMA or treatment of cells with the PKC inhibitor Ro-31-8220, had no substantial effect on invasion (Table I). Moreover, short pre-treatment of BWRacRho cells with PMA to activate PKC did not mimic the LPA effect in the absence of serum (Table I). PMA-treated cells did not produce pseudopodia and did not acquire invasive capacity, making it unlikely that PLC-induced DAG-mediated PKC activation plays an important role in LPA-induced invasion. To test whether Ca^{2+} -mobilization could be involved, ionomycin and thapsigargin were used to raise the intracellular Ca^{2+} concentration in BWRacRho cells. Different concentrations of ionomycin and thapsigargin, which were shown to induce adhesion of T-lymphoblastoid cells (Stewart *et al.*, 1998), did not induce the formation of pseudopods and resulted in only a slight increase in invasion of BWRacRho cells in the absence of serum (Table I). However, binding of intracellular Ca^{2+} by the Ca^{2+} chelator BAPTA-AM (20 µM) inhibited invasion by >60% (Table I), as well as the formation of pseudopodia (not shown). Apparently, Ca^{2+} is required for invasion, but increasing intracellular Ca^{2+} is not sufficient to induce invasion. Precisely how LPA- and SIP-induced PLC activation may contribute to T-lymphoma invasion awaits further studies.

Discussion

We have shown previously that the Rac-specific exchange factor Tiam1 and constitutively active V12Rac1 render

T-lymphoma cells invasive (Habets *et al.*, 1994; Michiels *et al.*, 1995). In this study, we show that activated V12Cdc42 also induces invasiveness, albeit somewhat less efficiently than V12Rac1. Furthermore, we show that invasion requires serum and that serum can be functionally replaced by the lysolipids LPA or S1P acting on their respective G protein-coupled receptors.

In epithelial cells, both V12Rac1 and V12Cdc42 are able to promote motility and invasion when homotypic E-cadherin-mediated adhesion is prevented (Hordijk *et al.*, 1997; Keely *et al.*, 1997). Cdc42 was shown to activate Rac in various cell types (Nobes and Hall, 1995; Van Leeuwen *et al.*, 1997), whilst Cdc42 and Rac share several downstream effectors (Hall, 1998) that may play a role in the induction of invasion. We show here that V14RhoA potentiates Rac/Cdc42-induced invasion but is unable by itself to substitute for Rac or Cdc42. These findings, together with those obtained with C3 transferase, indicate that RhoA activity is required but not sufficient for invasion. Although LPA and S1P activate RhoA, T-lymphoma cells expressing both V14RhoA and V12Rac1 displayed enhanced invasiveness but still required LPA or S1P for efficient invasion. We conclude, therefore, that LPA or S1P stimulate an additional RhoA-independent signaling pathway that is required for invasion.

LPA and S1P stimulate invasion

LPA and S1P activate their own G protein-coupled receptor (Guo *et al.*, 1996; Postma *et al.*, 1996). The receptor antagonist suramin abolished the stimulation of invasion by both LPA and S1P, while LPA and S1P evoke a rise in intracellular Ca^{2+} that is not subject to cross-desensitization. Thus, both LPA and S1P act through their respective receptors on the T-lymphoma cell surface.

Both LPA and S1P induce RhoA-dependent stress fiber formation in fibroblasts, and neurite retraction in neuronal cells (Postma *et al.*, 1996; Van Leeuwen, *et al.*, 1997; Wang *et al.*, 1997). LPA also stimulates RhoA-dependent invasion of hepatoma cells (Imamura *et al.*, 1993; Yoshioka *et al.*, 1995). Consistent with these results, we observed that both LPA and S1P stimulate RhoA-dependent invasion of T-lymphoma cells. This is in contrast to the reported inhibition by S1P of motility and chemotaxis of smooth muscle cells and melanoma cells (Sadahira *et al.*, 1992; Bornfeldt *et al.*, 1995). Inhibition of invasion of these cell types, as assayed in a Boyden chamber, correlated with a reduction in actin polymerization and formation of focal adhesions. This cell type-dependent discrepancy in S1P effects on tumor cell invasion is intriguing and deserves further investigation.

Possible PLC involvement in invasion

In fibroblasts, LPA activates various signaling pathways, including the G_i -Ras-ERK pathway and stimulation of RhoA, PLC and PLD (Moolenaar *et al.*, 1997). PT and the MEKK inhibitor PD98059 failed to inhibit invasion, which argues against a role for Ras-ERK signaling. The PLC inhibitor U-73122 abolished invasion of all T-lymphoma cell lines tested, including cells expressing both V12Rac and V14RhoA. Some caution is needed, however, since the U-73122 compound might have ill-defined side effects that are unrelated to PLC inhibition. PLC stimulation generates DAG and inositol 1,4,5-tris-

phosphate, which activate PKC and mobilize Ca^{2+} from intracellular stores, respectively. PKC recently has been shown to phosphorylate Tiam1 (Fleming *et al.*, 1997). However, PKC activation by PMA did not induce invasion of BWRacRho cells in the absence of serum or LPA. Moreover, inactivation of PKC by long-term treatment with PMA or by Ro-31-8220 treatment did not affect invasion of Tiam1-transduced cells, suggesting that PKC activation is not required for PLC-mediated invasion of T-lymphoma cells. LPA and S1P induce Ca^{2+} mobilization in the T-lymphoma cells through activation of distinct cell surface receptors, as is observed in other cell types (Guo *et al.*, 1996; Postma *et al.*, 1996). LPA and S1P activate RhoA, but cells expressing both V12Rac and V14RhoA still needed either of these lipids for strong invasion. These results indicate that activation of PLC by LPA or S1P is also required for invasion, independently of activation of RhoA. Whereas Ca^{2+} is a well-known regulator of cytoskeletal organization, Ca^{2+} mobilization by ionomycin or thapsigargin hardly induced invasion in serum-free conditions. However, the intracellular Ca^{2+} -chelator BAPTA-AM inhibited LPA-induced formation of pseudopodia as well as invasion, indicating that Ca^{2+} is required but Ca^{2+} mobilization is not sufficient for invasion. As was shown recently (Stewart *et al.*, 1998), Ca^{2+} might play a role in integrin-mediated adhesion, a prerequisite for invasion, but also seems to be involved in LPA-induced formation of pseudopodia. Rho-like GTPases and PLC-regulated phosphoinositide metabolism may also interact during invasion. Phosphoinositides have been implicated in the regulation of the actin cytoskeleton, for example by binding to actin-capping proteins (Hartwig and Barkalow, 1997; Toker and Cantley, 1997), and in intracellular targeting of signaling molecules, including activators of Rho-like GTPases (Michiels *et al.*, 1997; Toker and Cantley, 1997; Han *et al.*, 1998; Nimnual *et al.*, 1998).

Invasion and the actomyosin cytoskeleton

Invasion requires cells to adhere, to form cell protrusions, to detach, to contract and to regulate cell rigidity in order to extravasate. All these processes require a strict regulation of the actomyosin cytoskeleton in which Rho-like GTPases play an important role (Hall, 1998). LPA and S1P induced shape changes of T-lymphoma cells, resulting in the formation of pseudopodia. The lipid-induced pseudopodia formation was also observed in cells expressing V14RhoA, indicating that activation of RhoA is not sufficient to induce this response. The cytoskeletal rearrangements did not occur after inactivation of RhoA by C3 transferase or after inhibition of MLCK by KT5926. Since RhoA couples to inhibition of MLC phosphatase (Kimura *et al.*, 1996), this suggests that the morphological changes induced by LPA or S1P require actomyosin-mediated contractility regulated by RhoA, as well as a RhoA-independent PLC-mediated signaling pathway. The abolishment of shape changes and invasion by inhibitors of PLC, MLCK or RhoA underscores the importance of actin regulation for the invasion process. Although the cytoskeletal rearrangements induced by LPA or S1P are clearly important, they are not sufficient to achieve invasion. Shape changes and pseudopodia formation induced by both lipids also occurred in non-invasive control

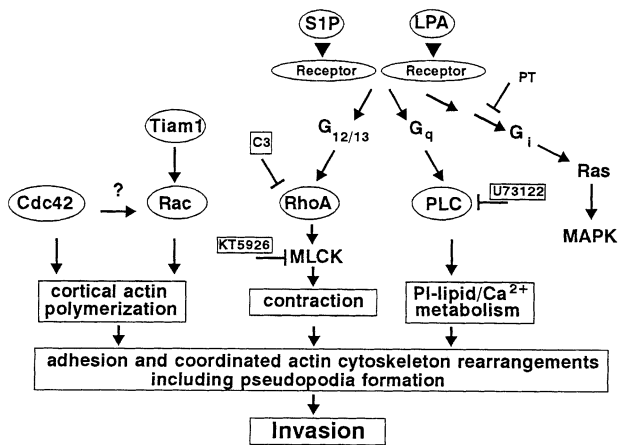


Fig. 7. Signaling pathways involved in T-lymphoma invasion. Invasion is mediated by the combined activities of Rac (or Cdc42), RhoA and PLC. Tiam1 activates Rac. LPA and S1P activate receptors which couple to heterotrimeric G proteins which activate RhoA and PLC. RhoA activation, together with a RhoA-independent PLC-mediated pathway, leads to pseudopodia formation, whereas Rac and Cdc42 activation promote actin polymerization and adhesion. PLC-mediated Ca^{2+} signaling and regulation of phosphatidylinositol lipids, which both are involved in cytoskeletal rearrangements, may play a role in pseudopodia formation. The coordinated activation of Cdc42, Rac, RhoA and PLC potentially regulates cell adhesion and local cytoskeletal rearrangements required for invasion. PT, pertussis toxin; C3, C3 transferase. The inhibitors that are framed abolish pseudopodia formation as well as invasion.

T-lymphoma cells. Based on these data, we propose that RhoA as well as a PLC-mediated pathway are required for the formation of pseudopodia, whereas Tiam1/Rac- or Cdc42-mediated pathways are necessary to allow adhesion and further cytoskeletal rearrangements (Figure 7). In lymphoid cells, Cdc42 has been shown to induce cell polarization (Stowers *et al.*, 1995), and both Cdc42 and Rac promote adhesion and lamellipodia formation in different cell types (Machesky and Hall, 1997; Michiels *et al.*, 1997; Van Leeuwen *et al.*, 1997). Actin cytoskeleton rearrangements as well as specific adhesion events will be required at different sites during the various stages of the invasion process.

Materials and methods

Cells and culture conditions

REF 208F cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated newborn calf serum. All transduced cells were derived from the non-invasive BW5147 T-lymphoma cells and were grown in suspension in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (FCS). Cell lines BW MLV3.1, BW MLV3.2 and BW MLV4.13 represent invasive variants of BW5147 cells that carry a Moloney murine leukemia proviral insertion in the *Tiam1* gene, resulting in high expression of truncated Tiam1 proteins (Habets *et al.*, 1994). BWRac cells express Myc-tagged V12Rac1 introduced by retroviral transduction (Michiels *et al.*, 1995).

Materials

LPA (1-oleoyl and 1-palmitoyl), phosphatidic acid and CCB were obtained from Sigma. PT was from List Laboratories; butan-1-ol from Merck; PMA, Ro-31-8220, thapsigargin, ionomycin, BAPTA-AM, PD98059 and KT5926 from Calbiochem. S1P (98% pure), C8-ceramide, sphingosine phosphatidylcholine, PLC inhibitor 1-{6-[(17 β -3-methoxestra-1,3,5(10)trien-17-yl)amino]hexyl}-1H-pyrrole-2,5-dione (U-73122) and its inactive congener 1-{6-[(17 β -3-methoxestra-1,3,5(10)trien-17-yl)amino]hexyl}-2,5-pyrrolidinedione (U-73343) were purchased from Biomol (Plymouth Meeting, PA). Suramin was obtained from Bayer

(Germany) and indo-1 acetoxymethylester (indo-1 AM) from Molecular Probes. Recombinant C3 transferase was kindly provided by S.Narumiya (University of Kyoto, Japan). S1P stock solutions (2 mM) were made in methanol, LPA stock solution (10 mM) in H_2O . U-73122 and U-73343 were dissolved in dimethylsulfoxide (DMSO; 5 mM).

Invasion assay

Invasion into REF monolayers was determined essentially as described (Habets *et al.*, 1994). REFs were grown to confluency in 24-well plates and washed twice with RPMI. A total of 7×10^5 T-lymphoma cells were added in 1 ml. After incubation for 4 h, non-invaded cells were removed by washing and mechanical agitation (four times) in phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The infiltrated cells were counted using a phase-contrast microscope, and the degree of invasion was expressed as the percentage of cells added. Less than 2% invasion was considered as background. Standard invasion assays were performed in RPMI containing 10% FCS, or RPMI with 0.5 mg/ml fatty acid-free bovine serum albumin (BSA, Sigma) to which LPA or S1P were added in various concentrations.

To determine the effects of inhibitors, 10^6 cells were pre-treated as follows: incubation in serum-free medium with 200 ng/ml PT for 2 h; in serum-containing medium with 1 μ M U-73122 or U-73343 for 5 min; with 5 μ M KT5926 for 10 min; with 1 μ M CCB for 30 min; with 1 μ M PMA for 24 h; with 5 μ M Ro-31-8220 for 30 min; or with 30 μ g/ml C3 transferase for 24 h. Subsequently, the cells were washed and assayed for invasive potential in serum-free medium in the presence of LPA. PD98059 (5 μ M) was added in serum-free medium for 30 min prior to addition of LPA. Cells were pre-treated with BAPTA-AM (20 μ M) for 10 min in PBS/20 μ M EGTA, washed and BAPTA-AM (20 μ M) was added again during the invasion assay. Other inhibitors and agonists were added to the indicated concentrations during the invasion assay. Viability of the cells was not affected, as determined by Trypan Blue exclusion.

Retroviral transduction

We previously described the cloning of Myc-tagged V12Rac1 in a pMFG plasmid, which was transfected into a BOSC23 packaging cell line to produce V12Rac1-transducing retroviruses (Michiels *et al.*, 1995). For the present study, Myc-tagged V14RhoA was cloned into a pMFG-IRES-LacZ retroviral vector (Staal *et al.*, 1996) and transfected into the BOSC23 packaging cell line to produce V14RhoA-IRES-LacZ-transducing retroviruses. Cells transduced with this construct express, in addition to V14RhoA, also the LacZ protein encoded on the same mRNA, since IRES represents an internal ribosomal entry site allowing translation of LacZ. Thus, X-gal staining is quantitatively correlated to V14RhoA expression, as was confirmed experimentally (not shown). V12Cdc42-expressing retroviral constructs will be described in detail elsewhere (F.Michiels, R.A.van der Kammen and J.G.Collard, in preparation).

BW5147 T-lymphoma cells (5×10^5) were infected by culturing for 18 h in 2.5 ml of cell-free BOSC23 supernatants, in the presence of 6 μ g/ml polybrene. Infection with V14RhoA-IRES-LacZ retrovirus yielded transductants that were selected by FACS sorting after viable staining for LacZ expression (Nolan *et al.*, 1988). Single cell clones were generated and analyzed by X-gal staining and Western blotting for V14RhoA (not shown). The single cell clone displaying the highest level of V14RhoA expression was employed as a BWRhoA cell line. To obtain BWRacRho cells, the BWRac line was transduced with V14RhoA-IRES-LacZ retrovirus or empty vector. After two cycles of FACS sorting, a bulk population of BWRacRho was obtained, as well as single cell clones. The empty vector yielded clones expressing only LacZ, in addition to V12Rac1, which did not affect invasive properties. Similarly, the BWRho cell line was also transduced with the V12Rac1 retrovirus. Successful transductants were selected by invasion selection on REFs (as in Michiels *et al.*, 1995) and also single cell clones were generated.

Western blot analysis, immunofluorescence and CLSM analysis

These were performed as described recently (Michiels *et al.*, 1997), using mouse monoclonal 9E10 antibodies to recognize the Myc epitope-tagged Rac, Rho and Cdc42 proteins. For actin staining, tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin was used.

Calcium measurements

T-lymphoma cells (10^7) were washed with RPMI (without serum) and loaded with indo-1 AM by incubation for 30 min in 1 ml of RPMI with 10 μ M indo-1 AM at 37°C. The cells were washed with PBS containing

1 mM CaCl₂ and 1 mM MgCl₂, incubated in quartz cuvettes (2×10⁶ cells/ml) and stimulated with LPA (5 μM) or S1P (1 μM), as indicated. Experiments were performed with a Perkin-Elmer LS3 spectrofluorometer, as described by Jalink *et al.* (1990).

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