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Autocrine Regulation of T Cell Motility by Calreticulin-Thrombospondin-1 Interaction

Shu Shun Li,*† Anna Forslöw,*† and Karl-Gösta Sundqvist2*†

The mechanisms regulating T lymphocyte migration within the extracellular matrix are not understood. We show in this study that the thrombospondin-1 binding site of calreticulin, spanning aa 19–32, is a major triggering factor for T cell motility and migration within a three-dimensional collagen type 1 matrix, and that exogenous motogenic factors such as chemokines can stimulate migration via a calreticulin-thrombospondin-1 pathway. Endogenous calreticulin binding to the N-terminal domain of endogenous thrombospondin-1 elicited a motogenic signal to the T cells through the C-terminal domain of thrombospondin-1 and its cell surface receptor integrin-associated protein (CD47). Our data further revealed that thrombospondin-1 was expressed on the cell surface with a high turnover, and that PI3K and the Janus family of tyrosine kinases were required for T cell motility mediated through calreticulin, thrombospondin-1, and CD47. These results unveil an autocrine mechanism of calreticulin-thrombospondin-1-CD47 interaction for the control of T cell motility and migration within three-dimensional extracellular matrix substrata. The Journal of Immunology, 2005, 174: 654–661.

Lymphocytes, the principal cells of the immune system, have a unique capacity to reposition themselves within the organism and to migrate to subcompartments of lymphoid and nonlymphoid tissues (1, 2). The migratory behavior of lymphocytes reflects the impact of various adhesive interactions and chemokines on the inherent locomotor capacity of the cells. The migration of lymphocytes and their subgroups is further controlled by the recognition of endothelial ligands by a selection of selectins, integrins, and other components (1–3). Naive T cells express homing molecules such that they extravasate and migrate almost exclusively in secondary lymphoid tissues, while effector T cells express adhesion molecules and chemokine receptors such that they extravasate and migrate in inflamed tissues (4).

It is highly likely that controlled T cell migration requires coordinated interaction with tissue components such as fibronectin (FN) and various types of collagen. Lymphocyte migration is usually studied in vitro models of three-dimensional (3D) extracellular matrix (ECM) such as Matrigel or collagen matrices (5). Lymphocyte migration in a 3D environment requires cellular competence to penetrate, the mechanism of which is not understood, although ECM-degrading metalloproteinases (MMPs) have been implicated in this context (6). The concept that ECM-degrading MMPs would constitute a penetration mechanism has, however, recently been questioned (7).

Chemokines are key mediators of lymphocyte migration by stimulating extravasation across endothelial cells and providing signals for adhesion, motility, and navigation within tissues (8–13). However, the mechanisms regulating chemokine-induced lymphocyte migration as well as the basic control of spontaneous T cell migration within 3D substrata are incompletely understood. Endogenous ligands with capacity to bind to cell surface receptors independent of the substrate may be attractive putative regulators of lymphocyte migration within 3D substrata. We have focused on the possibility that calreticulin (CRT), which is associated to CD91 (also named low density lipoprotein-related protein) on the cell surface (14), may play a role for T cell migration. The importance of CRT for lymphocyte functions is virtually unknown. It is interesting, however, that CRT has been shown to be involved in adhesive interactions of nonlymphoid cells to extracellular substrata by a number of mechanisms including regulation of expression of vinculin and N-cadherin (15), and modulation of the affinity of integrin for its ligand (16–19). Consistent with a role in cell-substrate adhesion, CRT-deficient embryonic stem cells were severely impaired in their ability to adhere to FN and laminin through cell surface integrins (16). Recent work on endothelial cells has shown that thrombospondin (TSP) mediates focal adhesion disassembly through interactions with cell surface CRT (20). The present studies of the role of CRT in T cell migration were based on the fact that T cells express TSP-1 (21), the TSP-1 binding site in CRT has been mapped to aa 19–36, and the CRT binding site in TSP-1 has been localized to the N-terminal domain of TSP-1 and characterized (22). We further reasoned that the chemokine stromal cell-derived factor-1α (SDF-1α), a highly efficacious lymphocyte chemoattractant (23, 24), which has merely one specific surface receptor (CXCR4) on T cells, would be well suited for analysis of the role of CRT and TSP-1 in T cell migration.

Materials and Methods

Chemicals and Abs

Collagen type 1 was prepared, as previously described (25). BSA, α2-macroglobulin, and brefeldin A were purchased from Sigma-Aldrich; Poly-L-lysine (m.w. 5300) was purchased from Miles-Yeda. Wortmannin, pertussis toxin, and AG490 were from Sigma-Aldrich; U0126 from Promega; and GM6001 from Chemicon International. Human plasma FN was purified, as described elsewhere (26). IL-2, IL-4, and the chemokine SDF-1α were from Genzyme Diagnostics. Anti-CD29 (clone Lia 1/2, IgG1) and
anti-CD3 (clone SK 7, IgG1) were obtained from BD Biosciences. Dynabeads were from Dynal Biotech. Mouse IgG and anti-CD54 (clone T29–33) were from DakoCytomation. FITC goat anti-mouse Ab was from Dakopatts. Anti-TSP-1 clone MBC 200.1 (also called TSP-Ab-9, IgG1, its epitope localizes in heparin binding domain of TSP-1), clone A6.1 (also called TSP-Ab-4, IgG1, its epitope localizes in collagen type III binding domain of TSP-1), and clone C 6.7 (also called TSP-Ab-3, IgG1, its epitope localizes in cell binding domain of TSP-1) were from NEO-MARKERS. Anti-FN (clone 1ST 1, IgG1) was obtained from Ser-Lab. Biotinylated peroxidase and avidin were from Vector Laboratories. The peptides RWI ESK HKF GFV TSP (the TSP-1 binding site in CRT, called peptide CRT19–36 in this work) (22), ELT GAA KRG SGR RLV KGP D (hep1), and RSK AST LGE RDL KPS AAV G (control peptide 1), RKFYYVMWKK (4N1K, control peptide 3) were synthesized by the Biomolecular Resource Facility, University of Lund, and RSK AGT LGE RDL KPS AAV G (control peptide 2), KRFYYVMWKK (4N1K), and KRFYGGMWKK (mutated 4N1K, control peptide 3) by Tri pep, Novum Research Park.

**Cell preparations and culture conditions**

Peripheral blood T cells were depleted of monocytes by treatment with carbonyl iron and magnetic removal of phagocytic cells and with anti-CD14-coated beads. Blood T cells and the birch (Bet v 1)-specific T cell clone AF 24 were stimulated with anti-CD3 or specific Ag and cultured in the presence of IL-2 and IL-4 for 24–48 h before the experiments. All cells were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 2 mM L-glutamine, 0.16% sodium bicarbonate, 10,000 U/ml benzylocillin, 10,000 μg/ml streptomycin, and 10% FCS, or in serum-free AIM-V medium (Invitrogen Life Technologies).

**Quantitative immunocytochemistry**

The expression of various Ags by T cells was analyzed using immunocytochemistry or flow cytometry after staining with mAbs. Abs were stained with poly-l-lysine (10 μg/ml) or FN (10 μg/ml) at 4°C overnight. T cells were attached to these slides in PBS or AIM-V medium at 37°C for 15 min, unless otherwise stated, and subsequently fixed in 2% paraformaldehyde (4°C for 20 min, unless otherwise stated) after which time the TSP-1-expressing T cells were detected with the mAbs and a complex of biotinylated peroxidase and avidin (Vector Laboratories). For detection of intracellular Ags, cells were fixed in 2% paraformaldehyde in Earle’s balanced salt solution (EBSS) buffer and permeabilized by washing in EBSS buffer containing 0.1% saponin. After three washes in EBSS, 2% FCS in EBSS was added for 10 min. Following three more washes in EBSS washing buffer containing 0.1% saponin, the cells were reacted with mAbs in washing buffer containing saponin. The cells were examined in a Nikon Eclipse E1000M microscope. The intensity of the immunocytochemical staining was quantified using the image processing and analysis program ImageJ analyzed by histogram of the distribution of gray values in each image or by measurements for each particle displayed in the images according to the following formula: I/mean gray value* × 100 (* the sum of the gray values of all the pixels in the selection divided by the number of pixels). Flow cytometry was conducted on cells in suspension and also performed with adherent cells after removal from the substrate by a cell scraper. Briefly, after blocking with γ-globulin (3 μl/106 cells) at 20°C for 15 min, T cells (2.5 × 106) diluted in FACS buffer (Tris-Hank’s, 0.2% HSA, 0.02% NaN3) were added to a microtiter plate and mixed with primary Ab. After 30-min incubation on ice, the cells were washed with FACS buffer and centrifuged through a layer of FCS. The cells were then incubated on ice for 30 min with 50 μl of secondary Ab (FITC-conjugated goat anti-mouse), washed as described above, and analyzed using a FACSscan (BD Biosciences). Dead cells were identified through addition of propidium iodide.

**Biotinylation and immunoprecipitation of cell surface TSP-1**

The surface membrane of intact lymphocytes was labeled with N-biotinyl-e-aminoacopinic acid-N-hydroxysuccinimide ester, as described by the manufacturer (Roche Molecular Biochemical). A total of 15 μl of N-biotinyl-e-aminoacopinic acid-N-hydroxysuccinimide ester was added to portions of 15 × 105 cells per test tube in 1.5 ml of biotination buffer. The reaction was stopped with 75 μl of stop solution per tube after incubation for 15 min at room temperature and centrifuged at 1500 rpm for 10 min. The supernatant was discarded, and 5 ml of cold PBS were added to each tube, followed by centrifugation at 1500 rpm for 10 min. The cells were lysed in 1 ml of lysis buffer (50 mM core buffer, 150 mM NaCl, 0.1 mg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1% Nonidet P-40, and 0.5% sodium deoxycholate) and incubated 30 min on ice. After incubation for 15 min, the cells were resuspended and centrifuged 12,000 × g for 10 min at 4°C, and the supernatants were transferred to clean Eppendorf tubes.

Immunoprecipitation was essentially conducted with protein G agarose beads, as described (Roche). The supernatants were mixed with 1 μg of Ab at 4°C overnight, followed by centrifugation at 12,000 × g at 4°C for 20 s. Then the supernatants were discarded and the beads were resuspended in 1 ml of washing buffer, and centrifuged again at 12,000 × g at 4°C for 20 s; the same procedure was repeated twice. After washing, 20 μl of reducing buffer (2×, containing 0.15 g of DTT in 5 ml of JB buffer) was mixed with the beads and heated at 95°C for 4 min and subsequently centrifuged at 7,000 × g for 10 min. The proteins were separated on a 6% SDS-PAGE gel. Proteins were transferred to the Hybond ECL membrane (Amersham) and detected using the BM chemiluminescence’s blotting kit (Roche).

**Cell attachment and migration assays**

Collagen type I was diluted in serum-free RPMI 1640 and H2O (8/1/1), applied in plastic petri dishes 1 ml/dish (30 mm; BD Biosciences), and allowed to polymerize at room temperature. The chemokines were dissolved in the gel, while Abs and peptides were present with the cells in migration experiments. A total of 1.0 × 105 cells in AIM-V medium was added to each well and allowed to migrate for different times. The cells were fixed in 2.5% glutaraldehyde and washed twice with PBS. Cell morphology and cell migration were routinely, unless otherwise stated, evaluated in seven fixed positions in each well and at 10-μm intervals throughout the gel by the use of an inverted microscope (Nikon Eclipse TE300) and a digital depth meter (Heidenheim ND221). The results are given as total number of infiltrating cells throughout the gel, as determined with >20 or 100 objectives or mean number of infiltrating cells/high power field (>100) per infiltration depth (50 μm for the first layer immediately beneath the gel surface, and 100 μm for other layers further down). To characterize the infiltrating cells, the collagen gels were slashed and the cells were collected and identified by using immunocytochemistry. Another approach was to fix the collagen gels containing infiltrating cells in paraformaldehyde. The cells were then identified in situ by immunocytochemistry. To study cell adhesion on ECM substrate, plastic petri dishes (90 mm; Heger) were coated with TSP-1 (1 or 10 μg/ml), BSA (10 μg/ml), or FN (10 μg/ml), and were extensively washed before use. The cells (10,000/position) in AIM-V medium were incubated on the substrates in a humidified CO2 incubator at 37°C for 15 or 30 min, after which time unbound cells were removed by gentle aspiration and bound cells were fixed in 2.5% cold glutaraldehyde for 10 min.

**Statistical analysis**

The results are shown as mean ± SD. The significance of the data was evaluated by Student’s t test, unless otherwise specified, and p < 0.05 was judged significant.

**Results**

The TSP-1 binding site in CRT triggers T cell migration into 3D collagen

The influence of CRT on T cell migration into collagen type I matrices was determined by counting of cells at different levels using a digital depth meter (Fig. 1). The experiments were conducted under serum-free (AIM-V) conditions to exclude any interference by exogenous proteins and peptides. CRT has previously been shown to be expressed on activated T cells (27) and also detected in 75–100% of the T cells in our experiments. As shown in Fig. 1, A–C, the TSP-1 binding site in CRT, which resides within residues 19–36, triggered motility in a high percentage of all T cells independent of the nature of the substrate (plastic or collagen), and a massive infiltration of T cells into 3D collagen type I, while control peptides of the same size did not. The TSP-1 binding site in CRT also provoked an extreme elongation of the infiltrating cells (Fig. 1D). Motile lymphocytes show a characteristic polarized locomotor morphology (Fig. 1D) that has frequently been applied as a measure of migrating cells (28, 29). They are easily distinguished from nonmotile ones, which show a spherical shape, as shown in Fig. 1D, a and c. The finding that the TSP-1 binding site of CRT induced T cell locomotor morphology and migration strongly indicated T cell migration is dependent on endogenous TSP-1.
The TSP-1 binding site in CRT augments the expression of TSP-1

Quantitative immunocytochemistry using mAbs to the C-terminal (TSP mAb-3), N-terminal (TSP mAb-9; data not shown), and collagen binding domains of TSP-1 (TSP mAb-4) showed that the TSP-1 binding site in CRT increased cell surface TSP-1 significantly and inhibited the internalization of TSP-1 (Figs. 2 and 7). Immunoprecipitation experiments with biotinylated cells demonstrated that cell surface TSP-1 consists of one major band of 170 kDa or multiple bands between 200 and 100 kDa, as well as some bands with lower molecular mass.

Immunocytochemistry staining of paraformaldehyde-fixed T cells demonstrated TSP-1 on the surface. In contrast, viable cells when reacted with Abs before fixation were found to have comparatively little cell surface TSP-1 antigenicity (~10% of the amount on fixed cells as determined by ImageJ quantification), whereas Abs to a control Ag (CD29) showed substantial cell surface reactivity. The finding that anti-TSP-1 Abs reacted strongly with nonfixed TSP-1 molecules in an ELISA (data not shown) excludes that fixation exposes antigenic determinants in the TSP-1 molecule. The data in Fig. 7A demonstrating that fixation did not permeabilize the cells further exclude that the preferential reactivity of anti-TSP-1 Abs with fixed cells reflects expression of intracellular Ags. In addition, the fact that immunoprecipitation of biotinylated cells also detected strong surface TSP-1 indicates that T cells express TSP-1 on their surface. Therefore, it is likely that the poor reactivity of anti-TSP-1 with live T cells in Fig. 6C reflects a labile association to the cell surface. Detection of pericellular FN is also routinely performed using paraformaldehyde-fixed cells (30).

Hep1 inhibits locomotor morphology and migration of T cells

Given the evidence in Fig. 1 that the TSP-1 binding site in CRT triggered T cell migration, we examined the role of endogenous CRT and TSP-1, particularly the N-terminal domain, for T cell migration, using a 19-aa sequence (17–35 aa) corresponding to the TSP-1 binding site in CRT, as compared with a control peptide (DC) (for sequences, see Materials and Methods). In the migration experiments, the peptides were present with the cells on top of the collagen at concentrations specified in A and B. A total of 93–100% of the cells in the collagen gel in separate experiments was CD3 positive.
T cell expression of TSP-1 and T cell migration are Zn$^{2+}$ dependent

To further analyze whether CRT induces T cell migration via T cell TSP-1, we made use of the observation that interactions between CRT and TSP-1 are Zn$^{2+}$ dependent (22). T cells were exposed to ZnCl$_2$ under serum-free conditions and analyzed with respect to migration into collagen. The influence of ZnCl$_2$ on T cell surface expression of TSP-1 was examined using immunocytochemistry and after biotinylation and immunoprecipitation with a mAb to TSP-1. We found that ZnCl$_2$ augmented T cell migration into 3D collagen (Fig. 4A) and cell surface expression of TSP-1 (Fig. 4, B and C). Immunoprecipitation of nonadherent biotinylated cells in suspension yielded a major band of 170 kDa; FN-adherent cells also showed two bands with a molecular mass of 110 and 100 kDa, respectively (data not shown). Several protease inhibitors were analyzed with respect to possible influence on cell surface TSP-1 expression without effect, indicating that the enhancement of TSP-1 expression by Zn$^{2+}$ was specific. One reasonable explanation for the enhancement of TSP-1 expression by ZnCl$_2$ is that Zn$^{2+}$ strengthens TSP-1 binding to CRT, as previously demonstrated (22), and inhibits TSP-1 internalization. According to the data in Fig. 2 and further discussed below, TSP-1 on the lymphocyte surface may have at least two alternative fates, either persistence on the cell surface or internalization/degradation. The evidence that ZnCl$_2$ both augmented TSP-1 binding to the T cell surface and potentiated migration affirms the conclusion that interactions between TSP-1 and CRT determine T cell migration.

SDF-1$\alpha$-induced T cell migration is dependent on CRT-TSP-1 interaction

To clarify the possible role of CRT and TSP-1 for chemokine-induced migration of T cells, we investigated whether the inhibition of T cell migration by the hep1 peptide also applied to migration induced by the chemokine SDF-1$\alpha$. It turned out that the hep1 peptide inhibited development of locomotor morphology induced by SDF-1$\alpha$, whereas a control peptide did not (Fig. 5A). It can further be seen in Fig. 5, B and C, that hep1 was also a potent inhibitor of SDF-1$\alpha$-induced T cell migration into a collagen type I matrix. We examined the influence of pre-exposure to exogenous TSP-1 on locomotor morphology and migration of T cells. It can be seen in Fig. 5D that exogenous TSP-1 inhibited development of motile forms, and T cell migration induced by SDF-1$\alpha$, while FN did not. It follows that the inhibitory influence of the hep1 peptide and TSP-1 was specific. The inhibition of migration by the hep1 peptide taken together with the finding that the TSP-1 binding site of CRT triggers migration (Fig. 1) indicates that CRT via its TSP-1 binding site plays a key role for chemokine-induced T cell migration.

The results shown in Fig. 3, that hep1 inhibited spontaneous T cell migration, most likely imply that the inhibitory effect of the same factors on chemokine-induced migration does not simply reflect interference with chemokine binding to receptors or chemokine signaling, but represents an inhibition of the effector mechanism of T cell motility. The inhibition of T cell migration by hep1 is thus independent of the nature of the stimulus that induces migration.

SDF-1$\alpha$ augments TSP-1 expression on the surface of T cells

Based on the evidence in Fig. 5 that TSP-1 is involved in chemokine-induced T cell migration, we studied whether the chemokine SDF-1$\alpha$ may influence TSP-1 expression in T cells using quantitative immunocytochemistry and immunoprecipitation of biotinylated cells. SDF-1$\alpha$ was found to augment TSP-1 expression on the surface of T cells and specific major TSP-1 bands with a molecular mass of 130 and 140 kDa as well as minor bands between 200 and 100 kDa (Fig. 6A). In addition, these Abs, but not control mAbs, detected some bands of low molecular mass that are likely to represent fragments of degraded TSP-1 (32). Brefeldin A inhibited
SDF-1α-induced TSP-1 expression (Fig. 7B), indicating that this TSP-1 expression is dependent on transport of endogenous TSP-1 to the lymphocyte surface. Noteworthily, SDF-1α did not augment CRT expression (data not shown), indicating that this chemokine specifically exerted its effect through up-regulation of TSP-1 expression.

Flow cytometry using viable cells was used in an attempt to confirm TSP-1 expression on the cell surface, although one must be aware that detection of antigenicity in viable cells is hampered by disappearance of TSP-1, as discussed above. This analysis showed that SDF-1α-induced TSP-1 expression on the lymphocyte surface in both adherent (detached from the substrate by a cell scraper) (Fig. 6C) and nonadherent cells (data not shown).

High turnover of T cell TSP-1

As shown above, T cells showed cell surface expression of TSP-1 (Fig. 2A). It is obvious from Fig. 7A that exogenous soluble TSP-1 in the culture medium did not contribute to the TSP-1 detected on the lymphocyte surface, but rather decreased cell surface TSP-1. Brefeldin A, an inhibitor of intracellular transport and secretion, was used to study TSP-1 expression by T cells. Fig. 7 shows the influence of inhibition of cellular protein transport on the expression of TSP-1 by nonpermeabilized and permeabilized T cells, respectively, including an SDS-PAGE gel showing TSP-1 expression by biotinylated cells. It is evident that inhibition of cellular protein transport for a period of 15 min markedly augmented intracellular TSP-1, while no TSP-1 was detectable on the cell surface. This result demonstrates that TSP-1 has a high turnover and is transported from an intracellular compartment to the cell surface. The pronounced intracellular increase of TSP-1 under serum-free conditions as a consequence of inhibition of protein transport shows that endogenous TSP-1 is the source of cell surface TSP-1 in T cells.

TSP-1 stimulates T cell migration through CD47

CD47 is a receptor for the C-terminal cell binding domain of TSP-1 (33). We investigated the possibility that CRT induction of T cell migration via the N-terminal domain of TSP-1 was mediated through interaction of the C-terminal domain of TSP-1 with CD47. It is apparent from Fig. 8 that a peptide from this domain KRFYV VMWKK (4N1K) was a potent stimulator of T cell migration into collagen, while a control peptide had no such effect (not shown in figure). Fig. 8 further demonstrates that a mAb to CD47, B6H12, previously generally found to block responses to CD47 ligands (34, 35), inhibited the peptide-induced stimulation of T cell migration. The same mAb to CD47 also inhibited CRT-induced T cell migration. These data indicated that TSP-1 induces T cell migration via interaction with CD47. We have also been able to induce T cell migration by a mAb to CD47, c1km1, which supports the conclusion that CD47 is involved in T cell migration (data not shown). Furthermore, C1km1 abrogated the inhibitory effect of B6H12 on migration, providing further evidence that CD47 is involved in T cell migration (data not shown). B6H12 in separate experiments exerted a 40–65% inhibition of SDF-1α-induced T cell infiltration into collagen, thus supporting the conclusion that CD47 is involved in T cell migration.

To begin defining a mechanism for the stimulation of T cell migration by CRT and TSP-1, we tested a number of well-characterized inhibitors of signal transduction enzymes with special reference to the fact that inhibitors of PI3K and the Janus family of
The N-terminal domain of TSP-1, and mAbs to TSP-1 on T cell studied the influence of the hep1 peptide, the CRT binding site of the role of CRT and TSP-1 as mediators of T cell migration, we interaction with TSP-1 induces T cell migration. To further analyze expression, thus providing support for the conclusion that CRT in- stimulated T cell migration and augmented cell surface TSP-1 ex-

was inhibited by hep1, it is highly unlikely that the inhibitory effect control of T cell motility. Because spontaneous T cell migration under serum-free conditions by the hep1 peptide and a mAb to chemokine-induced T cell migration and locomotor morphology was not varied between control T cells and T cells exposed to hep1, TSP-1, the intensity of all the TSP-1 bands between 200 and 100 kDa as well as the low molecular mass TSP-1. The intensity of staining with control IgG did not vary between control T cells and T cells exposed to hep1, TSP-1, or brefeldin A. The figures show densitometric quantification of the staining intensity of the cells (98% of the cells were CD3 positive) expressed in arbitrary units. The figure shows one representative experiment of three independent experiments. The data were analyzed with Mann-Whitney U test. Bars: 20 μm.

tyrosine kinases have been demonstrated to interfere with lympho-
cyte motility and homing (36, 37). The PI3K inhibitor wortmannin and the JAK tyrosine kinase inhibitor AG490 inhibited the induc-
tion of T cell migration by both the TSP-1 binding site of CRT and the CD47 binding site of TSP-1 (Fig. 8). Thus, T cell migration induced via CRT, TSP-1, or CD47, respectively, appears to use a common pathway involving PI3K and tyrosine kinase.

Discussion

In the present study, we first showed that the TSP-1 binding site of CRT, which resides in aa 19–36, was a potent triggering factor for T cell migration, and augmented TSP-1 expression on the T cell surface. Zn2+, which enhances binding of CRT to TSP-1, also stimulated T cell migration and augmented cell surface TSP-1 expression, thus providing support for the conclusion that CRT interaction with TSP-1 induces T cell migration. To further analyze the role of CRT and TSP-1 as mediators of T cell migration, we studied the influence of the hep1 peptide, the CRT binding site of the N-terminal domain of TSP-1, and mAbs to TSP-1 on T cell migration. The inhibition of spontaneous, Zn2+-dependent, and chemokine-induced T cell migration and locomotor morphology under serum-free conditions by the hep1 peptide and a mAb to TSP-1 (data not shown) indicates that endogenous TSP-1 via its N-terminal heparin binding domain plays a pivotal role for the control of T cell motility. Because spontaneous T cell migration was inhibited by hep1, it is highly unlikely that the inhibitory effect of these factors in the chemokine experiments is a consequence of interference with chemokine binding or chemokine signaling to the cells.

CD47 is a 50-kDa membrane glycoprotein that has five trans-
membrane-spanning regions and one Ig-like extracellular domain (38, 39) that plays a key role in host defense by participating in migration and activation of leukocytes in response to bacterial infection (40). CD47 has been shown to be a receptor for the C-terminal domain of TSP-1 (35), to stimulate T cell spreading (41), and to play an indirect role in chemotaxis of Jurkat cells to environmental TSP-1 (42).

The present results strongly implicate CD47 as the cell surface receptor through which CRT-TSP-1 interaction induces T cell migration. Thus, a CD47-binding peptide from the C-terminal domain of TSP-1 induces T cell migration. A mAb to CD47 inhibited T cell migration induced by this peptide as well as the TSP-1 binding site of CRT, and induction of T cell migration via CRT and CD47 showed the same pattern of sensitivity to inhibitors of signal transduction. The results suggest that T cell migration through CRT-TSP-1-CD47 is modulated via a common pathway of PI3K and the Janus family of protein tyrosine kinases. Interestingly, chemokine-mediated lymphocyte homing was also recently

![Image 328x441 to 534x742]

![Image 53x120 to 293x742]
reported to involve a tyrosine kinase pathway (37). PI3K has previously been shown to play a role in SDF-1α-induced lymphocyte polarization and chemotaxis (36) consistent with the present findings.

The present findings indicate that CRT-TSP-1-CD47 interaction plays a pivotal role in the control of T cell motility within 3D substrata. The fact that augmented TSP-1 expression was correlated to motility probably implies that the inducing effect on T cell motility by CRT is normally counterbalanced by internalization of TSP-1 due to the anchorage of CRT to CD91. It is also rather plausible that cell surface CRT expression may be a limiting factor for T cell migration. The present findings unveil a new mechanism for the control of T cell locomotor capacity and migration in addition to those of MMPs and direct adhesive contacts via βintegrins, the roles of which remain unclear (6, 7, 43, 44).

A model was taken shape, as depicted in Fig. 9, for the regulation of spontaneous T cell migration within 3D substrata as well as for T cell migration induced by various factors such as chemokines in which CRT and T cell TSP-1 are key mediators. This model is based on the present findings and on the fact that low density lipoprotein receptor-related protein CD91 and CRT form complexes in cells and interact with TSP-1 (14). According to this model, CRT interaction with the N-terminal domain of TSP-1 induces migration by increasing the strength of TSP-1 binding to the cell surface and formation of a ligand receptor complex with multipoint attachments to the lymphocyte surface. In other words, CRT triggers the binding of TSP-1 molecules to the lymphocyte surface, and over a certain threshold, TSP-1 induces T cell motility via interaction of its C-terminal domain with CD47. The chemokine SDF-1α also seems to potentiate T cell migration via CRT by increasing the number of TSP-1 molecules on the cell surface. An intriguing aspect of the present findings is that T cell TSP-1 participates in the control of T cell migration when bound to the lymphocyte surface. Therefore, this mediator of T cell migration does not restrict motile behavior by anchoring the cells in the same way as ECM components in the substrate. ECM substrata may, however, stimulate migration by up-regulating TSP-1 expression (21).

TSP-1 is a multicellular calcium-binding protein that participates in cellular responses to growth factors, cytokines, and injury (45). Our results indicate that chemokine-induced development of locomotor morphology and migration of T cells as well as spontaneous T cell migration in 3D substrata depend on endogenous TSP-1 mobilized to the cell surface. Chemokines and contact with substrata via β integrins may act in concert and regulate the transport of TSP-1 molecules onto the cell surface and/or augment cell surface TSP-1 expression. A reasonable interpretation of the present findings is that TSP-1 is continuously transported to the cell surface in a constitutive fashion and that CRT and chemokines either augment this transport or stimulate binding of TSP-1 to cell surface receptors. Further studies are needed to clarify the mechanisms that control TSP-1 expression on the cell surface.

The control of T cell migration by endogenous cell surface ligands such as CRT and TSP-1, which are not anchored to or presented as environmental ECM components, may be an advantage from a regulatory point of view. Ligands with these characteristics do not restrict the motility of the cells and will endow T cells with a freedom to migrate extensively, which is compatible with the high degree of recirculation and migration of this cell type. Therefore, CRT and T cell TSP-1 are attractive candidates as regulators of the motility of cells of the immune system in health and disease.

It is important to investigate further the cell surface receptor interactions of T cell TSP-1 and the behavior of TSP-1 in relation to these receptor interactions. The TSP-1 molecule interacts with multiple cell surface receptors, including αβ1, αβ2, and CRT via its N-terminal domain (22, 42, 46), and with CD47 via its C-terminal domain (47). As portrayed in Fig. 9, TSP-1 binding to different cell surface receptors leading to formation of a multireceptor complex (48–50) is likely to play a role for induction of locomotor morphology and migration of T cells.

In conclusion, the present results have uncovered an autocrine mechanism that may be of fundamental importance for the control of T cell motility within 3D substrata and within tissues in vivo. This mechanism is primarily dependent on the endogenous mediators CRT, T cell TSP-1, and CD47, and therefore is essentially autocrine in nature. Accordingly, exogenous factors such as the chemokine SDF-1α are shown to influence T cell migration via CRT-TSP-1 interaction. It is also likely that the cell surface receptor for CRT, CD91, may participate in the control of T cell migration, although the possible involvement of CD91 needs further investigation. The factors that determine CRT expression on the T cell surface and the behavior and fate of CRT and TSP-1 in lymphocytes should now be studied in detail.

**FIGURE 9.** CRT binding to TSP-1 determines the fate of TSP-1 on the cell surface and the motile response of T cells. A. Outlines of the two postulated major alternative fates of cell surface TSP-1 dependent on its interaction with CRT or TSP-1-binding CRT peptide and modulation by Zn²⁺ and SDF-1α. Low-affinity CRT-TSP-1 interaction favors internalization/degradation of TSP-1 whereas high-affinity interaction between CRT and TSP-1 favors persistence of TSP-1 on the cell surface and T cell migration. The CRT peptide (the TSP-1 binding site in CRT) exerts its stimulating effect on T cell motility without the counterbalancing effect of CD91, which anchors CRT. B. Schematic representation of associations of TSP-1 to CRT and CD91 (low density lipoprotein-related protein (LRP)). *, C1q is speculated to bind to a site spanning the N and P domains of CRT (14). Larger round ⬤ of TSP-1 molecule denote the C-terminal domain, and smaller ones the N-terminal domain.
References


