Dynamic recruitment of the adaptor protein LAT: LAT exists in two distinct intracellular pools and controls its own recruitment

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Summary

The integral membrane adaptor protein linker for activation of T cells (LAT) couples the T-cell receptor (TCR) with downstream signalling and is essential for T-cell development and activation. Here, we investigate the dynamic distribution of LAT-GFP fusion proteins by time-lapse video imaging of live T lymphocytes interacting with antigen-presenting cells. We show that LAT forms two distinct cellular pools, one at the plasma membrane and one that co-distributes with transferrin-labelled intracellular compartments also containing the TCR/CD3-associated ζ chain. The distribution of LAT between these two pools is dependent on LAT intracytoplasmic residues. Whereas plasma membrane-associated LAT is recruited to immune synapses after a few seconds of cell conjugate formation, the intracellular pool is first polarized and then recruited after a few minutes. We further show that LAT intracytoplasmic amino acid residues, particularly the Tyr136, 175, 195 and 235 residues, are required for its own recruitment to the immune synapse and that a herein-identified juxtamembrane LAT region (amino acids 32-104) is involved in the localization of LAT in intracellular pools and in T-cell signalling. Altogether, our results demonstrate that LAT controls its own recruitment at the immune synapse, where it is required as a scaffold protein for the signalling machinery. The results also suggest that the intracellular pool of LAT might be required for T-cell activation.

Movies and supplemental data available online

Key words: T-cell signalling, LAT, Raft, Immune synapse, Video imaging, Endosomes

Introduction

Engagement of the T-cell receptor (TCR) triggers signalling cascades that control T-cell activation. These cascades are initiated by protein tyrosine kinases, which cooperate to induce phosphorylation of the TCR-associated CD3-ζ chains and of various effectors. Among them is the linker for activation of T cells (LAT), an integral membrane adaptor protein whose cytoplasmic tail contains several tyrosine phosphorylation sites. Once phosphorylated by ZAP-70 (Zeta-associated protein), these sites bind Src-homology 2 (SH2)-domain-containing enzymes and enzyme-adaptor complexes. Thus, LAT might serve as a scaffold protein that orchestrates signalling pathways that are strictly required for T-cell activation (Rudd, 1999). The essential role of LAT in T-cell activation and development is underscored by studies performed on LAT-deficient cells and knockout mice showing impaired TCR signalling (Finco et al., 1998; Zhang et al., 1999a) and T-cell development (Zhang et al., 1999b), respectively.

Activation of T lymphocytes is associated with formation of an immune synapse at the interface between T cells and antigen-presenting cells (APCs). This formation is a dynamic process that involves spatio-temporal clustering of TCRs and adhesion and costimulatory molecules, re-modelling of the T-cell cytoskeleton, and recruitment of signalling molecules (Bromley et al., 2001; Delon and Germain, 2000). Several studies have also shown that cholesterol-sphingolipid raft domains are present in the immune synapse (Burack et al., 2002; Villalba et al., 2001; Viola et al., 1999). The importance of rafts in TCR signal transduction has been documented by studies showing that signalling proteins are recruited to these microdomains after TCR triggering and that disruption of these structures impairs T-cell activation. As the result of its palmitoylation on two cysteine residues, close to the transmembrane domain, LAT is constitutively targeted to raft domains and this localization seems to be crucial for its proper phosphorylation (Zhang et al., 1998). Thus, LAT might contribute to the recruitment of signalling proteins into rafts; however, the dynamics and mechanisms underlying LAT recruitment to the immune synapse are unknown.

Materials and Methods

Cells, antibodies and reagents

Jurkat JA16, LAT-GFP-transfected JA16 cells and the lymphoblastoid B-cell line Raji, as well as culture conditions, have been previously described (Montoya et al., 2002). LAT-negative Jurkat cells (JCAM2.5) were a kind gift of Dr Arthur Weiss (UCSF). Cells were maintained in RPMI supplemented with 10% FCS. To obtain human T-cell blasts, we cultured peripheral blood mononuclear cells with the toxic shock syndrome toxin 1 (TSST1) superantigen at 0.1 μg/ml for 3 days and interleukin 2 (IL-2; 20 UI/ml) for 6 further days. The anti-cis-Golgi monoclonal antibody (mAb) CTR433 was a kind gift from M. Bornens (Curie Institute, Paris, France); the anti-ZAP-70 and anti-PKC-θ mAbs were from Transduction Laboratories (Beckton Dickinson); the anti-ζ and anti-p56Lck mAbs were from Santa Cruz; the anti-phosphotyrosine (4G10) and anti-LAT mAbs were from Upstate Biotechnology; and the anti-GFP (clone 7.1 and 13.1) mAbs were from Roche or from Abcam (Ab290). Secondary Abs and the protocol for transferring-loading experiments were described elsewhere (Blanchard et al., 2002). TSST1 and *Staphylococcus* enterotoxin E (SEE) were obtained from Toxin Technology. The Orange Cell Tracker (CMTMR) and the cholera toxin-FITC were from Molecular Probes.

Expression of recombinant DNA constructs, immunoprecipitation and immunoblotting

LATΔCt was constructed by ligation of the *Xho*I-*Eco*RI fragment from murine LAT into pEGFP-N3 (Clontech). LATΔ32-104-GFP was obtained by PCR amplification of the LAT 32 N-terminus residues using the following primer pair: 5′-primer: GATGGAAGCA-GACGCCTTGAGC-3′; 3′-primer: TGAATTCAACTCACGGCA-GCGCACGCACAG-3′. The PCR amplification product was subcloned in PUC18 plasmid, followed by *Kpn*I and *Eco*R1 restriction. The restriction product was used to substitute for the N-terminus 104 residues of LAT similarly restricted by *Kpn*I and *Eco*R1 in the LATWT-GFP construct (Montoya et al., 2002). Stable JA16 clones expressing LATΔCt-GFP constructs were obtained by electroporation, as previously described (Montoya et al., 2002). LATY/F-GFP was obtained by PCR using the following primer pairs: 5′ primer: AAGCTTATGGAAGCAGACGCCTTGAGCCCG; 3′ primer: GGATCCGTTAAGCTCCTGTAGATTCTC; and cDNA from LATY/F 'knock-in' mice (E.A. and M.M., unpublished) as a template. The PCR product was verified by sequencing and cloned into the βDNA4 vector. 3×10^7 of stably transfected Jurkat cells were stimulated, lysed and analysed by immunoprecipitation and immunoblotting with 4G10 or anti-GFP, as previously described (Montoya et al., 2002).

Triton X-100 treatment

10^7 cells were transfected by electroporation using 20 μg of red-shifted GFP (RSGFP; Clontech). Expression of RSGFP was conducted for 36 hours at 37°C. 510^5 cells were collected, washed in PBS and put on silane-treated slides for 30 minutes at 4°C. Following washes in cooled PBS at 4°C, cells were incubated for 20 minutes at 4°C in PBS plus 1% Triton X-100, washed in PBS and fixed in 2% paraformaldehyde for 30 minutes at room temperature. Slides were next mounted and analysed by confocal microscopy.

Lipid raft isolation

Lipid raft isolation was performed as previously described (Drevot et al., 2002).

Conjugate formation, immunofluorescence and fluorescence quantitation

Conjugate formation and immunofluorescence experiments were as described elsewhere (Blanchard et al., 2002). Fluorescence images were acquired using a Leica TCS SP2 confocal scanning microscope equipped with a 100× 1.4 NA HCX PL APO oil immersion objective, and Ar and HeNe lasers emitting at 488 and 543 nm respectively. Double-staining images were obtained at zoom 4, with the intensity of excitation wavelengths and the power of photodetectors adjusted to avoid cross-talk. Quantification of fluorescent 12-bit images was performed with the Metamorph software (Universal Imaging). On a medial Z-section, fluorescence intensity was measured within regions encompassing the intracellular pool or total LAT.

Time-lapse confocal microscopy and video imaging

Time-lapse fluorescence confocal and video microscopy was performed as previously described (Montoya et al., 2002). See Movie 1A,B (http://jcs.biologists.org/supplemental/).

NF-AT activity and luciferase assay

The Luc reporter gene driven by the minimal human IL-2 promoter and three copies of the NF-AT-binding site was a kind gift of N. Clipstone. JCAM2.5 cells were transfected with a combination of reporter plasmid (5 μg), and plasmids encoding the different LAT-GFP chimera (15 μg) by electroporation. 18 hours after transfection, percentages of transfected cells (GFP-positive) were checked by FACS analysis. Transfected cells were incubated in standard round-bottom 96-well plates with Raji B cells (ratio 0.5 B cell for 1 T cell) or Raji B cells that have been pulsed with 1 μg/ml of SEE or PMA (50 ng/ml) plus ionomycin (10^{-6} M). Luciferase activity was assayed 6 hours later using a Promega luciferase assay kit according to the manufacturer's instructions.

Results

We have previously reported that LAT is dynamically recruited at the centre of the immune synapse upon TCR triggering, suggesting a role for TCR signalling in this process (Montoya et al., 2002). After TCR engagement, murine LAT is phosphorylated at Tyr136, 175, 195 and 235 residues with distinct and overlapping functions (Finco et al., 1998; Zhang et al., 1999a). We thus constructed a LAT deletion mutant coupled to GFP (LATΔCt; Fig. 1A) that is deleted at these four tyrosines but retains the transmembrane domain (TM; Fig. 1A), the two palmitoylated cysteine residues (CC; Fig. 1A), and the linker region formed between the TM and the tyrosine-rich region. In parallel, a construct deleted at the linker region between the CC motif and the tyrosine-rich region was developed (LATΔ32-104; Fig. 1A). Stable transfectants were obtained in Jurkat T cells. We first analysed LATWT-, LATΔCt- and LATΔ32-104-GFP cellular distribution. As shown in Fig. 1B, LATWT-GFP was detected at the plasma membrane and in intracellular compartments, which partially co-distributed with transferrin, suggesting its recruitment to recycling endosomes. Such a presence has already been described in intracellular vesicles for the ζ chain (Blanchard et al., 2002). As shown in Fig. 1B (lower panels), LAT constructs partially co-distributed with the ζ chain both at the plasma membrane and in transferrin-labelled endosomes. The mutant fusion proteins displayed similar expression levels, compared with LATWT-GFP, yet their subcellular distribution between the plasma membrane and intracellular pools appeared modified (Fig. 1B). The colocalization of LATΔCt-GFP with transferrin- and ζ-labelled intracellular compartments appeared increased and that with ζ-labelled plasma membrane was

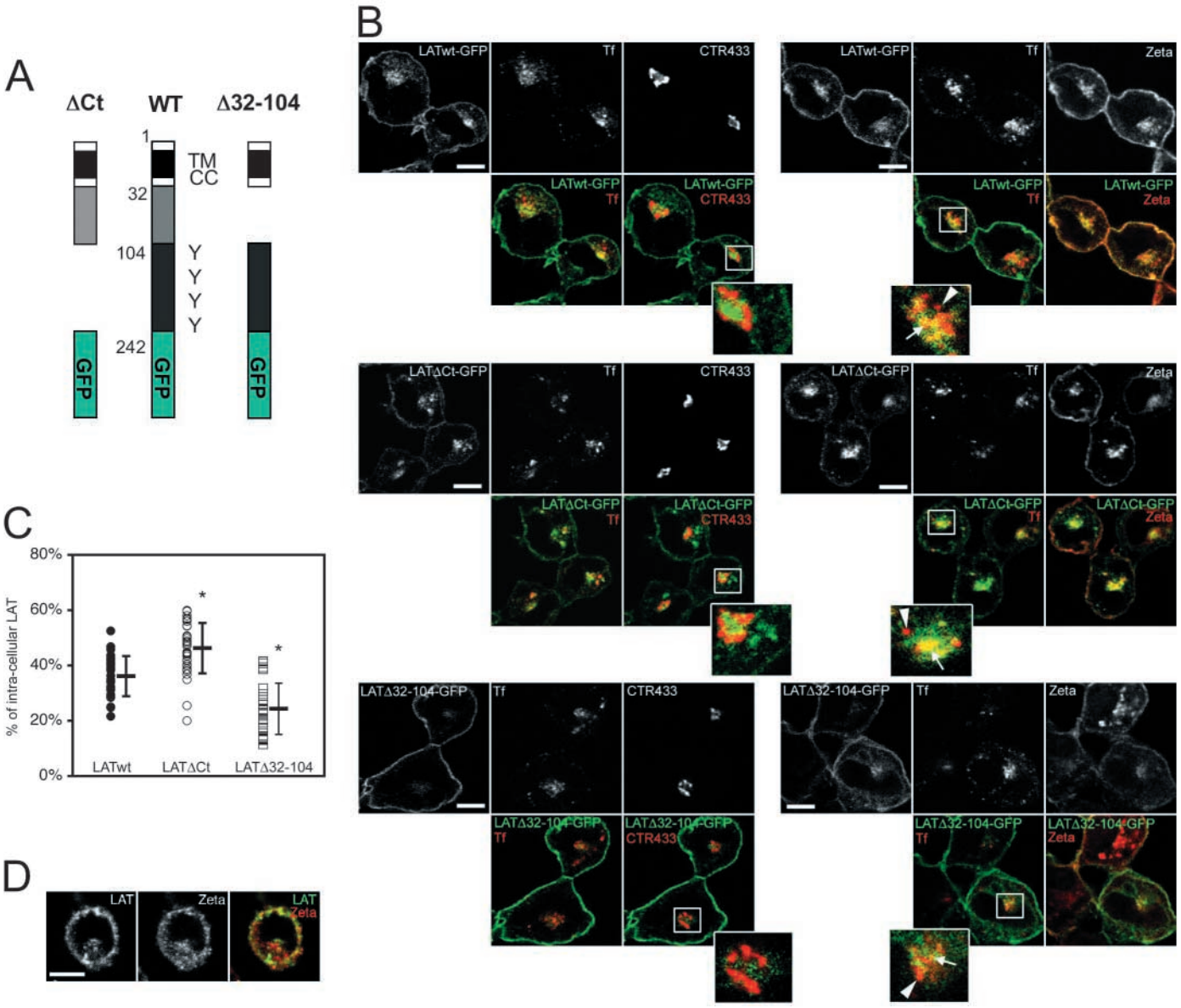

Fig. 1. Localization of LATWT-, LATΔ32-104-GFP and LATΔCt-GFP in T cells. (A) Schematic representation of the mouse LAT constructs. Domains include transmembrane (TM), palmitoylated cysteine residues (CC), green fluorescent protein (GFP) and tyrosine (Y) residues. (B) Jurkat cells expressing LATWT-GFP, LATΔ32-104-GFP or LATΔCt-GFP were pulsed with transferrin coupled to cyanine 3 (Tf) and labelled either with the mAb CTR433, which stains the cis-Golgi, or an anti-ζ mAb. Shown are single-colour or two-colour overlay images acquired with a confocal microscope at a medial Z-section. Insets display 3× enlargement of details comprised in the white box. White arrows point to co-localization areas and white arrowheads show vesicles where only Tf is found. Bar, 5 μm. (C) GFP fluorescence intensity of the intracellular and plasma membrane pools of LAT was measured on 12-bit confocal images. Percentage of intracellular versus total LAT is plotted for LATWT-GFP- (●, n=29), LATΔCt-GFP- (○, n=33) and LATΔ32-104-GFP- (□, n=28) expressing cells. Shown are mean±s.d., which are significantly different (P<0,0001, unpaired Student's t-test). (D) T-cell blasts were fixed and co-labelled with anti-LAT (left panel) and anti-ζ (right panel) Abs. Images show the presence of endogeneous intracellular pools of LAT and ζ. Bar, 5 μm.

reduced. LATΔ32-104-GFP fluorescence showed the reverse distribution, with increased plasma membrane and reduced intracellular staining. In contrast, neither of the constructs co-distributed with the Golgi marker (Fig. 1B). To characterize LAT distribution more precisely, we measured GFP fluorescence intensity of intracellular and plasma membrane pools of LAT and showed that the intracellular pool of LATΔCt-GFP was slightly but significantly more abundant than LATWT-GFP (46.2%±10.9 versus 36.3%±8.6) and that of LATΔ32-104-GFP was significantly lower (24.1%±9.3; Fig. 1C). This does not reflect a defect in transport of the mutant LAT from the Golgi to the plasma membrane as shown in Fig. 1B, but probably a higher rate of recycling. The partial LAT and ζ co-distribution was also observed in normal T-cell blasts (Fig. 1D), showing that LAT-GFP distribution is not an artefact due to GFP tagging or a specificity of the Jurkat T-cell line. In order to characterize better this intracellular pool of LAT, we co-labelled Jurkat T cells with antibodies directed against LAT

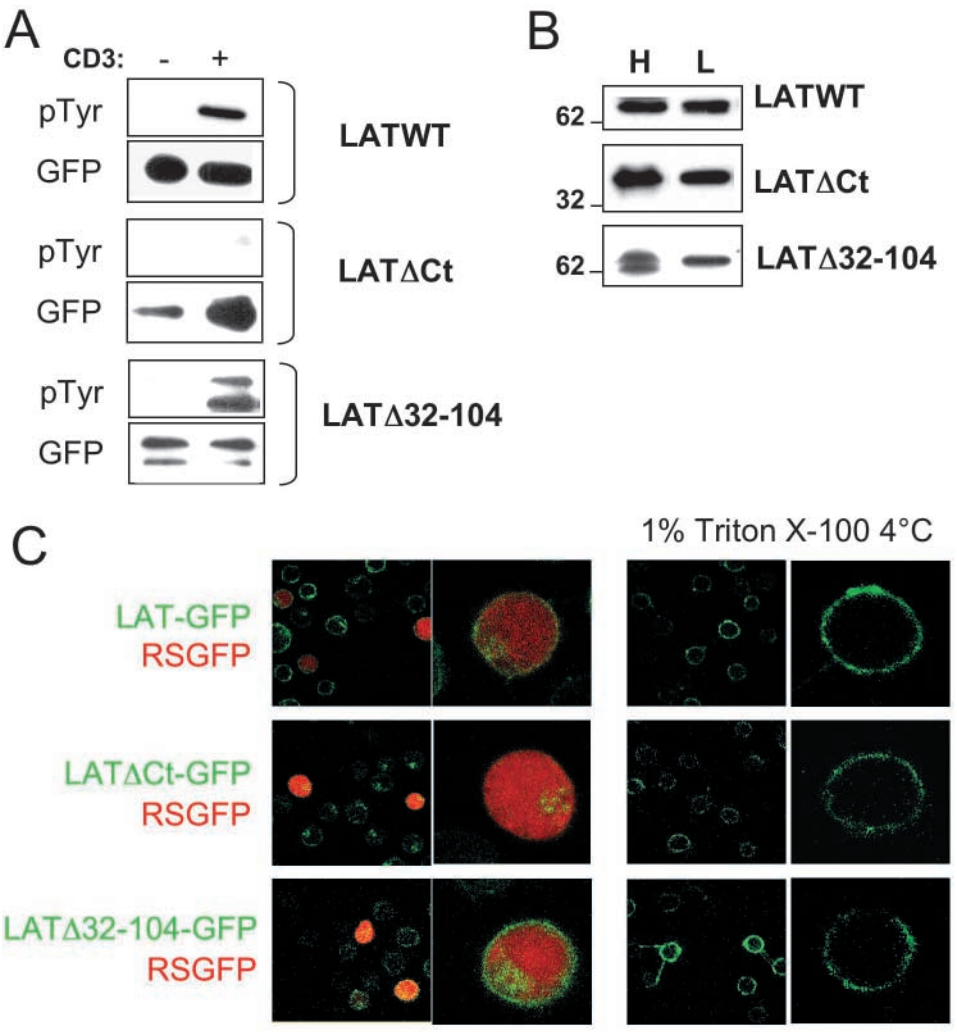

Fig. 2. Tyrosine phosphorylation and raft partition of LAT constructs. (A) LATWT-, LATΔ32-104- and LATΔCt-GFP cells were left unstimulated or triggered with anti-CD3 mAb for 2 minutes, and were then lysed and immunoprecipitated with anti-GFP. Immune complexes were sequentially analysed by anti-phosphotyrosine and anti-GFP immunoblotting. (B) Post-nuclear supernatants obtained from sonicated LATWT- and LATΔCt-GFP cells were lysed in Brij 98 at 37°C followed by sucrose gradient fractionation. Raft (L) and non-raft fractions (H) were collected and analysed by anti-GFP immunoblotting. (C) LATWT-, LATΔ32-104- and LATΔCt-GFP cells were transfected with the red-shifted GFP (RSGFP) construct. After 36 hours, cells were incubated or not in PBS 1%, Triton X-100 for 15 minutes at 4°C and mounted on slides. RSGFP and GFP staining was analysed by confocal microscopy.

and other signalling molecules as well as raft molecules (Fig. S1, http://jcs.biologists.org/supplemental/). Although the expression pattern of LAT, GM-1 and Lck were similar (intracellular compartment and plasma membrane), the co-distribution was only partial. As expected, LAT did not co-localize with two other signalling proteins (ZAP-70 and PKCθ) as these two proteins are cytosolic in resting T cells.

We then studied tyrosine phosphorylation of the constructs, induced by CD3 triggering. As expected, LATWT-GFP and LATΔ32-104-GFP, but not LATΔCt-GFP, were phosphorylated on tyrosine residues (Fig. 2A). Targeting of LAT to rafts is a prerequisite for its tyrosine phosphorylation and for efficient coupling to downstream TCR signalling. As shown in Fig. 2B, the three GFP fusion proteins partition in raft fractions isolated by Brij 98 extraction at 37°C followed by sucrose gradient. Confirming these results, partitioning of LAT fusion proteins in Triton X-100-insoluble membrane structures was also visualized in intact cells. Indeed, LATWT-, LATΔCt- and LATΔ32-104-GFP plasma membrane staining resisted a treatment consisting of a 15 minute incubation at 4°C in 1% Triton X-100, whereas the transiently transfected red-shifted GFP (RSGFP) did not (Fig. 2C).

We next studied the recruitment of the different LAT-GFP constructs at the contact zone between live T cells and *Staphylococcus* enterotoxin E (SEE)-pulsed Raji APCs by time-lapse imaging. As previously described (Montoya et al., 2002), a punctate central cluster appeared very rapidly (at 60 seconds) at the T/APC interface, which thereafter extended throughout most of the contact region (Fig. 3, upper panels). Notably, we consistently observed that the intracellular LAT-GFP-positive compartment 'polarized' in the vicinity of the APC after 2-3 minutes of T cell-APC contact (Fig. 3), in a SEE-dependent manner (not shown). The same results were obtained in transient transfection experiments excluding artefacts owing to stable transfection and clonal dilution of transfected cells (data not shown). LATΔ32-104-GFP was recruited at the immune synapse similarly to LATWT-GFP (Fig. 3). In contrast to LATWT-GFP and LATΔ32-104-GFP, LATΔCt-GFP did not form the typical punctate central cluster upon conjugate formation (Fig. 3). We conclude that the LAT C-terminus domain is required and sufficient for TCR-induced central clustering at the immune synapse. Yet, polarization and recruitment of the intracellular LATΔCt-GFP-labelled compartment towards the APC was observed (Fig. 3).

The fact that the LAT C-terminus is required for proper recruitment to immune synapses prompted us to perform site-directed mutagenesis of the four C-terminal tyrosine residues of LAT (LATY/F-GFP; Fig. 4A). This mutant showed the same expression level as LATWT-GFP (Fig. 4B, lower panel C). As expected, LATY/F-GFP displayed barely detectable tyrosine phosphorylation upon TCR triggering (Fig. 4B). No recruitment of LATY/F-GFP as a central membrane cluster in the contact zone was observed, and polarization of the intracellular LATY/F-GFP-labelled compartment did occur

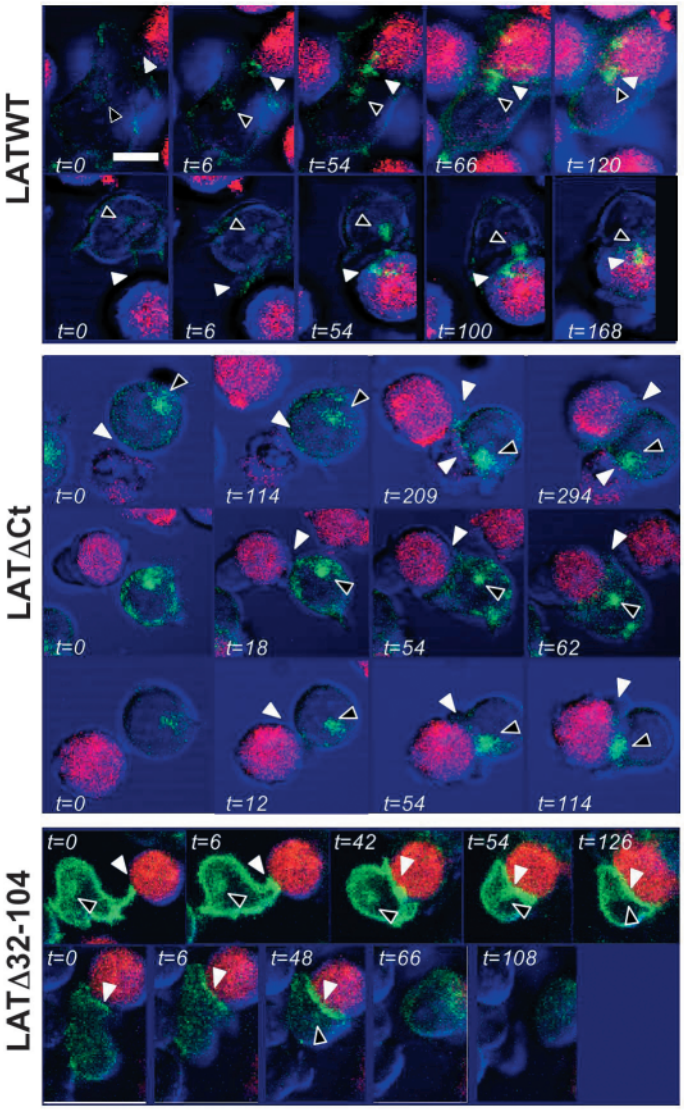

Fig. 3. Role of LAT C-terminal tail in TCR-induced recruitment of LAT to the immune synapse. APCs labelled with the CellTracker™ Orange were loaded for 20 minutes with 5 μg/ml SEE, mixed with LATWT-, LATΔ32-104- and LATΔCt-GFP T cells and monitored by time-lapse confocal microscopy. Images were taken at 10-second intervals. Representative images taken from the digital movies at the times indicated (seconds) are shown. White and black arrowheads point respectively to the initial site of contact and to the intracellular GFP-labelled compartment. Bar, 5 μm.

but was delayed (Fig. 4C; Movie 1A,B, http://jcs.biologists.org/supplemental/). This might be due to a partial dominant negative effect of the mutant on the reorientation of the T-cell microtubule-organizing center (MTOC). Membrane clustering and polarization of the intracellular GFP-labelled pool were quantified by counting fixed T cell-SEE-pulsed APC conjugates. In order to clearly discriminate between clustering of the plasma membrane pool and recruitment of the intracellular pool, we counted conjugates early after contact (3 and 7 minutes), in which the intracellular pool was not yet too close to the plasma membrane (Fig. 5A). LATWT-GFP clustered at the membrane in 85% and 90% of the analysed conjugates after 3 and 7 minutes of cell contact, respectively (Fig. 5A). LATΔCt-GFP and LATY/F-GFP clustered much less efficiently either at 3 minutes (10% and 23.3%, respectively) or at 7 minutes (22.5% and 29.3%) after cell contact (Fig. 5A). At 3 minutes, polarization of the intracellular GFP-labelled pool towards the APC was detected in up to 75% of conjugates involving LATWT-GFP-transfected cells, and fewer than 40% and 36% of conjugates involving LATΔCt-GFP and LATY/F-GFP, respectively. But at 7 minutes, the percentage of conjugates presenting with this polarization was the same for all LAT constructs, confirming that polarization of the mutated intracellular pool of LAT was only delayed.

To demonstrate further the role of LAT on its own recruitment to the immune synapse, we performed experiments in a LAT-negative T-cell line, JCAM2.5 (Finco et al., 1998) that we transfected back with the different LAT-GFP constructs. As shown in Fig. 5B and 5C, in transfected JCAM2.5 cells, the LATWT-GFP and LATΔ32-104-GFP constructs recapitulated the pattern of recruitment to the immune synapse formed between SEE-pulsed APCs and wild-type Jurkat cells described above. By contrast, the clustering of membrane-associated LATΔCt-GFP and LATY/F-GFP, as well as the polarization and recruitment of GFP-labelled intracellular pools, were both altered in the JCAM2.5 LAT-deficient cells. These results confirmed that the intracellular domain of LAT, and in particular the four tyrosine residues Tyr136, 175, 195 and 235, are required for the recruitment of the plasma membrane pool of LAT to the immune synapse. They also suggest that LAT controls the polarization of the MTOC towards the APCs.

We then studied T-cell activation in JCAM2.5 cells expressing the different mutants of LAT presented above. JCAM2.5 cells were co-transfected with the various LAT-GFP constructs and a NF-AT-luciferase reporter construct to determine NF-AT transcriptional activity. As shown in Fig. 6, LATWT-GFP restored NF-AT activation in JCAM2.5 cells stimulated by SEE-pulsed APCs. By contrast, neither LATY/F-GFP nor LATΔCt-GFP constructs were able to restore NF-AT activation in these cells, confirming the role of the intracellular Tyr residues in TCR activation, reported elsewhere (Zhang et al., 1999a; Zhang et al., 2000). More strikingly, the LATΔ32-104-GFP construct, which is predominantly expressed at the plasma membrane (Fig. 2) and is normally recruited at the immune synapse (Fig. 3), was unable to restore NF-AT transcriptional activity induced by triggering of the TCR (Fig. 6).

Discussion

LAT, which serves as a scaffold for signalling complexes in T cells, has been shown to be a key player of TCR-induced T-cell activation. This adaptor protein is modified after TCR activation both in terms of cellular localization and phosphorylation. However, the mechanisms involved in these modifications are still ill defined. We show herein that LAT forms two distinct cellular pools, one at the plasma membrane and one in intracellular compartments (Fig. 1; Fig. S1, http://jcs.biologists.org/supplemental/), which have already been described (Zhang et al., 1998) but that we characterized for the first time. Moreover, we shed some light on the control of this subcellular distribution by characterizing a region of

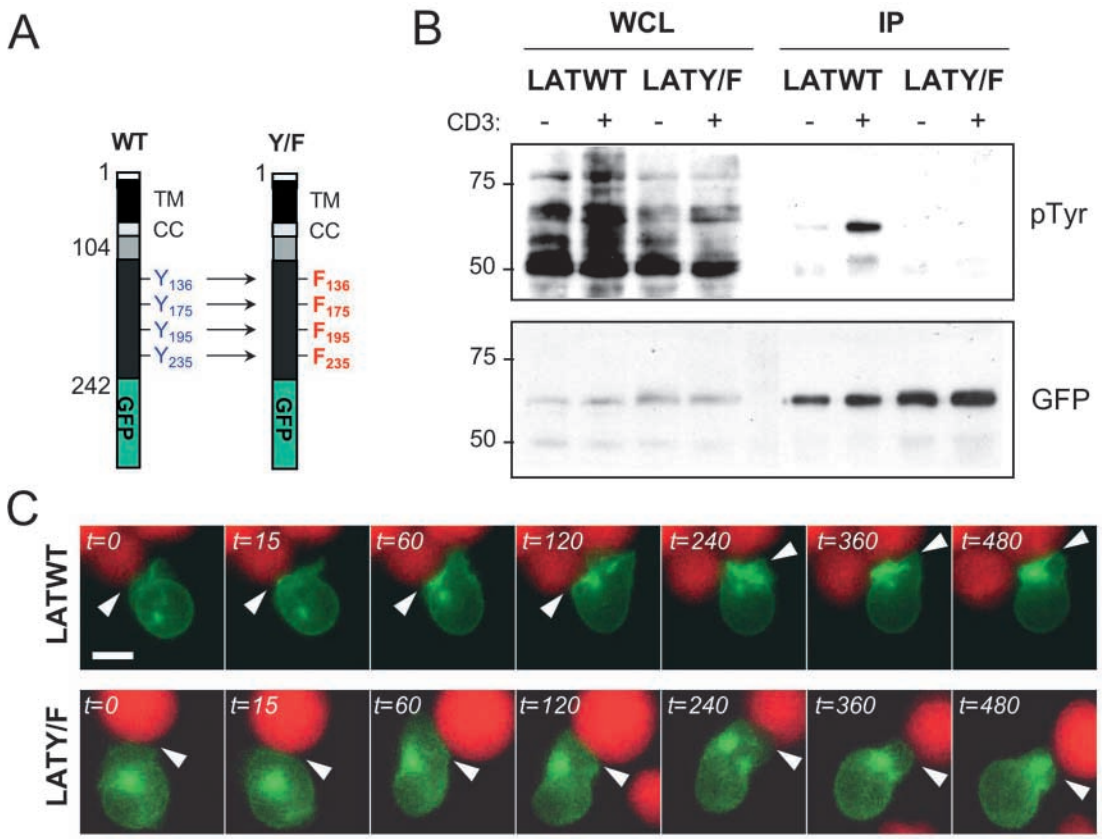

Fig. 4. Tyrosine residues 136, 175, 195 and 235 are required for TCR-induced recruitment of LAT to the immune synapse. (A) Schematic representation of the mouse LAT and LATY/F GFP chimaeras. (B) LATWT- and LATY/F-GFP cells were left unstimulated or triggered with anti-CD3 mAb for 2 minutes. Whole cell lysates (WCL) and GFP immunoprecipitates (IP) were sequentially analysed by anti-pTyr and anti-GFP immunoblotting. (C) LATWT- and LATY/F-GFP cells were mixed with CellTracker™ Orange-labelled APCs loaded for 20 minutes with 5 μg/ml SEE and monitored by time-lapse video microscopy. Images were taken at 15-second intervals. Representative images from the digital movies at the indicated times (seconds) are shown. White arrowheads point to the site of initial contact.

LAT that is required for its intracellular distribution. We also demonstrate, by dynamic studies, that both compartments are involved in TCR-induced recruitment of LAT to the immune synapse and that LAT controls its own recruitment. Finally, by analysing T-cell activation and LAT recruitment, we show that recruitment of both cellular pools of LAT seems to be required for TCR-induced T-cell activation, suggesting that LAT recruitment at the right place is the key event in T-cell activation.

We first characterized the intracellular LAT-GFP-labelled compartment and showed that it was present in recycling endosomes labelled with transferrin, suggesting that LAT might recycle in T cells. LAT-GFP only partially co-distributed with GM1, CD3-ζ and p56Lck (Fig. 1; Fig. S1, http://jcs.biologists.org/supplemental/) and displayed a subcellular distribution similar to the endogenous LAT (Fig. 1; Fig. S1, http://jcs.biologists.org/supplemental/) (G.B., unpublished), excluding artefacts due to the GFP tag. The absence of co-distribution of LAT with GM1 was somewhat surprising because LAT is present in rafts as shown by biochemical analysis. However, raft heterogeneity in the T-cell membrane has been described (Harder and Kuhn, 2000; Gomez-Mouton et al., 2001; Drevot et al., 2002), and LAT might be present in lipid microdomains that are not enriched in GM1. Finally, we also show that the 32-104 amino acids present in the intracytoplasmic domain of LAT are required for its distribution in endosomes containing ζ and transferrin, suggesting that motifs present in this region might control recycling of LAT.

We then dynamically studied the recruitment of these two cellular pools of LAT at the immune synapse and showed that they are differentially recruited. Whereas the membrane-associated LAT was found to be immediately recruited to the immune synapse formed between transfected T cells and SEE-pulsed APCs, the intracellular pool of LAT appeared first to be polarized and subsequently recruited towards the immune synapse in approximately 2-3 minutes (Figs 3, 4). These results are reminiscent of two studies by us and others showing respectively that endosomes labelled with ζ or p56Lck, which like LAT both co-distribute with transferrin, are recruited to the immune synapse with a kinetic similar to the one we observed (Blanchard et al., 2002; Ehrlich et al., 2002). Recruitment of these intracellular compartments probably witnesses the TCR-induced polarization of the MTOC towards the immune synapse (Kupfer et al., 1987; Lowin-Kropf et al., 1998), which drives with it intracellular organelles. Thus, our results together with the previous studies highly suggest that MTOC reorientation plays a role in translocation of intracellular pools

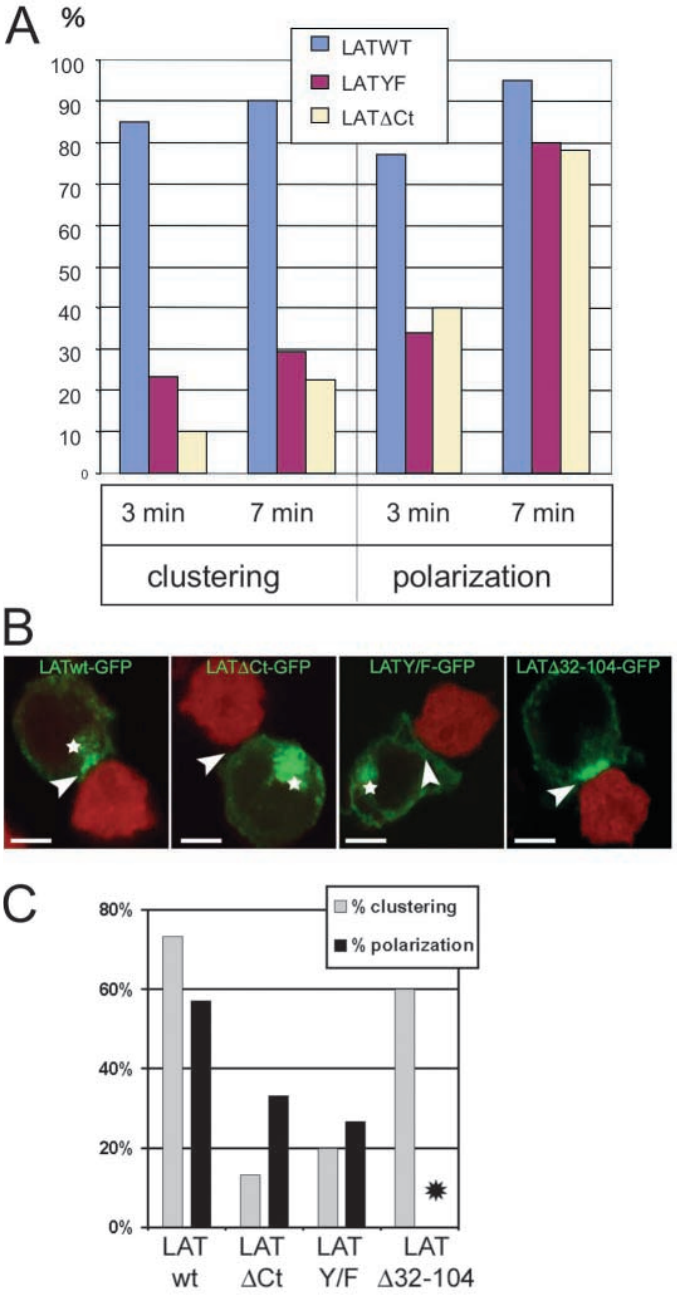

Fig. 5. LAT is required for its own TCR-induced recruitment to the immune synapse. (A) Transiently transfected LATWT-, LATΔCt- and LATY/F-GFP Jurkat cells were added to CellTracker™ Orange-labelled APCs for 3 minutes. Cells were then fixed and analysed by confocal microscopy. Histograms depict the percentage of T cells, after 3 minutes or 7 minutes of contact with SEE-pulsed APCs, showing either a clear clustering of LATWT-GFP (left panel) or a polarization of the intracellular LAT-containing compartment towards the APC (right panel). Quantification was performed on more than 35 conjugates for each LAT construct. Results of one representative experiment out of two are shown. Bar, 5 μm. (B) GFP fluorescence of LATWT-, LATΔCt-, LATY/F- and LATΔ32-104-GFP in JCAM2.5 cells interacting with SEE-pulsed Raji B cells. JCAM2.5 transiently reconstituted with the four LAT constructs were added to CMTMR-labelled (red) Raji B cells and shortly centrifuged to synchronize the contacts. After 8 minutes, cells were fixed and analysed by confocal microscopy. White arrowheads point to the site of initial contact and white stars show the LAT-containing intracellular compartment when visible. (C) Histograms depict the percentage of T cells showing either a clear clustering of LATWT-GFP (grey) or a polarization of the intracellular LAT-containing compartment towards the APC (black). Quantification was performed blindly on 15 conjugates for each LAT construct. Black star indicates the weak intracellular expression of LATΔ32-104-GFP, such that polarization could not be properly assessed. Bar, 5 μm.

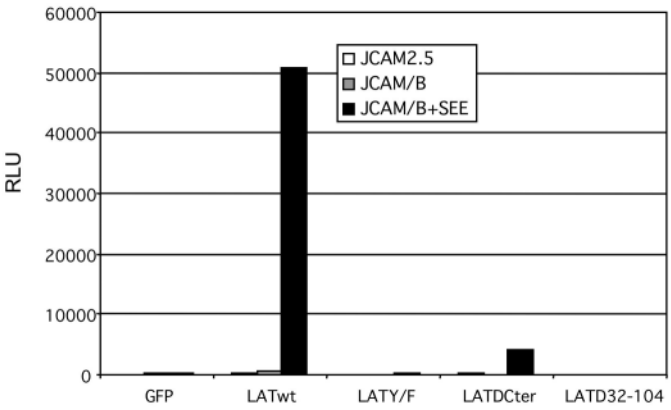

Fig. 6. NF-AT-dependent transcription in LAT-deficient cells reconstituted with different mutants. The NF-AT-Luc plasmid and the indicated LAT constructs were transfected into JCAM2.5 cells. 18 hours after transfection, transfected cells were incubated in standard round-bottom 96-well plates without or with Raji B cells (ratio 0.5 B cell for 1 T cell), either unpulsed or pulsed with 1 μg/ml of SEE. Luciferase activity was assayed 6 hours later using a Promega luciferase assay kit according to the manufacturer's instructions. The percentages and mean fluorescence of GFP-transfected cells were checked by FACS analysis to ensure similar transfection efficiencies. One representative experiment out of three is presented.

of signalling molecules such as p56Lck, ζ and LAT. It is noteworthy that signalling molecules control MTOC polarization itself. ZAP-70 activity is required for MTOC polarization of T cells towards the APC (Blanchard et al., 2002). More recently, LAT has also been shown to control this event (Kuhné et al., 2003). The absence of recruitment of LAT mutants that cannot be phosphorylated in their intracytoplasmic domain, which were used in this study, might be due to a more general defect in polarization of the T cells expressing these mutants.

The nature, mechanisms of constitution and role of such intracellular compartments in the context of T-cell activation is certainly important to evaluate further. A delayed recruitment of signalling molecules might contribute to the maintenance of signal transduction for a period sufficient to get full activation of T cells. Along this line, it is noteworthy that the LATΔ32-104-GFP mutant, which displays a much reduced intracellular localization, fails to restore NF-AT activation in LAT-deficient cells (Fig. 6), as well as activation of Ras-dependent signalling, as revealed by CD69 upregulation (C.H., unpublished). This absence of activation in LATΔ32-104-expressing T cells could either be due to altered subcellular localization of the mutated LAT and/or deficient recruitment of effector molecules by this

mutant. However, the LATΔ32-104-GFP mutant is phosphorylated on tyrosine residues in response to TCR activation (Fig. 2A) and is recruited to raft domains (Fig. 2B), similarly to the LATWT-GFP chimaera. Moreover, patterns of tyrosine-phosphorylated proteins that co-immunoprecipitate with LATΔ32-104-GFP and LATWT-GFP are the same (G.B., unpublished). We thus favour the hypothesis that accumulation of LAT in the intracellular compartment is involved in full LAT-dependent TCR signalling.

In the present study, we also demonstrate that LAT intracytoplasmic tyrosine residues strictly control clustering of the plasma membrane pool of LAT at the immune synapse. It has been shown that ZAP-70 is required for recruitment of LAT to the immune synapse (Blanchard et al., 2002). Here, we further extend this observation and show that LAT is required for its proper recruitment, because LAT constructs deleted of the C-terminus or mutated on the tyrosine residues Tyr136, 175, 195 and 235 failed to restore TCR-induced recruitment of LAT to the immune synapse in LAT-deficient cells (Fig. 5). Recently, Bunnell et al. showed in transfected Jurkat cells activated on anti-CD3-coated cover slips that clustering of LAT is inhibited by the Src-family kinase inhibitor PP2 (Bunnell et al., 2002). Altogether, these results suggest that LAT is probably actively recruited by mechanisms involving ZAP-70 and/or Src kinase activities. It is worth noting that recruitment of LAT and TCR fulfil different requirements since, in our model, ZAP-70 is required for LAT but not for TCR clustering at the synapse (Blanchard et al., 2002). Clustering of the plasma membrane pool of LAT at the immune synapse might result from an ‘active’ mechanism involving cytoskeletal remodelling. Indeed, LAT is involved in dynamic actin polymerization after TCR triggering, suggesting the existence of a link between LAT and the cytoskeleton (Bunnell et al., 2001).

We will now try to sort out which signalling pathways induced by LAT are required for LAT clustering at the plasma membrane and recruitment of the LAT endocytic pool. The respective roles of these two intracellular pools of LAT in TCR-induced T-cell activation will also be the subject of our investigations.

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References

Blanchard, N., DiBartolo, V. and Hivroz, C. (2002). In the immune synapse, ZAP-70 controls T cell polarization and recruitment of signalling proteins but not formation of the synaptic pattern. *Immunity* **17**, 389-399.

Bromley, S. K., Burack, W. R., Johnson, K. G., Somersalo, K., Sims, T. N., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M. and Dustin, M. L. (2001). The immunological synapse. *Annu. Rev. Immunol*. **19**, 375-396.

Bunnell, S. C., Kapoor, V., Trible, R. P., Zhang, W. and Samelson, L. E. (2001). Dynamic actin polymerization drives T cell receptor-induced spreading: a role for the signal transduction adaptor LAT. *Immunity* **14**, 315-329.

Bunnell, S. C., Hong, D. I., Kardon, J. R., Yamazaki, T., McGlade, C. J., Barr, V. A. and Samelson, L. E. (2002). T cell receptor ligation induces the formation of dynamically regulated signalling assemblies. *J. Cell Biol.* **158**, 1263-1275.

Burack, W. R., Lee, K. H., Holdorf, A. D., Dustin, M. L. and Shaw, A. S. (2002). Cutting edge: quantitative imaging of raft accumulation in the immunological synapse. *J. Immunol*. **169**, 2837-2841.

Delon, J. and Germain, R. N. (2000). Information transfer at the immunological synapse. *Curr. Biol*. **10**, R923-R933.

Drevot, P., Langlet, C., Guo, X. J., Bernard, A. M., Colard, O., Chauvin, J. P., Lasserre, R. and He, H. T. (2002). TCR signal initiation machinery is pre-assembled and activated in a subset of membrane rafts. *EMBO J*. **21**, 1899-1908.

Ehrlich, L. I., Ebert, P. J. R., Krummel, M. F., Weiss, A. and Davis, M. M. (2002). Dynamics of p56Lck translocation to the T cell immunological synapse following agonist and antagonist stimulation. *Immunity* **17**, 809-822.

Finco, T. S., Kadlecek, T., Zhang, W., Samelson, L. E. and Weiss, A. (1998). LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. *Immunity* **9**, 617-626.

Gomez-Mouton, C., Abad, J. L., Mira, E., Lacalle, R. A., Gallardo, E., Jimenez-Baranda, S., Illa, I., Bernad, A., Manes, S. and Martinez, A. C. (2001). Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. *Proc. Natl. Acad. Sci. USA* **98**, 9642-9647.

Harder, T. and Kuhn, M. (2000). Selective accumulation of raft-associated membrane protein LAT in T cell receptor signalling assemblies. *J. Cell Biol*. **151**, 199-208.

Kuhné, M. R., Lin, J., Yablonski, D., Mollenauer, M. N., Richie Ehrlich, L. I., Huppa, J., Davis, M. M. and Weiss, A. (2003). Linker for activation of T cells, ζ-associated protein-70, and Src homology 2 domain-containing leukocyte protein-76 are required for TcR-induced microtubule-organizing center polarization. *J. Immunol*. **171**, 860-866.

Kupfer, A., Swain, S. L. and Singer, S. J. (1987). The specific direct interaction of helper T cells and antigen presenting B cells: II. reorientation of the microtubule organizing center and reorganization of the membrane-associated cytoskeleton inside the bound helper T cells. *J. Exp. Med*. **165**, 1565-1580.

Lowin-Kropf, B., Shapiro, V. S. and Weiss, A. (1998). Cytoskeleton polarization of T cells is regulated by an immunoreceptor tyrosine-based activation motif-pendent mechanism. *J. Cell Biol*. **140**, 861-871.

Montoya, M. C., Sancho, D., Bonello, G., Collette, Y., Langlet, C., He, H. T., Aparicio, P., Alcover, A., Olive, D. and Sanchez-Madrid, F. (2002). Role of ICAM-3 in the initial interaction of T lymphocytes and APCs. *Nat. Immunol*. **3**, 159-168.

Rudd, C. E. (1999). Adaptors and molecular scaffolds in immune cell signalling. *Cell*, **96**, 5-8.

Villalba, M., Bi, K., Rodriguez, F., Tanaka, Y., Schoenberger, S. and Altman, A. (2001). Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells. *J. Cell Biol*. **155**, 331-338.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P. and Samelson, L. E. (1998). LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* **92**, 83-92.

Zhang, W., Irvin, B. J., Trible, R. P., Abraham, R. T. and Samelson, L. E. (1999a). Functional analysis of LAT in TCR-mediated signalling pathways using a LAT-deficient Jurkat cell line. *Int. Immunol*. **11**, 943-950.

Zhang, W., Sommers, C. L., Burshtyn, D. N., Stebbins, C. C., DeJarnette, J. B., Trible, R. P., Grinberg, A., Tsay, H. C., Jacobs, H. M., Kessler, C. M. et al. (1999b). Essential role of LAT in T cell development. *Immunity* **10**, 323-332.

Zhang, W., Trible, R. P., Zhu, M., Liu, S. K., McGlade, C. J. and Samelson, L. E. (2000). The association of grb2, gads and phospholipase C-γ1 with phosphorylated LAT tyrosine residues: the effect of tyrosine mutations on T cell antigen receptor-mediated signalling. *J. Biol. Chem.* **275**, 23355-23361.