Synergistic Assembly of Linker for Activation of T Cells Signaling Protein Complexes in T Cell Plasma Membrane Domains*

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Transmembrane adaptor molecule LAT (linker for activation of T cells) forms a central scaffold for signaling protein complexes that accumulate in the vicinity of activated T cell antigen receptors (TCR). Here we used biochemical analysis of immunoisolated plasma membrane domains and fluorescence imaging of green fluorescence protein-tagged signaling proteins to investigate the contributions of different tyrosine-based signaling protein docking sites of LAT to the formation of LAT signaling protein assemblies in TCR membrane domains. We found that the phospholipase Cγ docking site of LAT and different Grb2/Gads docking sites function in an interdependent fashion and synergize to accumulate LAT, Grb2, and phospholipase Cγ in TCR signaling assemblies. Two-dimensional gels showed that Grb2 is a predominant cytoplasmic adaptor in the isolated LAT signaling complexes, whereas Gads, Crk-1, and Grap are present in lower amounts. Taken together our data suggest a synergistic assembly of multimolecular TCR-LAT signal transduction complexes in T cell plasma membrane domains.

Activation of the T cell antigen receptor (TCR)

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‡The abbreviations used are: TCR, T cell antigen receptor; LAT, linker for activation of T cells; NFAT, nuclear factor of activated T cells; PLCγ, phospholipase Cγ; wt, wild type; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; TLA, TCR-LAT signaling assembly; DRM, detergent-resistant membrane; BAPTA-AM, bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester; SH, Src homology.

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stably express wt LAT, LAT all Y→F, LAT Y6F, LAT Y7F/Y8F, LAT Y6F/Y7F/Y8F, and LAT F6Y/F7Y/F8Y were described previously (11) and were maintained in full medium, 1 mg/ml G418. We generated new JCaM2 cell lines stably expressing LAT Y7F/Y8F/Y9F and LAT F4Y/F6Y/F8Y/F9Y as described below. All the JCaM2 lines used in this study were verified by Western blot and fluorescence-activated cell sorter analysis to express the similar levels of LAT and CD3 (not shown).

Anti-CD3 monoclonal antibody TR66 was from Antonios Lanzavecchia (Institute for Research in Biomedicine, Bellinzona, Switzerland). Antibodies and reagents were purchased from the following sources: monoclonal antibody against TCR γ-chain were from Santa Cruz Biotechnology; Grb2, PLC-γ, and ZAP-70 antibodies were purchased from Transduction Laboratories. Rabbit polyclonal serum against CD3-ε was obtained from the University Hospital, Basel, Switzerland. Phycoerythrin-conjugated ω-human CD3 antibodies were from BD Pharmingen, and α-LAT rabbit antiserum from Upstate Laboratories. Secondary horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were obtained from Bio-Rad and Amersham Biosciences.

Expression Constructs—The expression construct of LAT F4Y/F6Y/F7Y/F8Y/F9Y in pcd6 was generated by PCR-mediated mutagenesis from wt LAT cDNA clone without introducing a Myc tag, cloned into pcd6, and verified by DNA sequencing. WT LAT-EYFP and LAT ALL Y→F-EYFP fusion constructs were generated by PCR using the Expand high fidelity PCR system (Roche Diagnostics), introducing a Myc tag, and the Sal1 and AgeI restriction sites of the expression vector (pCDEF). The Sal1 site was cleaved and the sequence from the vector was ligated into the PCR fragment digested with Sal1 and AgeI. Primers were designed to introduce stop codon at the end of the cDNA for LacZ expression. Sequences were verified by sequencing, and all plasmids were purified using the Qiagen columns.

Transfections and Fluorescence Microscopy—JCaM2 cell lines were transfected by electroporation in ice-cold cytomix electroporation buffer (120 mKCl, 0.15 mNaCl, 10 mM K-PO4 buffer, 25 mM Hepes, pH 7.6, 2 mM EGTA, 5 mM MgCl2, 5 mM reduced glutathione, 2 mM ATP) at 960 μF, 280 mV in a 0.5-ml volume at a cell density of 1.5 × 107/ml in a Bio-Rad Gene pulser. After 5 min on ice, cells were collected, transferred into full medium, and incubated overnight at 37°C. To select stable transfectants, cells were plated for selection in 2 mg/ml G418 at a density of 8000 and 4000 cells/well in 96-well plates. For immunofluorescence analysis, the cells were incubated with one α-CDS-coated dynabead per cell at 37°C for 7 min in RPMI-Hepes, 1% fetal calf serum. Subsequently the conjugates were washed twice in phosphate-buffered saline, and 5 × 104 cells in 15 μl of phosphate-buffered saline were placed on a polyDlysine (Sigma)-coated microscope slide for 30 s on ice. Subsequently the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline at room temperature for 15 min. The cells were subsequently washed in phosphate-buffered saline and mounted in fluoromount G (Southern Biotechnologies). Immunofluorescence was performed on a Zeiss Axioplan 2 fluorescence microscope, and digital images were obtained using a 12-bit Spot camera (Diagnostic Instruments).

Immunosolation—M-450 goat α-mouse magnetic beads (Dynal) were coated with TR66 α-CDS monoclonal antibody following the instructions of the manufacturers. 1–4 × 106 Jurkat cells per data point were pelleted at 2000 rpm, 10 s at 4°C in an Eppendorf table top centrifuge. Loose cell pellets were mixed with α-CDS beads (1:2 ratio of beads:cells), incubated at 0°C for 2 min, and subsequently incubated at 37°C for 0 min, 3 min, 7 min, respectively. Cell conjugates were washed on a magnetic stand (5 mM NaCl, pH 7.2, 250 mM sucrose, 5 mM MgCl2, 10 mM NaF, 1 mM vanadate) and suspended in 1 ml of H-buffer containing CLAP protease inhibitors (100 μg/ml each of chymostatin, leupeptin, antipain, pepstatin, Sigma) and 0.2 mM PMSF. The cells were N2-cavitated using a nitrogen cavitation bomb (model 4639, Parr Instrument Company) equilibrated at 4°C, 50 bar for 10 min. The homogenate was filled to 10 ml with H-buffer; beads were subsequently removed with a magnet (Dynal) and washed three times in 10 ml of H-buffer for 3 min each at 0°C. Beads were analyzed by Western blotting using ECL chemiluminescence (Amersham Biosciences). In some experiments non-reducing SDS-PAGE was used. ECL-exposed x-ray films were scanned, and images were mounted using Photoshop (Adobe) software.

Pharmacological Treatments—For disruption of the actin cytoskeleton by latrunculin, bead cell conjugates were formed at 37°C for 7 min and subsequently treated with latrunculin (Calbiochem) at 12 μM concentration for 3 min. This treatment causes complete disappearance of phalloidin-actin staining in Jurkat cells (18) and leads to rapid rounding of the cells.

For depletion of intracellular Ca2+ stores, cells were incubated in RPMI, 1% fetal calf serum, 1 mg/ETGA, 50 μM BAPTA-AM (Sigma) for 30 min at 37°C as described previously (19). Cell bead conjugates were formed in RPMI, 1% fetal calf serum, 1 mg/ETGA, 50 μM BAPTA-AM, and isolations were performed as described above.

Detergent-insoluble Membrane Fractions—1 × 106 JCaM2 cells stably expressing either LAT ALL Y→F or WT LAT were lysed in 300 μl of ice-cold HNE (10 mM Hepes, pH 7.0, 150 mM NaCl, and 5 mM EDTA) homogenized by 1% Triton X-100 and passage through a 22-gauge needle, and incubated on ice for 15 min. The extract was adjusted to 40% OptiprepTM (Nycomed, Pharma), transferred to an SW55 centrifuge tube (Beckman Coulter), and overlaid with 1 ml of 35, 30, 25, and 0% OptiprepTM in HNE. The gradients were spun at 40,000 rpm for 3 h, and 600–μl fractions were trichloroacetic acid-precipitated and analyzed by Western blotting.

Two-dimensional Gel Analysis and Identification of Proteins by MALDI-MS—Immunosoluates of 4 × 106 Jurkat cells were prepared as described above except that prior to the homogenization, bead/cells conjugates were incubated with non-cell-permeable cross-linker bis(sulfosuccinimidyl) suberate (Pierce), 1 mg/ml, in H buffer on ice for 10 min at 4°C in order to increase the yields of the isolation and to cross-link the antibody chains to the beads. Two-dimensional gels of the isolates were run using Bio-Rad mini two-dimensional gel apparatus according to the instructions of the manufacturers, using carrier amiline mixtures, pH ranges 5–7, 3.5–10 (Amersham Biosciences), and 5–7 (Serva for the isoelectric focusing gels) (20). Proteins were stained using a colloidal Coomassie Blue staining kit (Novex).

MALDI-MS analysis was performed as described (21) with minor modifications. Briefly, spots were excised, destained with 30% (v/v) acetonitrile in 0.1 mM ammonium bicarbonate, and dried in a Speedvac evaporator. The dried gel pieces were reswollen with 5 μl of 5 mM ammonium bicarbonate, (pH 8.8) containing 50 ng of trypsin (Promega, Madison, WI), centrifuged for 1 min, and left at room temperature for about 12 h. After digestion, 5 μl of water was added; 10 min later 10 μl of 75% acetonitrile containing 0.3% trifluoroacetic acid was added, centrifuged for 1 min, and the content was vortexed for 20 min. For MALDI-MS 1.5 μl from the separated liquid was mixed with 1 μl of saturated d-cyano cinnamic acid in 50% acetonitril, 0.1% trifluoroacetic acid in water and applied to the MALDI target. The samples were analyzed in a time-of-flight Bruker mass spectrometer (Reflex III) equipped with a reflector and delayed extraction. An accelerating voltage of 20 kV was used. Des-Arg-1 Bradykinin (Sigma) and ACTH (18–38) (Sigma) were used as standard peptides. Calibration was internal to the samples. The peptide masses were matched with the theoretical peptide masses of all proteins from all species of the SWISS-Prot data base. For protein search, monoisotopic masses were used and a mass tolerance of 0.0075% was allowed. The protein search was performed with a software, developed by Roche (Basel, Switzerland), which is similar to the PepTident software on the ExPaSy server (https://www.expasy.org/sprot/peptident.html).

RESULTS

LAT Lacking Tyrosine-based Protein Docking Sites Fails to Accumulate at the Site of TCR Engagement—Earlier work of our laboratory showed that TCR-LAT signaling assemblies (TLAs) form at the contact zone to α-CDS antibody-coated TCR-activating beads (15). To visualize LAT accumulation, JCaM2 cells were reconstituted with fluorescent LAT-EYFP and the cells were stimulated with α-CDS dynabeads for 7 min. LAT distribution was monitored by fluorescence microscopy. In line with earlier reports using green fluorescence protein-tagged LAT, we observed a strong accumulation of wt LAT-EYFP in the plasma membrane contacting the TCR activating bead (Fig. 1A) (16, 17).

An EYFP fusion of a LAT mutant with all tyrosines replaced by phenylalanine (LAT ALL Y→F-EYFP) was efficiently targeted into the plasma membrane. In contrast to wt LAT-EYFP, we observed no enrichment of LAT ALL Y→F-EYFP at the contact region to TCR-activating beads. This showed that LAT accumulation at TCR activating beads depends on tyrosine-based docking signals for signaling proteins (Fig. 1B).

PLCγ and Grb2 Binding Sites on LAT Contribute to TCR Signaling Complex Formation in Membrane Domains—Next, we studied which tyrosine-based protein docking motifs of LAT

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are required for the formation of TLAs in plasma membrane domains. We employed a biochemical analysis of TCR signaling domains that were immunoisolated by a previously established procedure (14). Briefly, α-CD3 antibody-coated magnetic beads are used to trigger TCR in Jurkat T cells. Subsequently, bead/cell conjugates are homogenized mechanically by nitrogen cavitation, and plasma membrane fragments attached to the beads are retrieved. By Western blot analysis of the α-CD3-isolated membranes we previously demonstrated signaling-dependent accumulation of LAT-based signaling protein assemblies at the site of TCR engagement (15). Here, we assayed formation of TLAs mediated by LAT variants with specific mutations in tyrosine-based protein docking sites (Fig. 2A). We compared TCR signaling protein assemblies from JCaM2 cell lines stably reconstituted with wt LAT, which fully restores TCR signal transduction, and with LAT containing Tyr to Phe substitution of all tyrosines (LAT All Y→F), which does not transduce TCR signals (11). Western blots showed that similar amounts of TCR chain and ZAP-70 are recruited into the immunoisolated TCR signaling domains of all cell lines studied. LAT, Grb2, and PLCγ were enriched in immunoisolated TCR signaling complexes of wt LAT-reconstituted cells. In line with our fluorescence data, lower amounts of the LAT All Y→F mutant were recovered in α-CD3 immunoisolates (Fig. 2B). In all our experiments the amounts of LAT All Y→F in α-CD3 immunoisolated signaling domains were significantly reduced. Occasionally, the residual LAT All Y→F reached relatively high levels in the isolates, most likely because of higher plasma membrane recovery in the isolated membrane fragments. Non-complexed soluble Grb2 and PLCγ are not associated with signaling complexes and are thus barely detected in the isolates from LAT All Y→F-expressing cells.

We compared α-CD3 isolates of wt LAT and LAT All Y→F-expressing JCaM2 cells with JCaM2 cells expressing a LAT variant carrying a Y7F/Y8F mutation deleting two Grb2/Gads binding sites. This mutant was previously shown to exhibit a mild reduction of the TCR signal transduction capabilities of LAT (11, 12). Here, this mutant showed no reduction of LAT and PLCγ accumulation in TCR signaling domains, whereas Grb2 appears to be slightly reduced (Fig. 2B).

Analysis of the JCaM2 cells reconstituted with LAT mutant Y6F, which disrupts the PLCγ binding motif of LAT, showed no reduction of LAT accumulation and no significant change of Grb2 recruitment into the isolates. In line with immunoprecipitation data, we observed a strong reduction of PLCγ in the isolates from LAT Y6F-expressing cells (11). Above-background levels of PLCγ were consistently observed in the isolates from LAT Y6F-expressing cells (Fig. 2B). Consequently, we studied α-CD3 isolates formed by the LAT Y6F/Y7F/Y8F variant, in which Tyr-7 and -8 Grb2/Gads docking sites and the Tyr-6-based PLCγ docking site are both disrupted. Interestingly, in JCaM2 cells expressing the LAT Y6F/Y7F/Y8F variant not only PLCγ but also Grb2 incorporation into immunoisolated LAT/TCR signaling complexes was strongly reduced. Moreover, LAT recruitment was diminished. Taken together, these data show that the Tyr-6 of LAT not only has an important role for PLCγ recruitment and activation of sustained Ca2+ fluxes but that it also cooperates with tyrosine 7 and 8-based Grb2/Gads binding sites to mediate LAT and Grb2 accumulation in immunoisolated TCR signaling domains.

Next, we analyzed signaling complexes formed by LAT Y7F/Y8F/Y9F mutant, in which in addition to the Tyr-7 and -8 Grb2/Gads binding sites a Grb2 binding motif at Tyr-9 is dis-
Grb2-EYFP accumulation at the site of TCR activation, whereas no accumulation was detected in LAT All Y F-expressing JCaM2 cells. LAT F6Y/F7Y/F8Y/F9Y reconstituted Grb2-EYFP accumulation into TLAs. Bar, 10 µm.

It is possible that the weak enrichment of LAT All Y F in anti-CD3 immunoprecipitates results from a failure of raft targeting of LAT All Y F caused by defective LAT palmitoylation. Targeting of LAT into Triton X-100 detergent-resistant membranes (DRMs) strictly depends on its palmitoylation (22). Thus we tested the palmitoylation status of LAT All Y F of TX-100 DRMs from JCaM2 expressing LAT All Y F and wt LAT. Optiprep™ gradient centrifugation of the TX-100-extracted cells showed that the DRM fraction (fraction 2) of wt and All Y F LAT contained very similar relative amounts of LAT (Fig. 2C). Our results show that LAT All Y F as well as wt LAT are efficiently palmitoylated.

The Tyrosine 6-, 7-, and 8-based Docking Sites of LAT Are Not Sufficient for Complete Signaling Complex Assembly—Our data show that tyrosines 6, 7, and 8 are essential for the formation of TCR-LAT signaling complexes. Moreover, these sites have previously been shown to comprise the minimal docking site requirement for LAT to be capable of transducing Ca2+ fluxes and Ras-dependent Erk kinase and NFAT activation (11). Therefore, we asked whether these tyrosines are sufficient to mediate LAT recruitment to TCR signaling domains. We transiently transfected different JCaM2 cell lines expressing LAT variants with Grb2-EYFP. In line with our biochemical analysis (15) and fluorescence imaging of TLAs (16), we observed an accumulation of Grb2-EYFP at the contact zone of JCaM2-wt LAT cells to α-CD3-coated beads. As expected, LAT All Y F-expressing JCaM2 did not exhibit Grb2-EYFP accumulation at the bead/cell contact zone, because of the lack of Grb2 binding motifs in this LAT variant. LAT F6Y/F7Y/F8Y/F9Y mediated no detectable accumulation of Grb2-EYFP at the site of TCR activation, despite its two adjacent Tyr-7 and -9 Grb2/Gads binding sites. In JCaM2 lines expressing LAT F4Y/F6Y/F7Y/F8Y/F9Y, which additionally restores Grb2 binding motifs at Tyr-4 and -9, Grb2 accumulation at the TCR activating bead was significantly restored.

These data were corroborated by our biochemical analysis of immunoprecipitated LAT signaling domains (Fig. 4). Accumulation of LAT F6Y/F7Y/F8Y in α-CD3 immunoprecipitates was clearly detectable when compared with signaling complexes from All Y F LAT-expressing cells but significantly lower than in isolates from wt LAT cells. Moreover, Grb2 recovery in the isolated signaling domains from the LAT F6Y/F7Y/F8Y JCaM2 line was clearly reduced when compared with isolates from wt LAT-expressing JCaM2 cells. Immunoisolation of F4Y/F6Y/F7Y/F8Y/F9Y LAT cells accumulated LAT to wt LAT levels and to a large extent reconstituted Grb2 accumulation. Despite intense efforts it was not possible to resolve the individual contributions of LAT Tyr-4- and -9-based Grb2 binding sites to this enhancement, because the differences between signaling complexes formed by LAT variants carrying the Tyr-4-, -6-, -7-, -8, Tyr-6-, -7-, -8, and Tyr-4-, -6-, -7-, -8, -9 docking sites were too subtle and variable to be unambiguously resolved.

Taken together our data show that, albeit their important contribution to signaling protein assembly, the protein docking motifs of LAT comprising tyrosines 6, 7, and 8 are not sufficient to fully reconstitute LAT-TCR signaling domain formation and that additional Grb2 binding sites outside this motif contribute to the recruitment of Grb2 and LAT into TCR signaling domains.

Multiple SH2/SH3 Cytoplasmic Adaptors Are Present in Immunoprecipitated TCR-LAT Signaling Assemblies—Multiple cytoplasmic adaptors can be resolved on two-dimensional gels of isolated Jurkat cell TLAs (Fig. 5). We identified SH2/SH3 adaptors Grb2, Gads, Grap, and Crk-1 by MALDI-MS on tryptic digest of excised protein spots. Moreover, spots for Gads and Grb2 were verified by Western blotting (data not shown). Importantly, Grb2 is a very abundant cytoplasmic adaptor, whereas Gads is relatively minor and Grap and Crk-1 are present in intermediate amounts, showing that cytoplasmic adaptors strongly differ in their relative abundance in TLAs. LAT was not resolved on our gels, possibly because of its highly acidic isoelectric point or because of the hydrophobic nature of its membrane anchor.
Role of Ca^{2+} Fluxes and Actin in LAT Signaling Complex Formation—The activation of PLCγ leads to inositol 1,4,5, PI3 release and triggers intracellular Ca^{2+} fluxes. To test whether intracellular Ca^{2+} fluxes influence the formation of TLAs we performed immunooisolation using wt LAT-expressing JCaM2 cells that were treated with the intracellular Ca^{2+} chelator BAPTA as described previously (19). Western blot analysis of the respective immunooisolates from BAPTA-treated and control cells revealed no significant difference in the amount of LAT, PLCγ, and Grb2 (Fig. 6A). This suggests that the formation of the TLAs does not require the induction of Ca^{2+} fluxes.

Moreover, we addressed the possibility that the formation of TLAs is because of their mutual anchoring in close vicinity by the actin cytoskeleton. We treated Jurkat cells with the drug latrunculin A, which rapidly and efficiently disrupts the actin cytoskeleton (23). It was not possible to form conjugates of α-CD3 beads with Jurkat cells treated with latrunculin A. This is most likely because of the rounding of the cells following addition of the latrunculin. However, we succeeded in obtaining immunooisolates from Jurkat cells that were treated with latrunculin after conjugate formation and triggering for 7 min (Fig. 6B). We observed a clear reduction of TCR and ZAP-70 yields in the isolates from latrunculin-treated cells. By loading two times the equivalents of immunooisolated material from latrunculin-treated cells on the Western blot, we normalized the immunooisolates to essentially equal amounts of TCR and ZAP-70. Under these conditions, the amounts of LAT, PLCγ, and Grb2 in the isolates are very similar to the non-treated cells. This showed that the ratio of TCR/ZAP-70 to LAT complexes is not affected by disruption of the actin cytoskeleton, demonstrating that anchoring of LAT in the vicinity of TCR is not directly mediated by the actin cytoskeleton. However, the yields of signaling complexes were reduced upon latrunculin treatment, possibly because of the strong reduction of bead/cell contact area or reduced TLA anchoring in the plasma membrane domain TCR activating bead.

DISCUSSION

Using imaging and biochemical analysis of TCR signaling membrane domains, we studied the role of the tyrosine-based protein docking sites of LAT in the accumulation of LAT and cytoplasmic signaling proteins in the vicinity of activated TCR. We analyzed signaling assemblies formed by LAT variants that carry different combinations of tyrosine-based signaling protein docking sites.

Non-autonomous Signaling Protein Docking Sites on LAT: Structural Implication—Our study showed that different docking sites for the signaling proteins PLCγ and Grb2/Gads synergize in formation and/or stabilization of TCR-LAT signaling complexes. This synergy may be caused by cooperative stabilization of these assemblies by networks of intermolecular interactions between signaling proteins. A prototypic model for such multimolecular structures is the tetrameric complex formed by LAT, Gads, SLP-76, and PLCγ (6, 12, 24). However, Gads and SLP-76 are present in the isolated complexes in relatively low amounts (Fig. 5 and Ref. 15), possibly because of their early dissociation from the TCR-LAT signaling complexes (19). Therefore, other molecular interactions are also likely to play a role in complex assembly.

Our two-dimensional gel analyses showed strong differences in the relative amounts of different SH2/SH3 adaptor in the immunooisolated signaling complexes. We identified the adaptors Crk-I, Grap, and, in particular, Gads as relatively minor components, whereas Grb2 is by far the most abundant member of the SH2/SH3 adaptor family (for review on cytoplasmic adaptors, see Ref. 25). Possibly, Grb2 has an important role, beyond SOS recruitment and Ras activation, by organizing the structure of LAT signaling complexes. This is corroborated by the important contribution of multiple Grb2 binding motifs of LAT to signaling complex formation and downstream NFAT and Erk activation.

Moreover, our analysis showed that the PLCγ docking site Tyr-6 contributes to TLA assembly. It remains to be shown whether the enzymatic activity of PLCγ is required for this process. However, our experiments blocking Ca^{2+} fluxes and the efficient TLA formation mediated by the LAT F6Y, which inefficiently transduces Ca^{2+} fluxes, suggest that Ca^{2+} fluxes are not required for complex assembly. It remains to be tested whether diacylglycerol production by PLCγ plays a role in complex formation, for example by activating protein kinase C, and/or whether PLCγ plays a role in the structure of signaling assemblies. A role of Tyr-6 was also implicated by recent photobleaching experiments showing a stable anchoring of wt LAT-GFP in TCR signaling assemblies, whereas LAT F6Y-GFP and LAT F6Y/F7Y/F8Y/F9Y-GFP readily exchanged with their environment (17).

It has recently been shown that phosphorylation of different LAT tyrosines occurs in a non-autonomous fashion (13). In line with the issues discussed above, tyrosine-phosphorylated docking motifs may become stabilized by tight association of their specific binding partners upon complex assembly. Alternatively, phosphorylation and binding of signaling proteins may lead to conformational changes on LAT or the whole signaling complexes. This synergy may be caused by cooperative stabilization of these assemblies by networks of intermolecular interactions between signaling proteins. A prototypic model for such multimolecular structures is the tetrameric complex formed by LAT, Gads, SLP-76, and PLCγ (6, 12, 24). However, Gads and SLP-76 are present in the isolated complexes in relatively low amounts (Fig. 5 and Ref. 15), possibly because of their early dissociation from the TCR-LAT signaling complexes (19). Therefore, other molecular interactions are also likely to play a role in complex assembly.
assemblies, causing exposure and availability of tyrosines for further phosphorylation by kinases. Therefore, it is also possible that the phosphorylation events on LAT occur in an ordered fashion, explaining the interdependence of the different tyrosine-based docking sites in TLA formation.

Further evidence for a structural basis of a cross-talk between signaling protein docking sites of LAT in the signaling response stems from the observation that different tyrosine protein docking sites had to reside on the same LAT molecule in order to restore signaling function in JCaM2 cells (11). To resolve the mechanisms for the synergistic interactions of signaling protein docking to TLA assemblies, causing exposure and availability of tyrosine-based docking sites in TLA formation.

Non-autonomous Signaling Protein Docking Sites on LAT: Functional Implications—Formation of the LAT signal assembly by respective LAT mutants correlated well with their capability to transduce different downstream signaling reactions. LAT tyrosines 6, 7, and 8 comprise a minimal set of docking motifs to enable transduction of Ca\(^{2+}\) fluxes, Erk and NFAT activation. However, the additional presence of the Grb2 docking sites tyrosines 4 and 9 was required to restore NFAT and Erk activation to the same levels as transduced by wt LAT (11). This correlated well with the incomplete accumulation of LAT F6Y/F7Y/F8Y and Grb2 into CD3 immune isolates, whereas LAT signaling assembly formation was almost completely reconstituted by LAT F4Y/F6Y/F7Y/F8Y/F9Y. Disruption of the Tyr-6 PLC\(\gamma\) binding motif of LAT has little effect on Grb2 and LAT recruitment into TLAs but impedes induction of Ca\(^{2+}\) fluxes and abolishes NFAT and Erk activation (12). A residual amount of PLC\(\gamma\) is recovered in isolates of LAT Y6F-expressing cells, possibly caused by indirect association via Gads/SLP 76. This residual PLC\(\gamma\) may be sufficient for the induction of weak or aberrant Ca\(^{2+}\) fluxes, which have been described as JCaM2 cells expressing LAT Y6F (11, 12).

Because these experiments were performed in the JCaM2 leukemic T cell line, it will be very important to study the physiological roles of TLA assembly in T lymphocytes. Possibly the synergistic contribution of signaling protein docking to TLA formation reflects a mechanism to define a sequence and/or a threshold of tyrosine phosphorylation on LAT required to trigger specific downstream responses.

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