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Brief communication

Evidence of LAT as a dual substrate for Lck and Syk in T lymphocytes

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Abstract

LAT is a linker protein essential for activation of T lymphocytes. Its rapid tyrosine-phosphorylation upon T cell receptor (TCR) stimulation recruits downstream signaling molecules for membrane targeting and activation. LAT is physically concentrated in cholesterol-enriched membrane microdomains and is known a substrate for Syk/Zap70 kinase. In this study, we demonstrate that LAT serves as a dual substrate for both Lck and Syk kinases. LAT phosphorylation is absent in Lck-deficient J.CaM1.6 cells and Lck is co-precipitated with LAT in pervanadate-activated Jurkat cells. Further, the in vitro kinase assay using purified Lck and LAT shows that Lck directly phosphorylates LAT. Both Lck and Syk, phosphorylate the ITAM-like motifs on LAT at Y171Y191, which is essential for induction of the interaction of LAT with downstream signaling molecules such as Grb2, PLC-γ1 and c-Cbl, and for activation of MAPK-ERK. Collectively, our data indicate that LAT is an immediate substrate for Lck in one of the earliest events of T cell activation.

Keywords: LAT; Lck; Syk; Zap70; T lymphocytes

1. Introduction

Upon ligand engagement of TCR, Src tyrosine kinases Fyn and Lck are immediately activated, resulting in phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within the CD3 cytoplasmic domains [1,2]. The phosphorylated ITAMs attract signaling molecules such as Syk/Zap70 kinases, PLC-γ1, Vav, SLP-76 and Grb2 for membrane targeting, phosphorylation and activation [1–5]. LAT, a 36 Kd type III membrane protein, is an essential adaptor for activation of T cells [6–11]. It contains motifs similar to the CD3 ITAMs at tyrosine residues Y171 and Y191 [11]. Like CD3, the phosphorylated LAT binds to SH2 domain-containing proteins including PLC-γ1, p85 subunit of PI3 kinase and Grb2 to transmit activation signal into the nucleus.

LAT is phosphorylated at reduced levels upon T cell activation in Zap70- or Syk-deficient Jurkat T cells [11,12], indicating that it is a substrate for the Syk family kinases. Several findings, however, suggest that tyrosine kinases other than the Syk family kinases may also involve in the LAT phosphorylation. First, the LAT phosphorylation can be induced by CD28 ligation in the absence of Zap70 and Syk activation [13]. Second, in the Zap70/Syk-deficient cells, the LAT phosphorylation is reduced, but not completely abolished. Last, LAT and Src kinases Fyn and Lck are palmitoylated, and are components of cholesterol-enriched membrane microdomains or rafts, together with PKCθ, CD4 and the TCR/CD3 complex [14–18]. In the rafts, both LAT and Lck are able to bind to CD4 [19]. The co-localization with Lck in the rafts raises a possibility that LAT may serve as an immediate substrate for Lck, prior to Syk/Zap70 activation.

In this study, we present evidence of LAT as a dual substrate for Lck and Syk that phosphorylate LAT at Y171Y191, resulting in recruitment and activation of downstream signaling molecules. Our data suggest that LAT is an immediate substrate for Lck in one of the earliest events of T cell activation.

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2. Materials and methods

2.1. Cells and antibodies

HEK293 cells were cultured in DMEM medium with 10% fetal bovine serum (FBS) and 50 μg/ml of gentamycin. Jurkat E6-1 and J.CaM1.6 cells were obtained from ATCC (Bethesda, MD) and J.CaM2.5 cells (LAT-deficient) were maintained in RPMI-1640 with 10% FBS and 50 μg/ml of gentamycin. Anti-HA, anti-CD3ζ and anti-pERK mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PLC-γ1, ERK1, Fyn and Zap70 mAbs were obtained from Transduction Laboratories (San Diego, CA), anti-FlagM2 from IBI (New Haven, CT) and OKT3 hybridoma cell line from the ATCC. Anti-phosphotyrosine 4G10 and anti-LAT were purchased from Upstate Biotechnology (Lake Placid, NY).

2.2. Plasmids and recombinant protein

LAT, Grb2, Fyn, Lck, Syk and Zap70 cDNAs were obtained from a cDNA library derived from human lymph nodes using high fidelity PCR. The primer sequences used for PCR cloning will be provided upon request. The amplified fragments were inserted in the expression plasmid pCEF with epitope tagging of Flag, hemagglutinin (HA) and GST or polyhistindine (His). The cDNA sequences were confirmed by automated sequencing analysis. The kinase activities of the cloned Lck and Fyn were verified by their ability to phosphorylate CD3ζ chain in co-transfected HEK293 cells. The kinase activity of the cloned Zap70 was determined by its ability to phosphorylate a known substrate SLP76 in the presence of Lck, and Syk kinase activity was determined by its ability to phosphorylate LAT. Amino acid substitutions of Y171Y191 to F171F191 on LAT were generated by PCR-based mutagenesis approach. Three murine Lck constructs including the wild type Lck ($mLck_{WT}$), a constitutively kinase-active Lck ($mLck_{KA}$) and the kinase mutant Lck ($mLck_{KM}$) were kindly provided by Dr. Dan Littman (NYU, New York, NY). The His- and Flag-tagged LAT protein was purified from the bacterial expression plasmid pQE30-LAT plasmid transformed E.coli. The purification of the recombinant protein was performed using Ni-agarose beads.

2.3. Transfection, immunoprecipitation and immunoblot

Transient transfection of HEK293 cells was performed using SuperFect Reagent (Qiagen Inc.). J.CaM2.5 T cells were transfected with LAT-Flag and the $LAT_{F171F191}$-Flag constructs using GenePorter reagent (GTS, San Diego, CA) and selected with puromycin (1 μg/ml). Total puromycin-resistant cell populations were used for immunoprecipitation and immunoblot analysis.

T cell stimulation with OKT3 was performed following the protocol described by Zhang et al. [11]. T cell activation with pervanadate (0.01 mM sodium vanadate/4.5 mM H_2O_2, freshly prepared) was conducted at 37 °C for 10 min. To detect LAT tyrosine phosphorylation and interaction with Grb2 in co-transfected cells, the cellular protein extracts were prepared in lysis buffer A (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 and 0.5% deoxycholate) containing PMSF (1 mM), aprotinin (10 μg/ml), leupeptin (10 μg/ml), NaF (5 mM) and sodium vanadate (1 mM). The immunoprecipitation and immunoblot procedures were described previously [11].

2.4. In vitro kinase assay

HA tagged Src kinases Fyn and Lck were transfected into HEK293 cells. 24 h post-transfection, the cells were lysed in lysis buffer B (1%NP-40, 20 mM Tris–HCl pH 8.0, 150 mM NaCl) in the presence of protease inhibitors (PMSF 1 mM; aprotinin 10 μg/ml, leupeptin 5 μg/ml and sodium orthovanadate 1 mM). The lysates were clarified by centrifugation at 14,000 rpm for 10 min, 4 °C and the supernatants were precipitated with anti-HA and protein A agarose beads for 2 h at 4 °C. The beads were subsequently washed with the same buffer for twice and followed by two washes with the kinase buffer (25 mM HEPES pH 7.5; 5 mM MnCl2; 5 mM MgCl2; 0.1 mM sodium orthovanadate). The kinase reaction was performed by resuspending the beads in 15 μl of the kinase buffer, addition of 5 μl (0.1 μg) of the purified recombinant LAT protein and 3 μl of 10 mM cold ATP, and incubation for 30 min at 25 °C. The samples were analyzed by immunoblot using anti-pY mAb 4G10.

3. Results and discussion

To investigate the possibility that LAT serves as the substrate for Src kinases in addition to Syk family kinases, transient co-transfection of LAT with Fyn, Lck, Zap70 or Syk in HEK293 cells was performed to examine the in vivo phosphorylation of LAT. The LAT phosphorylation was observed when co-transfected with either Fyn or Syk, while the phosphorylation of LAT was not seen when co-transfected with either Lck or Zap70 (Fig. 1a). Since the activity of Zap70 is induced by Lck, and the activity of Lck requires stimulation of T cells, we next co-transfected LAT and Zap70 with or without various Lck constructs that are derived from murine Lck including wild type Lck ($mLck_{WT}$), a constitutive kinase-active form of Lck ($mLck_{KA}$) and a kinase-inactive mutant ($mLck_{KM}$). As shown in Fig. 1b, the LAT phosphorylation was induced by $mLck_{KA}$ alone, but not by $mLck_{WT}$ nor by $mLck_{KM}$. The combination of $mLck_{KA}$ and Zap70 also induced LAT phosphorylation but did not significantly enhance the phosphorylation intensity as compared with the $mLck_{KA}$ induced LAT phosphorylation (Fig. 1b). The LAT substrate specificity for Src kinases was investigated further in an in vitro system using recombinant LAT protein and HA-tagged Fyn and Lck protein purified from transfected HEK293 cells. Substantial phosphorylation of the recombi-