Ezrin is a substrate for Lck in T cells

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Abstract **We evaluated the role of Lck tyrosine kinase, an early effector of T cell activation, in regulation of the membrane–cytoskeleton linker protein ezrin. Ezrin was constitutively tyrosine phosphorylated in wild-type and CD45-deficient Jurkat T cells, but not in Lck-deficient cells. However, phosphorylation was evident in cells, in which Lck activity had been restored by transfection. Phosphorylation was reduced by the Src family kinase inhibitor PP2 and increased by the tyrosine phosphatase inhibitor pervanadate, implying continuous tyrosine phosphorylation and dephosphorylation. Lck phosphorylated ezrin in vitro, and the major phosphotyrosine was identified as Y145. These results identify ezrin as the first cytoskeletal substrate for Lck.**

Key words: T lymphocyte; Lck; Actin cytoskeleton; Adhesion

1. Introduction

T cell activation is initiated by recognition of an antigen/MHC protein complex by the T cell receptor and a co-receptor, such as CD4. Following receptor engagement, an intracellular signal is generated by a set of receptor-associated protein tyrosine kinases (PTKs). Among them the Src family PTK Lck holds a key position as an early effector of signaling. The PTK substrates are in turn involved in further signal propagation [1]. One of the early events after the onset of a T cell signal is tyrosine phosphorylation of the membrane–microfilament linker protein ezrin [2]. Recent findings argue for a functional link between Lck and ezrin at the membrane–cytoskeleton interface during the T cell activation [3,4]. However, many aspects in this connection are poorly characterized, including the possible direct regulation of ezrin by Lck.

The function of ezrin, or other ezrin–radixin–moesin (ERM) family proteins, i.e. radixin or moesin, is regulated in a complex manner, including phosphorylation of a C-terminal threonine, PIP_2 binding and tyrosine phosphorylation [5]. Studies of the tyrosine phosphorylation of the ERM members have been focused on ezrin. In epithelial cells phosphorylation is induced upon stimulation of their PTK pathways with growth factors, such as epidermal (EGF), platelet-derived (PDGF) or hepatocyte growth factors (HGF) [6–8]. Both in T cells and in other cell types, the increase in tyrosine phosphorylation is transient, indicating a dephosphorylation of ezrin by a counteracting protein tyrosine phosphatase (PTPase). The present study was undertaken to evaluate the dependence of the tyrosine phosphorylation of ezrin on the enzymatic activity of Lck.

2. Materials and methods

2.1. Cells

The Jurkat clones E6-1, J45.01 [9] and JCaM1.6 [10] were obtained from the American type culture collection (ATCC, Rockville, MD, USA), and JCaM1.6/Lck [11] was kindly provided by Dr. A. Weiss, UCSF, CA, USA. For experiments the cells were grown to a density of $0.5–1.0\times10^6$ cells/ml.

2.2. Antibodies

The ezrin rabbit antibody has been described [12]. The rabbit antibody to an N-terminal peptide of Lck was from Dr. T. Mustelin (Burnham Institute, La Jolla, CA, USA). The horseradish peroxidase (HRP)-conjugated monoclonal antibody (mAb) PY99 against phosphotyrosine was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the HRP-conjugated secondary antibody to rabbit IgG was from Amersham (UK).

2.3. Measurement of PTK activity

Cells were lysed in ice-cold TKB buffer (20 mM HEPES, pH 7.5, 1% NP-40, 150 mM NaCl, 5 mM EDTA) supplemented with 1 mM sodium orthovanadate and protease inhibitors. Lck was immunoprecipitated with rabbit anti-Lck and protein A-Sepharose. The beads were washed with the PTK reaction buffer (10 mM HEPES, pH 7.2, 5 mM $MgCl_2$, 3 mM $MnCl_2$). The reaction was done with 3 μM ATP, 1 μg of the substrate poly(Glu/Tyr 4:1) and 5 μCi of [γ-^{32}P]ATP. After 15 min at 37°C the reaction was stopped with 20 mM HEPES, pH 7.4, 40 mM EDTA, 2 mg/ml bovine serum albumin on ice. After centrifugation the substrate was precipitated with trichloroacetic acid and filter-bound radioactivity measured. Specific counts were obtained by subtracting background values (pre-immune serum) from total radioactivity.

2.4. Immunoprecipitation and immunoblotting

5×10^6 cells were lysed in RIPA buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40, 0.1% SDS), supplemented with 1 mM sodium orthovanadate and protease inhibitors. The lysates were cleared, pre-absorbed with protein A-Sepharose, and

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Abbreviations: PTK, protein tyrosine kinase; ERM, ezrin–radixin–moesin; PTPase, protein tyrosine phosphatase

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incubated with anti-ezrin or pre-immune serum. The immune complexes were bound to protein A-Sepharose, washed and eluted with 1% SDS. Immunoprecipitates were separated by SDS–PAGE and transferred to nitrocellulose filters. Phosphotyrosine was detected with PTyr mAb PY99-HRP. After stripping, ezrin was detected with rabbit antibody and HRP-conjugated anti-rabbit IgG. Bound HRP was detected using ECL. To test the effect of PP2, 5×10^6 cells at the density of 10^6 cells/ml were treated with 50 μM PP2 at 37°C for 10 min. Control samples were treated with the PP2 solvent dimethyl sulfoxide (<0.1%).

2.5. Phosphorylation of ezrin in vitro

Placental ezrin was purified as described [6,13]. 2 μg of ezrin was phosphorylated in the PTK reaction buffer by purified human Lck (Upstate Biochemical, Lake Placid, NY, USA) in the presence of 3 μM ATP and 10 μCi of [γ-^{32}P]ATP for 15 min at 37°C.

2.6. Tryptic phosphopeptide mapping and Edman sequencing

In vitro phosphorylated ezrin was blotted on a polyvinylidene difluoride filter. The filter piece containing phosphoezrin was excised, blocked in 0.5% polyvinylpyrrolidone-360/100 mM acetic acid and washed with 10% acetonitrile/H_2O. Ezrin was digested overnight at +37°C with 1 μg of sequencing grade modified trypsin in 10% ACN/50 mM NH_4HCO_3, pH 8. Another 1 μg of trypsin was added and the incubation prolonged by 5 h. After the second digestion, residual radioactivity was still retained in filter. The digest was dried and oxidized with performic acid and dissolved in the HTLE electrophoresis buffer (formic acid:acetic acid:H_2O, 50:156:1794, pH 1.9). Phosphopeptides were separated on a cellulose thin-layer plate, first by electrophoresis and then by ascending chromatography in *n*-butanol:pyridine:acetic acid:H_2O, 750:500:150:600 v/v/v/v. Phosphopeptides were detected by phosphoimager and recovered by elution with HTLE buffer. Peptides were coupled to Sequelon-AA membranes, and Edman degradation was performed on an ABI sequencer model 477A. Released phenylthiohydantoin amino acid derivatives from each cycle were spotted on TLC plates, and the radioactivity was quantified by digital imaging after the subtraction of background. The phosphorylated amino acid was deduced by comparing to the predicted tryptic peptides of ezrin.

2.7. Recombinant DNA constructs and fusion proteins

GST–ezrin fusion proteins were generated from full-length human ezrin cDNA. For generation of the Y145F mutant fusion protein, a double PCR method was used as described in [8]. All products were verified by DNA sequencing. The fusion proteins were expressed in *Escherichia coli* and purified using Glutathione Sepharose (Amersham Pharmacia).

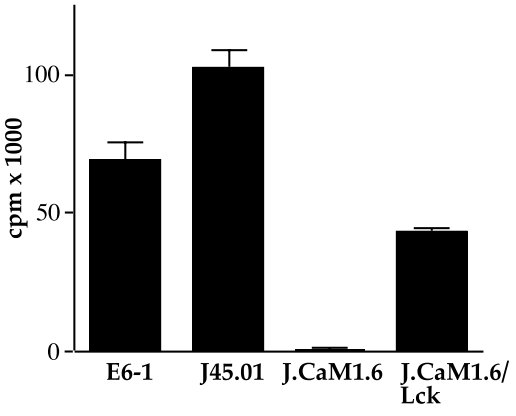

Fig. 1. Lck activity in Jurkat clones. Immunocomplexed Lck from different Jurkat clones was assayed for kinase activity using poly(Glu/Tyr 4:1) as a substrate. The results are expressed as mean specific counts ± S.D. of three experiments.

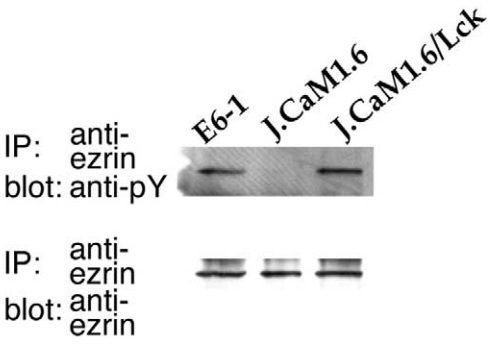

Fig. 2. Phosphorylation of ezrin in Jurkat clones with different Lck activity. Ezrin was immunoprecipitated from Jurkat clones E6-1, J.CaM1.6 and J.CaM1.6/Lck, and sequentially detected by pTyr mAb and ezrin antiserum.

3. Results

3.1. Lck activity and expression of CD45 in Jurkat clones

The human T lymphoma cell line Jurkat and established Jurkat clones with different Lck and CD45 activities were used to evaluate the role of these signaling enzymes in the regulation of ezrin in T cells. The wild-type clone E6-1, its mutant derivatives JCaM1.6 lacking Lck activity, J45.01 defective in the expression of CD45, and JCaM1.6/Lck, in which Lck activity has been restored by transfection of mouse Lck, were used. In order to set a firm basis for the studies, we first compared the PTK activity of Lck and the expression of CD45 in the Jurkat clones.

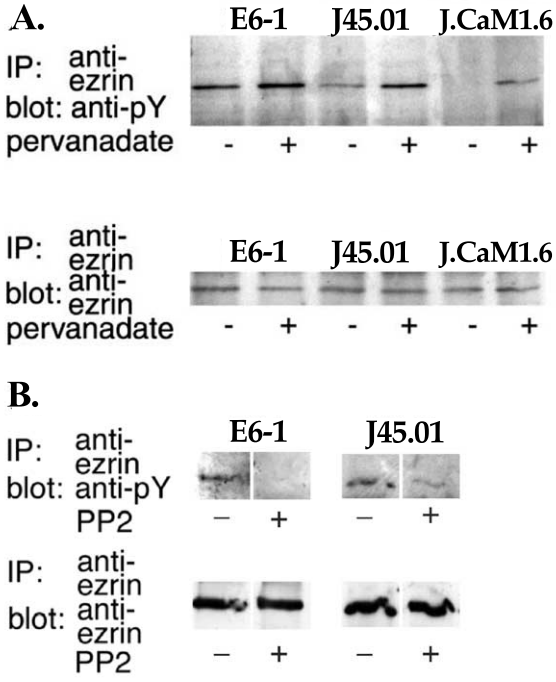

Fig. 3. Effects of pervanadate and PP2 on ezrin phosphorylation. A: Ezrin was immunoprecipitated from Jurkat clones. The level of tyrosine phosphorylated ezrin in control (−) or pervanadate-treated cells (+) was evaluated by pTyr mAb reactivity. Ezrin antiserum blot verifies loading of immunoprecipitates. B: Immunoprecipitated ezrin from untreated cells (−) or cells treated with with Src family kinase inhibitor PP2 (+) was evaluated by pTyr mAb reactivity. The loading control as in A.

High Lck activity was detected from clones E6-1 and J45.01, whereas no activity was detectable from clone JCaM1.6 (Fig. 1). As in a variety of CD45-deficient cells [14], the total activity of Lck was even higher in J45.01 cells than in the wild-type cells. In JCaM1.6/Lck cells the activity was restored. The specific counts between JCaM1.6/Lck and other clones cannot be directly compared because immunoprecipitations were performed with anti-human Lck antibody, which may not react with equal affinity with mouse Lck expressed in JCaM1.6/Lck cells. In additional assays a major part of the Lck activity was found to be associated with the membrane fraction in E6-1 and J45.01 cells (not shown). These results confirmed the Lck-negativity of the clone JCaM1.6 and the localization of a significant Lck activity at the cell membrane in clones E6-1 and J45.01.

The level of cell surface expression of CD45 was evaluated by flow cytometry using mAb MEM-28, which recognizes all CD45 isoforms. The expression of CD45 on the J45.01 cells was about 7% of that on the clones E6-1 and JCaM1.6 (not shown).

3.2. Tyrosine phosphorylation of ezrin in vivo

Ezrin was immunoprecipitated from each Jurkat clone, probed with the anti-PTyr mAb PY99 on a Western blot filter and subsequently reprobed with ezrin antiserum to confirm that an equal amount of protein was precipitated in each sample (Fig. 2). A basal level of tyrosine phosphorylation was detected in ezrin from clone E6-1, whereas no constitutively phosphorylated ezrin was detected in the JCaM1.6 cells. In the JCaM1.6/Lck clone, in which Lck activity was reconstituted, a significant amount of phosphotyrosine containing ezrin was demonstrated, indicating a role of Lck in the phosphorylation of ezrin in T cells.

CD45 is the major tyrosine phosphatase of the T cell membrane [15]. Therefore, we studied ezrin phosphorylation by using the CD45-low Jurkat clone J45.01. In line with Lck activity, a constitutive phosphorylation was observed (Fig. 3A). However, no hyperphosphorylation was detected. On the other hand, when the cells were treated with 50 μM of pervanadate, a PTPase inhibitor [16], the level of the tyrosine phosphorylation of ezrin was elevated in both E6-1 and J45.01

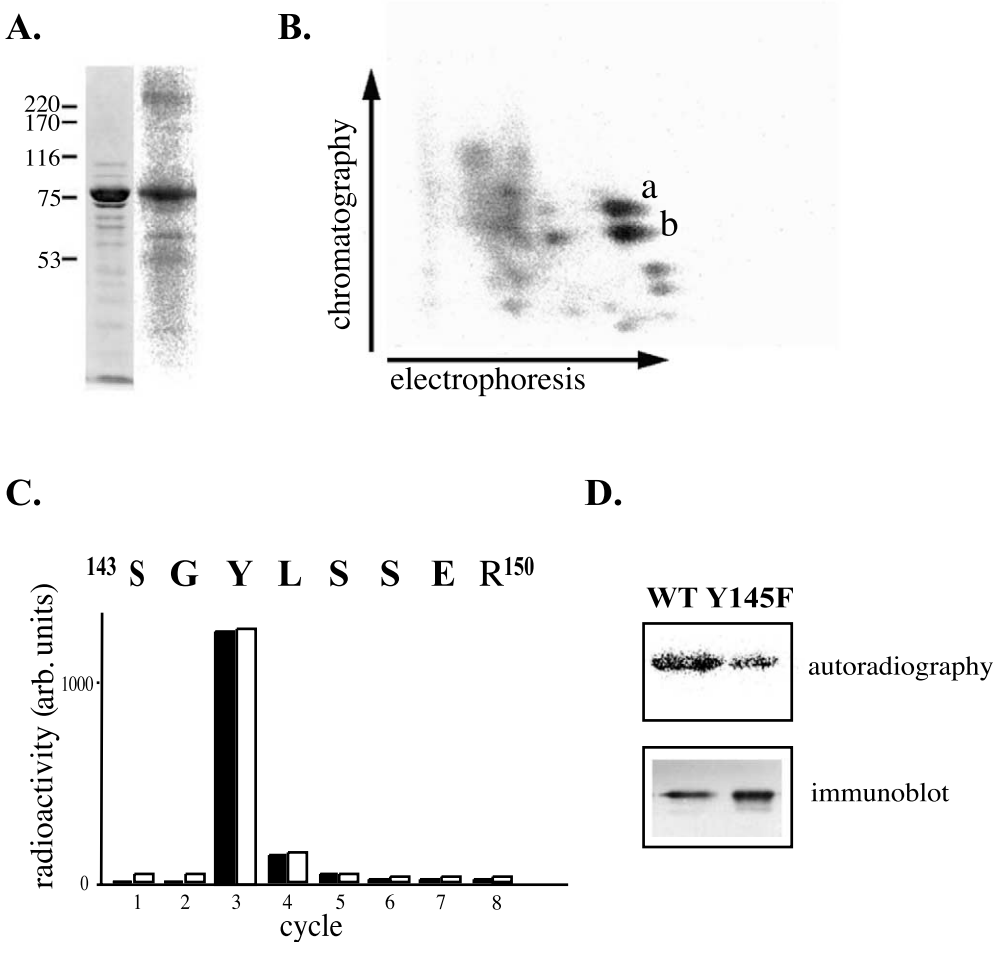

Fig. 4. Lck-catalyzed phosphorylation of ezrin in vitro and identification of the major phosphorylation site. Placental ezrin, visualized by Coomassie staining (A, left lane) was phosphorylated by recombinant Lck (A, right lane). Two major phosphopeptides (a and b) from phosphopeptide mapping (B) were eluted and analyzed by quantification of ^{32}P label released by successive cycles of Edman degradation (C). Both peptides showed the same pattern (a = black bar, b = white bar). The phosphorylated amino acid was identified as tyrosine 145 by comparing to predicted tryptic peptides of ezrin sequence. D: The wild-type and Y145F ezrin were produced as GST fusions and phosphorylated by Lck. The mutant form was less efficiently phosphorylated than the wild-type protein. The amount of fusion proteins used in the experiment was detected by Western blotting. A representative of three individual experiments is shown.

cells (Fig. 3A). This effect suggests that ezrin is constitutively phosphorylated and dephosphorylated in these cells and that pervanadate blocks the dephosphorylation step. However, we have observed that the pervanadate treatment causes an almost two-fold increase in the activity of Lck in E6-1 cells (data not shown), which also might contribute to the increased phosphorylation. The increase seen in ezrin phosphorylation in J45.01 cells in the presence of pervanadate could be due to more efficient phosphorylation by Lck or to the inhibition of some other phosphatase than CD45. Interestingly, pervanadate induced a detectable tyrosine phosphorylation of ezrin also in JCaM1.6 cells (Fig. 3A), indicating that an alternative phosphorylation mechanism, in addition to Lck, exists in Jurkat cells.

Experiments with the Src family selective PTK inhibitor PP2 [17] provided further evidence for a direct role of Lck in the constitutive phosphorylation of ezrin. When the E6-1 or J45.01 cells were treated for 10 min with 50 μM PP2, the level of tyrosine phosphorylation of ezrin was reduced (Fig. 3B). The immediate decrease on the phosphorylation further suggests that tyrosine phosphorylation of ezrin is efficiently counteracted by some PTPase(s) in the Jurkat cells.

3.3. Phosphorylation of ezrin in vitro

To study whether ezrin can directly be phosphorylated by Lck, ezrin was purified from human placenta and mixed with purified Lck in an appropriate PTK reaction buffer in the presence of [γ-^{32}P]ATP. After 15 min, the reaction was stopped, proteins were separated on SDS–PAGE and analyzed. As shown in Fig. 4A, ezrin was readily phosphorylated by Lck in this reaction.

The Lck-catalyzed phosphoezrin was subjected to two-dimensional tryptic phosphopeptide mapping. The signal was accumulated in two major phosphopeptides with significantly less activity in other peptides (Fig. 4B). The two phosphopeptides (a and b), which contain about 65% of the total radioactivity, were further analyzed by Edman sequencing. The majority of radioactivity was released by both peptides at the third cycle of sequencing (Fig. 4C). Based on the predicted tryptic cleavage sites in ezrin amino acid sequence, these peptides represent modifications of the same tryptic peptide, ^{143}SGYLSSER^{150}, the only peptide which contains a tyrosine at position three. Thus, tyrosine 145 is the major residue phosphorylated by Lck. Similar finding with two spots corresponding to the same peptide was found when the Src-phosphorylation site in the PDGF beta-receptor was mapped [18]. The result was further verified by producing wild-type and mutant Y145F ezrin as GST fusion proteins, which were subjected to in vitro phosphorylation by Lck. The wild-type was more efficiently phosphorylated than the mutant form (Fig. 4D).

4. Discussion

The Src family kinase Lck is an important signalling molecule mediating downstream effects of TCR activation. Several lines of evidence indicate that Lck acts as a direct regulator of ezrin in Jurkat T cells. Ezrin is constitutively tyrosine phosphorylated in the Jurkat clones E6-1 and J45.01, both of which express a high PTK activity of Lck at the membrane, whereas constitutive tyrosine phosphorylation of ezrin in the clone JCaM1.6, which completely lacks Lck activity, is typically not detected. In this clone, reconstitution of Lck activity by stable transfection restored the constitutive tyrosine phosphorylation of ezrin. The decrease in the phosphorylation during a short treatment of cells with the Src family specific PTK inhibitor PP2 also argues for a role of Lck. Finally, in vitro studies show that ezrin is a direct substrate for Lck.

Previous studies with A431 epidermoid carcinoma cells have identified Y145 and Y353 as the major sites of tyrosine phosphorylation of ezrin by the EGF receptor kinase [19]. We have now shown that Y145 also acts as a target for Lck, indicating that the phosphorylation by a receptor-type PTK and by a Src family PTK bear overlapping functions in the regulation of ezrin. Currently, not much is known on the role of Y145 phosphorylation. Mutagenesis of both Y145 and Y353 decreases the motogenic and morphogenic responses of epithelial cells to the growth factor HGF [8]. Although Y145 is the major site of Lck-catalyzed phosphorylation, it is apparent that other tyrosine residue(s) are also phosphorylated to some extent, as Lck could phosphorylate Y145F-mutated ezrin, albeit at a significantly lower level than the wild-type protein. At present, we have no evidence for Y353 as a target site for Lck, as (1) Y353 was not identified in the phosphopeptide mapping, and (2) we did not see a decrease in Lck-catalyzed in vitro phosphorylation of a double Y145F–Y353F mutant GST–ezrin (data not shown).

The cytoskeletal effects exerted by the Lck activation in early T cell stimulation are, at least partially, mediated by activation of the Rho family GEF Vav [20,21]. Whether tyrosine phosphorylation of ezrin is an alternative mechanism to regulate dynamic changes of the actin cytoskeleton, as has been suggested in EGF-, PDGF- or HGF-treated epithelial cells [6–8], is still unclear. Alternatively, Lck-induced tyrosine phosphorylation of ezrin may be linked to its other functions, including participation in signaling pathways that control proliferation and apoptosis [5].

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