

T Cell Receptor Signalling Results in Rapid Tyrosine Phosphorylation of the Linker Protein LAT Present in Detergent-Resistant Membrane Microdomains

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Stimulation of human T cell line Jurkat results in rapid tyrosine phosphorylation of a 35-38 kDa protein that is found in large and buoyant detergent-resistant membrane microdomains containing also glycosylphosphatidylinositol (GPI)-anchored proteins, glycolipids and Src-family protein tyrosine kinases ("GPI-microdomains"). The pp35-38 was found to be identical to LAT, a recently cloned key component of the T-cell receptor signalling pathway. Moreover, a modified form of protein tyrosine kinase Lck (pp60) was newly detected in the GPI-microdomains of the anti-CD3-stimulated Jurkat cells. These data support the idea that GPI-microdomains play important roles in immunoreceptor signalling. © 1998 Academic Press

Engagement of the T-cell antigen receptor (TCR) complex by natural ligands (complexes of MHC proteins with peptides on the surface of antigen presenting cells) or by suitable antibodies triggers signalling cascades resulting either in cell division and differentiation, or initiation of effector functions or even in functional silencing or apoptotic death, depending on the nature of the cell and on circumstances (e.g. co-operation with signals from other receptors) (1, 2). The earliest events initiating the complex signalling pathways are based on activation of two protein tyrosine kinases (PTK) of the Src family, Lck and Fyn. These enzymes appear to phosphorylate tyrosine residues in the ITAM

motifs characteristic for the intracellular tails of CD3 chains (ϵ , γ , δ , ζ) present in the TCR complex. Phosphorylated ITAMs then serve as binding sites for the tandem SH2 domains of ZAP-70, a Syk family PTK. ZAP-70 bound in this way to the TCR/CD3 complex is then activated by tyrosine phosphorylation (probably mediated by Lck or Fyn) and phosphorylates further downstream substrates. The cytoplasmic substrates of the active Src and Syk family kinases include further enzymes (PLC γ , PI3-K) as well as several other regulatory proteins (e.g. Grb2, Vav, SLP-76, Cbl, Grap, SOS). Recently an important substrate of the activated ZAP-70, p36-38, was cloned and named LAT (linker for activation of T cells) (3). This transmembrane protein has a very short extracellular domain (4 amino acids) and the intracellular domain contains 10 tyrosine residues which, after phosphorylation by ZAP-70 and Syk PTKs, bind to SH2 domains of several proteins such as Grb2, Grap, PI3-K and PLC- γ 1. Overexpression of a LAT mutant lacking a critical tyrosine residue inhibited TCR signalling, indicating the critical importance of this protein (3).

Plasma membranes of many cell types, including T cells, appear to contain specific areas (microdomains) rich in glycosphingolipids, cholesterol and GPI-anchored proteins but poor in phospholipids and most transmembrane proteins (4, 5). Importantly, these microdomains (to be called here "GPI-microdomains") are also rich in PTKs of the Src family, and they contain trimeric G-proteins and other so far poorly identified cytoplasmic proteins (6-8). The GPI-microdomains are relatively resistant to solubilization at low temperature by some detergents and due to their low buoyant density can be isolated by density gradient ultracentrifugation (9). Their existence seems to explain conveniently the somewhat paradoxical signalling capacity of GPI-anchored proteins and glycolipids upon their cross-linking by antibodies (10). However, the biological role

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Abbreviations: CIAP, calf intestinal alkaline phosphatase; GPI, glycosylphosphatidylinositol; mAb, monoclonal antibody; PI3-K, phosphatidylinositol 3-kinase; PLC, phospholipase C; pp, phosphoprotein; PTK, protein tyrosine kinase; TCR, T-cell receptor.

of these Src-family PTK rich membrane specializations has remained unclear. Recently, it has been suggested that they could play a role in immunoreceptor signaling. According to this idea, immunoreceptors aggregated as a result of interactions with their natural ligands or antibodies may associate with the GPI-microdomains and the PTKs contained in them can phosphorylate the ITAMs within the immunoreceptor complexes (11, 12). In the present study we sought support for this attractive idea by examining whether some components of the GPI-microdomains become rapidly tyrosine phosphorylated upon TCR crosslinking in Jurkat cells.

MATERIALS AND METHODS

Reagents, cells and antibodies. Chemicals used and luminography films were from Sigma (St. Louis, MO), anti-Ig peroxidase conjugates from Bio-Rad (Hercules, CA), enhanced-chemiluminescence Western blotting kit from Amersham Buchler (Braunschweig, FRG), nitrocellulose membranes from Schleicher and Schuell (Dassel, FRG). CIAP was from USB (Cleveland, OH). Jurkat cells were from ATCC. MAbs to human proteins CD3 (MEM-92; IgM and MEM-57; IgG2a), CD53 (MEM-53; IgG1), CD59 (MEM-43; IgG2a and MEM-43/5; IgG2b) and to phosphotyrosine (P-TYR-01 and P-TYR-02; both IgG1) were previously produced and characterized in our laboratory. Rabbit antiserum to LAT (3) was kindly provided by Dr. L. Samelson, rabbit antisera to Lck and Fyn by Dr. A. Veillette, rabbit antibodies to Lnk (provided by Dr. I. Štefanová), and G_{β} were from Santa Cruz Biotechnology (Santa Cruz, CA).

Jurkat cell stimulation and solubilization. Stimulation of tyrosine phosphorylation in Jurkat cells was performed as described before (13) using the IgM CD3 mAb MEM-92 at approx. concentration of 50 μ g/ml. The cells were solubilized for 30 min. at 0°C in lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 8.2, 5 mM iodoacetamide 1 mM Pefabloc, 2 mM EDTA, 1 mM Na_3VO_4 and 1% NP40), and the suspension (including insoluble nuclei and cytoskeleton) was used for density gradient ultracentrifugation.

Density gradient ultracentrifugation. The detergent cell lysate was adjusted to 40% (w/v) in sucrose, 1 ml of it was overlaid by 3.5 ml of 30% sucrose in the lysis buffer and 0.5 ml lysis buffer and ultracentrifuged at 250 000xg for 18 h at 2°C. Afterwards, the gradient was separated into fractions that were used for further work.

Dephosphorylation. The GPI-microdomains obtained by density gradient ultracentrifugation were transferred (by gel filtration) into the phosphatase buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 1% Triton X-100), 15 units of CIAP were added to 50 μ l of this solutions and incubated 1 h at 37°C.

Immunoisolation. The GPI-complexes were immunoisolated from the NP40 lysate of the stimulated cells using the solid-phase immunoisolation technique as described before (13). Alternatively, the buoyant density gradient fractions containing the GPI-complexes were slowly filtered through a 100 μ l column of an anti-phosphotyrosine immunosorbent (P-TYR-01 mAb coupled to CNBr-activated Sepharose 4B); the column was washed with 10 volumes of the lysis buffer and the specifically bound phosphoproteins were eluted by 0.1 M glycine-NaOH buffer pH 11.5.

Analytical methods. SDS PAGE (in the Laemmli buffer system) and Western blotting were performed in standard way using non-reduced samples, semi-dry blotting on nitrocellulose and indirect immunoperoxidase staining with luminographic detection.

RESULTS

Tyrosine-Phosphorylated Proteins in the Buoyant GPI-Microdomains

The most striking change in tyrosine phosphorylation of Jurkat cell proteins elicited by anti-CD3

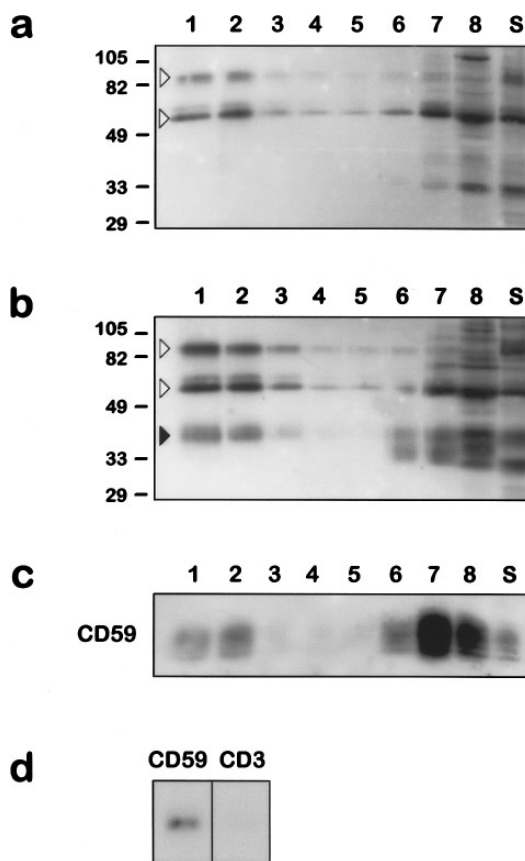


FIG. 1. Phosphoproteins in the buoyant GPI complexes. (a) Detergent extract of unstimulated Jurkat cells was fractionated by sucrose density gradient ultracentrifugation and the fractions (numbering: fraction 1, the top of the gradient; fraction 9, the bottom; S, the sediment) were analyzed by SDS PAGE (under non-reducing conditions on a 10% gel) followed by electroblotting and anti-phosphotyrosine immunostaining (mixture of P-TYR-01 and P-TYR-02). The arrowheads indicate the major phosphoproteins present in the buoyant fractions corresponding to the GPI-complexes. Positions of the M_r standards (in kDa) are indicated at the left margin. (b) As in (a) but using Jurkat cells stimulated for 1 min. by the anti-CD3 mAb. Note the third major phosphoprotein (pp35-38) detected in the buoyant fractions (marked by the closed arrowhead). The profile of the phosphoproteins in these fractions was identical whether non-reduced or reduced samples were used (not shown). (c) Distribution of a GPI-anchored protein, CD59, in the same fractions shown in (a) (detected by Western blotting using mAb MEM-43/5). Only the relevant part of the blot (around 20 kDa) is shown. (d) Co-isolation of pp35-38 on the CD59 immunosorbent (immobilized mAb MEM-43) as compared to a negative control (CD3; mAb MEM-57) immunosorbent. Only the relevant part of the blot (around 30-40 kDa) is shown; immunostained by P-TYR-01.

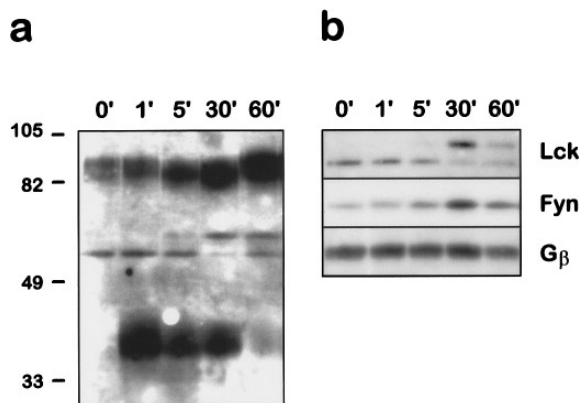


FIG. 2. Kinetics of the changes detected in the buoyant fractions (combined fractions corresponding to 1 and 2 in Fig. 1a, b) following anti-CD3 Jurkat cell treatment. (a) Detection of tyrosine-phosphoproteins. Duration of the treatment in min. is indicated at the top; immunostaining as in Fig. 1a, b; positions of the M_r standards are shown at the left margin (kDa). (b) The same samples as in (a) but immunostained with antibodies to Lck, Fyn and β subunits of trimeric G-proteins (equal loading control). Only the relevant parts of the blots are shown.

mAbs was rapid phosphorylation of a 35-38 kDa pro savedYYtein. Density gradient ultracentrifugation of detergent (NP-40) cell lysates revealed that a significant fraction of this phosphoprotein was found in large, buoyant complexes containing also a fraction of the GPI-anchored protein CD59, a typical marker of the GPI-microdomains (Fig. 1c). The pp35-38 copurified with CD59 from NP40 lysate of the anti-CD3 activated Jurkat cells on a CD59 immunosorbent (Fig. 1d) confirming that it is a component of the GPI-microdomains.

It should be noted that two tyrosine-phosphoproteins (pp55/60, pp80-80) were observed as constitutive components of the buoyant GPI-microdomains in unstimulated Jurkat cells; the intensity of their tyrosine phosphorylation did not change dramatically upon anti-CD3 treatment (Fig. 1a, b). Tyrosine phosphorylation of the pp35-38 was characterized by a rapid onset with a maximum achieved already

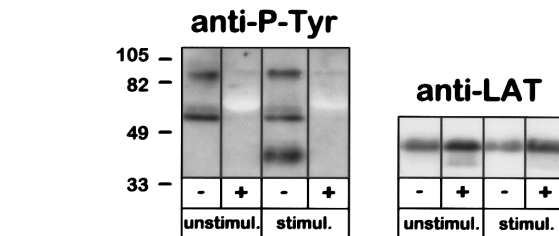


FIG. 4. Dephosphorylation of the tyrosine-phosphorylated proteins. Combined buoyant ultracentrifugation fractions (corresponding to No. 1 and 2 in Fig. 1a, b) of unstimulated and 1 min. anti-CD3-stimulated cells were mock-treated (-) or phosphatase treated (+), subjected to SDS PAGE, electroblotting and immunostaining with anti-phosphotyrosine or anti-LAT Abs.

after 1 min. followed by a slow decrease over 60 min. (Fig. 2).

Identification of pp35-38 as LAT

Rapid anti-CD3-induced tyrosine phosphorylation and M_r of the pp35-38 indicated that it could be identical to one of the two recently cloned proteins, Lnk (14) or LAT (3). While no Lnk could be demonstrated in the buoyant GPI-microdomains of the unstimulated or anti-CD3 stimulated Jurkat cells, LAT was clearly found in the GPI-microdomains before and after anti-CD3 stimulation (Fig. 3). The intensity of the LAT zone as detected by Western blotting in the buoyant fractions of the density gradient apparently decreased after anti-CD3 stimulation (Fig. 3); this could be because of adverse effect of the phosphorylation on the reactivity of the protein with the antiserum. Indeed, if the buoyant complexes obtained from the stimulated cells were subjected to dephosphorylation, the intensity of the immunostaining increased and was similar to that in unstimulated cells (Fig. 4). Interestingly, electrophoretic mobility of the LAT present in GPI-complexes was slightly different from the LAT forms found in the dense fractions of the gradient (Fig. 3). The M_r of the pp35-38 (immunostained by anti-phosphotyrosine) and that of LAT

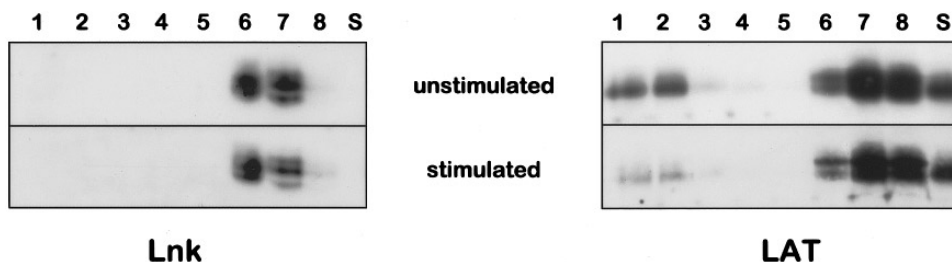


FIG. 3. Identification by Western blotting of Lnk (left) and LAT (right), respectively, in the density gradient fractions (as in Fig. 1) of unstimulated and 1 min. anti-CD3 stimulated Jurkat cells. Only the relevant parts of the blots (around 30-40 kDa) are shown.

(immunostained by anti-LAT) exactly matched (Fig. 5). Moreover, LAT could be isolated on anti-phosphotyrosine immunosorbent after dissociation of the isolated GPI-microdomains (obtained from the stimulated cells) by their incubation with 1% laurylmalto-side at 37°C (Fig. 6); however only a fraction of total LAT bound to the immunosorbent indicating that only a part was tyrosine-phosphorylated. These data unambiguously prove the presence of LAT in the GPI-microdomains of the Jurkat cells and strongly indicate that the major phosphoprotein detectable in the GPI-microdomains of anti-CD3 stimulated Jurkat cells is identical to tyrosine-phosphorylated LAT. The presence of a significant fraction of LAT in the GPI-microdomains was demonstrated also in the thymoma cell line HPB-ALL (T. Brdička, unpublished).

The Nature of the Other Phosphoproteins Detectable in the GPI-Microdomains

Another striking change in the pattern of tyrosine-phosphorylated proteins present in the Jurkat cell GPI-complexes upon the anti-CD3 treatment is the appearance of a pp60, in addition to the constitutively present pp56. The apparent M_r of pp56 and pp60 and kinetics of their changes in the GPI-microdomains upon anti-CD3 treatment exactly matched the behavior of Lck as detected by Western blotting (Fig. 1a, b).

The identity of the last major phosphoprotein component of the GPI-microdomains, pp80-85, remains so far unknown. We were unable to demonstrate the identity of this phosphoprotein with HS1, ezrin, p85 subunit of PI3-K and CD44 (J. Černý, P. Angelisová, unpublished).

DISCUSSION

In the present study we identified the recently cloned T cell protein LAT, an essential component of

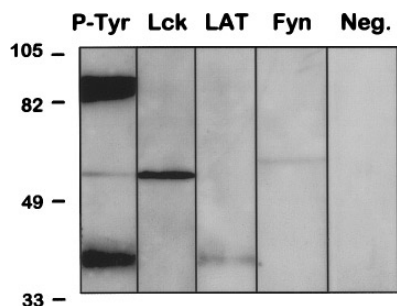


FIG. 5. Comparison of M_r of the proteins detectable in the gradient buoyant fractions of the anti-CD3 (1 min.) stimulated Jurkat cells by Western blotting. The proteins detected by immunoperoxidase staining of the blot (Neg. is a negative irrelevant control) are indicated at the top, positions of M_r standards are shown.

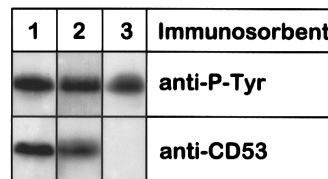


FIG. 6. Binding of LAT to the anti-phosphotyrosine immunosorbent. The buoyant GPI-complexes of the anti-CD3-stimulated (1 min.) cells were dissociated by laurylmalto-side and filtered through a minicolumn of a control (anti-CD53; MEM-53) or anti-phosphotyrosine (P-TYR-01) immunosorbent (see Materials and Methods). The original sample (1), flow-through fraction (2) and the fraction eluted by pH 11.5 (3) were subjected to SDS PAGE, electroblotting and immunostaining with anti-LAT antiserum. Only the relevant parts of the blots (around 35-40 kDa) are shown.

the early part of the TCR/CD3 signalling pathway, as a component of the GPI-microdomains of Jurkat T cells and demonstrated that it becomes rapidly phosphorylated upon anti-CD3 treatment. This finding further emphasizes the potential importance of the GPI-microdomains in TCR/CD3 signalling: in addition to the Src kinases they are now demonstrated to contain another key molecule, LAT. Moreover, the Lck PTK present in the GPI-microdomains responds to the anti-CD3 stimulation by a modification shifting its apparent M_r to approx. 60 kDa, indicating functional involvement in the process of signalling. We hypothesize that the TCR signalling is initiated by rapid attachment of the ligated TCR complex to the GPI-microdomains rich in Lck and Fyn PTKs; these enzymes phosphorylate the ITAM sequences of the CD3 chains (including ζ) which then bind the PTK ZAP-70. Activated ZAP-70 then phosphorylates the LAT present also in the GPI-microdomains and the tyrosine-phosphorylated LAT serves as a hub for assembly of several SH2-containing proteins. It will be of interest to identify the last major phosphoprotein (pp80-85) contained in the GPI-complexes which may be another important signalling molecule.

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REFERENCES

1. Weiss, A., and Littman, D. R. (1994) *Cell* **76**, 263-274.
2. Wange, R. L., and Samelson, L. E. (1996) *Immunity* **5**, 197-205.
3. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Triple, R. P., and Samelson, L. E. (1998) *Cell* **92**, 83-92.
4. Brown, D. A. (1993) *Curr. Op. Immunol.* **5**, 349-354.

5. Simons, K., and Ikonen, E. (1997) *Nature* **387**, 569–572.
6. Cinek, T., and Hořejší, V. (1992) *J. Immunol.* **149**, 2262–2270.
7. Dráberová, L., and Dráber, P. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3611–3615.
8. Parolini, I., Sargiacomo, M., Lisanti, M. P., and Peschle, C. (1996) *Blood* **87**, 3783–3794.
9. Brown, D. A., and Rose, J. K. (1992) *Cell* **68**, 533–544.
10. Robinson, P. J. (1991) *Immunol. Today* **12**, 35–41.
11. Field, K. A., Holowka, D., and Baird, B. (1997) *J. Biol. Chem.* **272**, 4276–4280.
12. Marano, N., Crawford, M., and Govindan, B. (1997) *Mol. Immunol.* **34**, 967–976.
13. Černý, J., Stockinger, H., and Hořejší, V. (1996) *Eur. J. Immunol.* **26**, 2335–2343.
14. Huang, X., Li, Y., Tanaka, K., Moore, K. G., and Hayashi, J. I. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 11618–11622.