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Biological significance of differential tyrosine phosphorylation of ITAM sequences in T cell receptor ζ subunit

Ph.D. thesis

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INTRODUCTION

T cells recognize antigen by means of membrane-bound T cell antigen receptor (TCR). The TCR consists of two structurally and functionally distinct modules: the antigen binding module which consists of the clonotypic αβ or γδ heterodimers and a signalling module composed of the CD3 γ, δ, ε and ζ/ζ or ζ/η invariant chains. None of the invariant chains possesses intrinsic enzyme activity. However, each subunit contains one or multiple copies of a recurrent cytoplasmic sequence that fully accounts for their individual signal transducing capacity. These conserved sequences referred to as the immunoreceptor tyrosine-based activation motifs (ITAMs) are found in one copy in each CD3 subunits and in three copies in the ζ chain.

The mechanisms of ITAM-mediated signal transduction have been extensively studied. Upon recognition of the peptide-MHC complex by TCR, the initial activation starts with the phosphorylation of ITAMs within CD3 and ζ chains by *Src* family kinases. Phosphorylated ITAMs recruite src-homology 2 (SH2) domain-containing signal and adaptor molecules to the TCR complex facilitating the transmission of downstream signals. It is well documented that differently phosphorylated forms of the ζ chain subunit can be found in T cells at distinct differentiation stages or activated under different conditions. However the cellular components that participates in the development of the differently phosphorylated forms of the TCR ζ chain have not been identified.

A single TCR complex contains 10 ITAMs - 3 in each of the 2 ζ chains and 4 in the CD3 subunits. Despite extensive investigation it is not yet clear whether the presence of 10 functional units in TCR is required for signal amplification or ITAM-specific functions exists.

AIMS OF THE STUDY

Our aim was to answer the following main questions:

1. What is the contribution of *Src* family tyrosine kinases and CD45 protein

tyrosine phosphatase to the generation of the differently phosphorylated forms of the TCR ζ chain?

2. Are the three ITAMs within ζ chain required to activate distinct signalling patways or they are functionally redundant?

SUMMARY OF THE RESULTS

1.Contribution of Src family tyrosine kinases and CD45 protein tyrosine phosphatase to the generation of the differently phosphorylated forms of the TCR ζ chain

In order to find out which enzymes may be involved in the generation of the various phospho-forms of the ζ chain, we determined which ITAMs, and individual tyrosine residues in the human chain can be phosphorylated *in vitro* by Lck and Fyn protein tyrosine kinases (PTKs). Because the phosphorylation status of the proteins is determined by the concerted action of kinases and phosphatases we assessedwhether CD45 protein tyrosine phosphatase (PTPase) may contribute to the formation of ζ chain phosphorylation pattern.

O All the ITAM motifs and all tyrosine residues located within the ITAMs of the ζ chain were phosphorylated by *Src* family kinases, $p56^{lck}$ and $p59^{fyn}$ *in vitro*. In contrast, a peptide, containing a tyrosine residue located outside the ITAMs was not phosphorylated. Intensity of the phosphorylation obtained by the two enzymes was different, as $p56^{lck}$ gave much stronger signal than $p59^{fyn}$.

O We determined whether the CD45- a PTPase that acts as a positive regulator of the early events in TCR signaling- may have a substrate preferency for individual tyrosine residues within the ζ chain ITAMs. For this purpose we used mono- and biphosphorylated peptides corresponding to the ITAMs of ζ chain and short phosphopeptides corresponding to half- ζITAM sequences as substrates for CD45 *in vitro*. We found that CD45 discriminates between the individual phosphotyrosine

residues of the ζ chain ITAMs, suggesting that this phosphatase may be involved in the regulation of the phosphorylation patern of the ζ chain.

The *in vivo* contribution of the *Src* PTKs and CD45 in generation different phosphorylation forms of the TCR ζ chain should be the subject of future investigations.

2. Biological significance of differential tyrosine phosphorylation of ITAM sequences in TCR ζ chain

In order to determine whether the three ITAMs found in the ζ chain and the two *YxxL* segments within each ITAM are functionally equivalent or they are able to activate distinct signaling pathways, we introduced nonphosphorylated, mono- and biphosphorylated peptides corresponding to the ITAMs of ζ chain peptides in permeabilized cells and we determined their effect on early events of TCR signalling cascade, i.e. tyrosine phosphorylation and association of tyrosine phosphoproteins with Grb-2 adaptor molecule.

O We compared different permeabilizing reagents and permeabilisation conditions in order to find a procedure that does not affect the basal level of tyrosine phosphorylation and the cell responsiveness to TCR stimulation. We found that cells permeabilized with L-α lysophosphatidylcholine under strictly controlled temperature conditions are suitable tools for intracellular tyrosine phosphorylation studies. Cell permeabilization with L-α lysophosphatidylcholine was efficient and reproducible and did not affect the cell responsiveness to anti-TCR stimulation.

O When synthetic peptides corresponding to the non-phosphorylated, monophosphorylated and biphosphorylated forms of each ζ ITAMs were introduced into permeabilized T cells, we found that differently phosphorylated ζITAMs induced phosphorylation of partially distinct substrate sets. The intensity of phosphorylation was different among ITAMs with the biphosphorylated forms of the first and second ζITAMs ($\zeta(1)y^Py^P$ and $\zeta(2)y^Py^P$) being the most potent inductors of tyrosine phosphorylation, followed by the C-terminal phosphorylated form of ITAM1 ($\zeta(1)yy^P$), the

biphosphorylated form of the third ITAM ($\zeta(3)y^Py^P$) and by the monophosphorylated forms of ITAM2 ($\zeta(2)y^Py$ and $\zeta(2)yy^P$). The monophosphorylated forms of ITAM3 ($\zeta(3)y^Py$ and $\zeta(3)yy^P$) and the N-terminal phspho-form of ITAM1 ($\zeta(1)y^Py$) showed the poorest capacity to induce tyrosine phosphorylation of intracellular proteins.

O We assesed whether the ITAM-induced tyrosine phosphorylation was mediated by one of the tyrosine kinases involved in TCR signaling. For this purpose we used cell lines deficient in one of the kinases (the JCaM 1.6. Lck-deficient variant of Jurkat and P116- a Syk/ZAP deficient variant of Jurkat) and also we determined the effect of ITAM peptides on Lck and ZAP-70 phosphorylation.. We found significant differences in both the intensity and the pattern of ITAM-induced tyrosine phosphorylation in kinase-deficient cell lines as compared to wild type Jurkat. This indicates that the effect of phospho-ITAMs was at least partially mediated by these two kinases. Notably, the phosphorylation of a protein with molecular weight (MW) of 150 kDa could not be induced in any of the kinase deficient cell lines. Although we have not identified this protein, according to its MW it can be phospholipase Cγ whose tyrosine phosphorylation requires both $p56^{lck}$ and *Syk* family PTKs activity. Furthermore, the phospho-ITAMs had different abilities to enhance tyrosine phosphorylation of Lck and ZAP-70 kinases in Jurkat T cells. Since the activity of these kinases is controlled by tyrosine phosphorylation, it is possible that the ITAM-induced increase in tyrosine phosphorylation of these kinases up-regulated their activity. Further investigations are necessary to confirm this hypotesis.

O We assesed the involvement of protein tyrosine phosphatases (PTPs) in the phospho-ITAM induced increase in the tyrosine phosphorylation of cellular proteins by stimulating the cells with phospho-ITAMs in the presence of a PTP inhibitor-phenylarsine oxide (PAO). The pattern of peptide-induced phosphorylation in the presence or absence of PAO was similar however, the relative intensity of tyrosine phosphorylation decreased in the presence of PAO. The latter finding indicated a contribution of phosphatase(s) to this process. The use of PAO at 15 mM, was previously reported to abrogate the activity of CD45 PTP-ase in Jurkat T cells (143). Since a known role of CD45 is to activate $p56^{lck}$ and $p59^{fyn}$ PTKs by dephosphorylating their negative

regulatory site, the reduction in the tyrosine phosphorylation of certain bands observed in cells stimulated with phospho-ITAM peptides in the presence of PAO might be due to a decreased activity of *Src* family tyrosine kinases.

O In order to determine the effect of phospho-ITAMs on adaptor protein mediated pathways, we analyzed whether T cell stimulation with phospho-ITAM peptides results in changes in Grb-2 association with tyrosine phosphorylated proteins. For this purpose we used Grb2-agarose beads to affinity purify Grb-2 associated proteins from lysates of phospho-ITAM stimulated Jurkat cells. We found that peptides corresponding to ITAM1 and ITAM2 of the ζ chain can either induce or enhance Grb-2 association with distinct sets of tyrosine phosphorylated proteins. In contrast the $\zeta(3)y^{P}y^{P}$ was unable to induce changes in Grb-2 association with tyrosine phosphorylated proteins. Therefore, it is possible that the third ITAM of the ζ chain is unable to couple TCR to Grb-2 mediated patways.

3. Effect of TCR ζ chain ITAM phosphopeptides on tyrosine phosphorylation in B cells

Signal transduction following ligation of TCR or BCR is mediated by common families of intracellular enzymes and signalling molecules and by conserved sequences involved in protein-protein interaction (ITAM and proline-rich motifs, SH2 and SH3 domains) (164). These similarities prompted us to determine whether the ζ chain ITAMs are functional only in T cells or they may induce tyrosine phosphorylation events in B cells as well. Similarly to T cells, the peptides corresponding to the differently phosphorylated ITAM sequences from the ζ chain increased tyrosine phosphorylation of cellular proteins in B cells in a primary structure and phosphorylation pattern-dependent manner. However, we found significant differences between the ability of the first and third ζ ITAM phosphopeptides to induce tyrosine phosphorylation in B cells as compared to T cells. These differences may result from phosphopeptide interaction with distinct sets of SH2 domains containing proteins that are selectively expressed in B cells such as the members of *Src* family tyrosine kinases Blk and Lyn and the *Tec* family tyrosine kinase Btk (53, 164).

CONCLUSIONS

⇨ All the tryrosine residues located within the three ζ ITAM sequences are substrates for *Src* family kinases Lck and Fyn suggesting that the *Src* kinases are not the only components regulating the generation of different phosphorylation patterns in the ζ chain.

⇨ CD45 PTPase can discriminate between individulal phosphotyrosine residues of the ζ chain ITAMs indicating that CD45 may be involved in the formation of the multiple phosphorylated forms of ζ chain.

⇨ Cells permeabilized with L-α lysophosphatidylcholine under strictly controlled temperature conditions are suitable tools for intracellular tyrosine phosphorylation studies.

⇨ Differently phosphorylated forms of the ζ-ITAMs are capable of inducing protein-tyrosine phosphorylation of distinct substrate sets, when introduced into permeabilized T cells. The $p56^{lck}$ and ZAP-70/Syk PTKs were involved in phospho-ITAMs induced tyrosine phosphorylation, as it has been shown in enzyme deficient cells.

⇨ The three ζ-ITAMs and their various phosphorylated forms differ in their capacity to couple TCR to downstream adaptor protein mediated signalling pathways.

⇨The first and second ITAM sequences of the ζ chain may have similar but not totally overlapping functions in TCR signalling. In contrast, the third ITAM may have distinct functions since the peptides corresponding to its different phospho-forms shows the poorest capacity to induce tyrosine phosphorylation in T cells and they are unable to induce changes in Grb-2 association with cellular phosphoproteins.

➪ There are differences between the ability of the ζ ITAM phosphopeptides to induce tyrosine phosphorylation in B cells as compared to T cells. These differences may result from phosphopeptide interaction with distinct sets of SH2 domains containing proteins that are selectively expressed in B cells such as the members of *Src* family tyrosine kinases Blk and Lyn and the *Tec* family tyrosine kinase Btk.

LIST OF PUBLICATIONS AND ABSTRACTS

Publications connected to the thesis

1. **V. Chiţu**, Z. Hegedüs, G. K. Tóth., É. Monostori, Studies on the role of TCR-ζ chain phosphorylation in T-cell receptor signalling, *1996/97 International Training Course - Proceedings of the Closing Seminar*, 18-29, (1997).

2. Tóth K. G., I. Laczkó, M. Hollosi, **V. Chiţu**, Z. Hegedüs, É. Monostori, Synthesis of phosphorylated T-cell receptor/CD3 ζ-chain sequences- conformational effects and immunological investigations. *Journal of Peptide Science, Special Issue.*4, 60 (1998).

3. Z. Hegedüs, **V. Chiţu**, G.K. Tóth., Csaba, F., Kalman, M., G. Váradi, Andó, I., Monostori, E., Contribution of kinases and CD45 phosphatase to the generation of tyrosine phosporylation patterns in the TCR-ζ chain, *Immunology Letters*, 67,31-39, (1999).

4. **V. Chiţu**, D. Demydenko, G.K. Tóth., Z. Hegedüs, É. Monostori, Conditions for permeabilization of cells used for intracellular tyrosine phosphorylation studies. *BioTechniques*, 27, 435-437, (1999).

Publications not connected to the thesis

1. **Chiţu, V.**, Veliceasa, D., Diaconu, C.C, Târdei, G., Măgureanu, C., Cernescu, C., "Studiul interactiunii antigen-anticorp folosind peptide sintetice ce mimeaza bucla V3

din glicoproteina de suprafata a HIV-1" [Studies on the antigen-antibody interaction using synthetic peptides that mimic the V3 loop of HIV-1 surface glycoprotein], *Studii si Cercetari de Virusologie*, **25**, 31 (1995).

2. Târdei, G., Ruţă, S. M., **Chiţu, V.**, Cernescu, C. E., Argumente serologice pentru definitia de caz într-o epidemie de meningoencefalita arbovirala [Serological arguments for case definition in an arboviral meningo-encephalitis epidemic], *Studii si Cercetari de Virusologie* 27:25-35, (1997).

3. Tsai, T. F., Popovoci F., Campbell G. L., Nedelcu N. I., for the Investigative Team (V. Laurenţia, L. Spăntulescu, **V. Chiţu**, S. Ruţă, G. Târdei, D. Crăciun, D. Nicolaiciuc, D. Piţigoi, C. Ceianu, G: Nicolescu, A. Ungureanu, L. A. Vladimirescu, V. Deubel, B. LeGuenno, L. Han, H. Savage, L. Tengelsen, E. A. Henchal, F. Knauert, J. A. Mangiafico, C. Rossi), West Nile encephalitis epidemic in Southeastern Romania, *The Lancet*, **352**, 767-771, (1998).

Abstracts connected to the thesis

1. Z. Hegedűs, **V. Chiţu**,. G.K. Tóth, F. Csaba, M. Kalman, G. Váradi, I. Andó, É., Monostori: Contribution of Kinases and CD45 Phosphatase to the Generation Of Tyrosine Phosporylation Patterns in the TCR-ζ Chain, *Israeli-Hungarian Workshop in Molecular Immunogenetics*, Sept. 1997, Szeged, Hungary.
2. **Chiţu V.**, G.K. Tóth, K. Székely Szűcs, Z. Hegedűs and É. Monostori: The Effect of TCR ζ Chain Derived Synthetic Phospho-Peptides on the Early Signallling Events in Permeabilized T Lymphocytes. *Annual Workshop of the Hungarian Society of Biochemistry*, May 11-14. 1998, Sarospatak, Hungary.
3. G.K. Tóth., I. Laczkó, M. Hollosi, **V. Chiţu**, Z. Hegedűs, É. Monostori, Synthesis of phosphorylated T-cell Receptor/CD3 ζ-chain Sequences- Conformational Effects and Immunological Investigations. *The 25th European Peptide Symposium*, Aug. 30- Sept 4, 1998, Budapest, Hungary.

Abstracts not connected to the thesis

4. Diaconu C. C., Măgureanu C., **Chiţu V.**, Veliceasa D., Cernescu C. E., Study of the Polyclonal Antibody- Antigen Interactions Using HIV-1 Subtype- Specific Peptides, *Fourth European Summer School of Immunology*, Sept. 1995, Prague, Czech Republic.
5. **Chiţu, V.**, Veliceasa, D., Diaconu, C.C, Târdei, G., Ruţă S. M., Cernescu, C., Variations in the Anti V3 IgG Immune Response in HIV-1 Infected Children, *FEBS Course An Introduction to Animal Cell Culture for Biochemists*, National Cell and Tissue Culture Centre, Jun. 1996, Dublin, Ireland.
6. **Chiţu V.**, Veliceasa, D., Diaconu, C.C, Târdei, G., Magureanu, C., Cernescu, C.,: The Longitudinal Study on the Distribution of Different HIV-1 Subtypes in Bucharest, *The Second National Congress on HIV and AIDS Infection with International Participation*, 20-23 Oct. 1998, Bucharest, Romania.
7. Diaconu C. C., **Chiţu V.**, Târdei G., Bleotu C., Ruţă S. M., Grancea C., Zaharia C., Cernescu C. E., Quantitative semiautomated fluorescence-based assays for biological evaluation of new anti-HIV agents, *Future Trends in Quantitative Cytology for Clinical and Research Applications*, Hortobagy-EPONA, Hungary, May 1999.

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