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

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Contents

1. Preface

The phrase "T cell activation" usually refers to the immune activation of mature T lymphocytes in peripheral blood, lymphatics, or tissue. During immune activation T cells undergo a sequence of genetic and phenotypic changes, which results ultimately in T cell clonal expansion and in the induction of T cell effector function. The physiological stimulus that activates T cells is foreign antigen in association with the major histocompatibility molecules, presented to the T cell by another cell. Multiple receptors on the surface of the T cell mediate the interaction between the T cell and the antigen-presenting cell. The antigen specificity is dictated by the T cell antigen receptor (TCR).

During the past 10 years an enormous progress has been made in the understanding the mechanisms of TCR-mediated signal transduction. The catalytic event of this progress was the recognition that components of antigen and IgFc receptors contain a common sequence motif in their cytoplasmic tails. The motif, described first in 1989 by Michael Reth has come to be known as the immunoreceptor tyrosine-based activation motif (ITAM). Its predominant occurrence in the signalling subunits immunoreceptor complexes and its location in the cytoplasmic tails suggested a role in interaction with cytoplasmic signaling effectors. Recently the ITAMs have been found in invertbrate signal transducing proteins, proving the conservative nature of signal transduction pathways. To date, the mechanism of ITAM-mediated signal transduction has been revealed. Following TCR triggering the tyrosine residues within ITAMs become rapidly phosphorylated. Tyrosine-phosphorylated ITAMs recruits intracellular signalling proteins *via* their src-homology 2 (SH2) domains and a cascade of biochemical events is initiated by sequential molecular interactions.

2. Introduction

2.1. Structure of T Cell Antigen Receptor

T cells recognize antigens by means of membrane-bound T-cell receptor complex (TCR). Two distinct forms of TCR complexes- TCR αβ and TCR γδ– define the αβ and γδ T-cell lineages. The TCR complex consists of two structurally and functionally distinct modules: an antigen binding module which consits of the clonotypic αβ or γδ heterodimers and a signalling module composed of the CD3 γ, δ, ε and ζ/η invariant chains (1,2).

2.1.1. The antigen binding module

The antigen binding module consists of the clonotypic TCR αβ or TCR γδ disulphide-bound heterodimers (reviewed in 1). In the adult, most of the T lymphocytes express TCR heterodimers containing the α and β chains, whereas a minor population expresses an alternative TCR isoform consisting of γ and δ chains (1, 2).
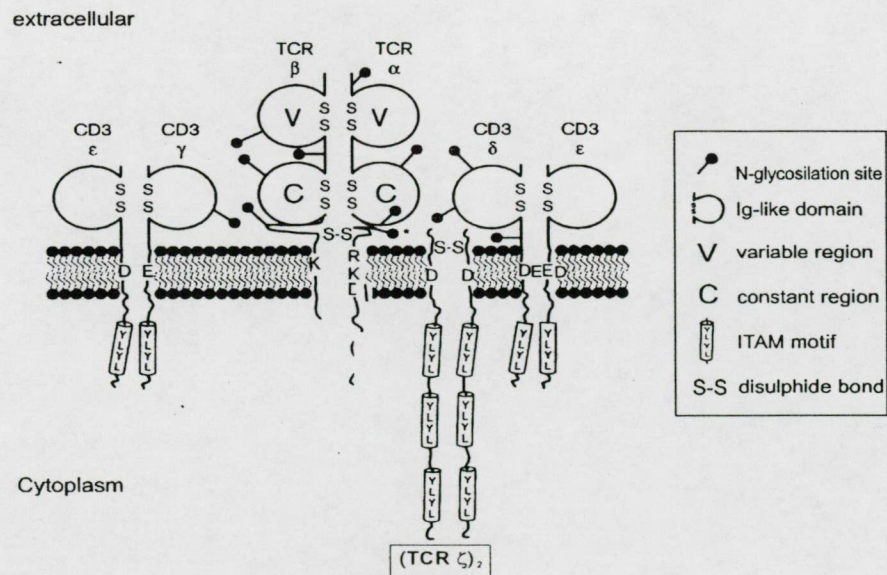

Fig. 2.1. Structure of theTCR-complex *(copied from ref. 7).*

The TCR α, β and γ, δ chains are type I transmembrane glycoproteins. Each chain comprise an amino-terminal, clonally variable (V) region, an α-helicoidal transmembrane region, and a carboxi-terminal (C) constant region (Fig. 1) (1,2). Three peptide loops homologous to immunoglobulin (Ig) complementarity-determining regions (CDRs) are found at the membrane distal end of the V regions, where they collectively form the binding site for antigenic peptides that are bound to products of the major histocompatibility complex (MHC). The Vα and Vβ CDR 3 are structurally hypervariable whereas the diversity of the Vα and Vβ CDR1 and CDR2 loops is rather limited. The C regions consist of four functional domains. The most amino-terminal domain contains two cysteine residues spaced appropriately for the formation of an intrachain disulphide bonded loop, which probably folds into a tertiary structure similar to an Ig constant region domain. A short hinge region comprises the second part of the C region and contains a cysteine residue most likely involved in the disulphide linkage of the two chains. The third part of the C region is the transmembrane domain, composed of predominantly hydrophobic aminoacid residues. An unusual feature of these transmembrane portions is the presence of a lysine residue (β and γ chains) or a lysine and an arginine residues (α and δ chains) positively charged side chains which may be crucial for the noncovalent association with the negatively charged residues found in the transmembrane portions of the CD3/ζ polypeptides (Fig. 1). The carboxy terminal part of the C region forms a short (5-12 aminoacids) cytoplasmic tail, that is unable to couple the extracellular part to the intracellular signalling machinery.

2.1.2. Signalling module components

The components of the TCR signalling module are the CD3 and ζ/η invariant polypeptides (1-4, 7). The CD3 γ, δ and ε chains exist as noncovalently associated γδ and εδ heterodimers in the TCR complex (Fig. 1). In contrast the ζ chain is expressed as a disulphide bound homodimer in 90% of TCRs or as a ζη heterodimer in the remaining 10% of TCRs (1,2).

Unlike the antigen binding subunits, these polypeptides have longer cytoplasmic tails (44 - 155 aminoacids) that are responsible for coupling the antigen-binding TCR αβ or γδ heterodimers to intracellular signalling pathways. Each CD3 or ζ/η subunit contains one or multiple copies of a recurrent cytoplasmic sequence (3, 4). These conserved

sequences, referred to as the immunoreceptor tyrosine-based activation motifs (ITAMs), are defined as two exactly spaced YxxL sequences (YxxL/Ix$_{6-8}$YxxL/I; in a single-letter amino acid code, where x denotes nonconserved residues) (3,4). The motif is found the signalling components of other immunoreceptor complexes such as the B-cell receptor (BCR) subunits Igα and Igβ, and the Fc receptors (FcRs) (Fig.2.2) (5).

Recently the ITAMs have been found in invertebrate proteins such as the A74 protein, a transmembrane signal transducing molecule involved in the cellular immune defense reactions in *Halocynthia roretzi*,, suggesting the conservative nature of signal transduction pathways throught the evolution (6). Furthermore, the motif is also found in certain viral proteins, including bovine leukemia virus gp30, Epstein-Barr virus proteins LMP2A and EBNA2 and simian immunodeficiency virus protein Nef, suggesting that these molecules could provide a signaling function in viral pathology (8).

	Sequence	Chain
TCR	DK-QTLLPNDQLYQPLKDRED-DQYSHL	CD3γ
	DT-QALLRNDQVYQPLRDRDD-AQYSHL	CD 3δ
	ER-PPPVPN-PDYEPIRKGQR-DLYSGL	CD3ε
	EPPAYQQGQNQLYNELNLGRR-EEYDVL	ζ(1)
	KPRR-KNPQEGLYNELQKDKMAEAYSEI	ζ(2)
	ERRRGK-GHDGLYQGLSTATK-DTYDAL	ζ(3)
BCR	DAGDEY-EDENLYEGLNL-DDCSMYEDI	Igα (MB1)
	DSKAGM-EEDHTYEGLDI-DQTATYEDI	Igβ (B29)
FcR	AAITSYEKSDGVYTGLSTRNQ-ETYETL	FcεRIγ

Fig. 2.2. ITAMs in the mammalian antigen and Fc receptors. The consensus residues are indicated by boxes.

a) The CD3subunits

The CD3 complex comprises three distinct members that are the 25-28 kDa glycosylated γ chain, a 20 kDa glycosylated δ chain, and a 20 kDa nonglycosylated ε chain (1) (Fig. 2.1.). The invariable γ, δ, and ε genes are members of the immunoglobulin supergene family. They are highly homologous to each other and are found in a cluster on human chromosome 11 (1, 2, 7). The γ, δ and ε chain proteins each include an N-terminal extracellular region with a single Ig-like domain, a short connecting sequence, a transmembrane segment, and a cytoplasmic tail (Fig. 2.1.).

There is no variability or polymorphism identified in the extracellular domains of the CD3 proteins or their genes, and therefore it is not likely that these proteins contribute to antigen recognition (2). The transmembrane segments of all three chains contain a negatively charged aspartic acid residue (Fig. 2.1.). This unusual feature may be important

for the physical association or functional interactions of the CD3 proteins with the TCR α and β chains, since the latter polypeptides each contain a positively charged lysine residue in their transmembrane domains (2, 7). The cytoplasmic domains of the CD3 subunits range from 44 to 81 aminoacid residues and each contains one copy of an ITAM motif (2, 3).

b) The ζ chain

The ζ chain is a 16 kDa nonglycosylated polypeptide that belongs to a family of structurally and functionally related molecules that includes ζ, η (an alternatively spliced form of ζ) and FcεRIγ (2, 7). The ζ gene is found on the human chromosome 1, separately from other TCR and CD3 genes. The ζ and η chains are structurally different from the CD3 proteins (2). Unlike the other CD3 proteins, the ζ gene products do not belong to the IgG superfamily. They have a very short extracellular part (nine aminoacids) and long cytoplasmic tails (113 aminoacids and 155 aminoacids for ζ and η respectively) (2,7). The transmembrane domains contain a negatively charged aspartic residue (similar to the CD3 chains). Both ζ and η chains have multiple ITAMs in their cytoplasmic tails (two for η and three for ζ chain). In addition, the ζ chain contains a GTP/GDP binding site that may also have a role in signal transduction (9).

2.1.3. Composition and membrane organization of the TCR

Currently, it is accepted that the T cell receptor is composed of at least six different polypeptide chains consisting of the clonotypic heterodimer ($\alpha\beta$ or $\gamma\delta$), the noncovalently associated CD3 chains (CD3γ, δ, ε) and the ζζ or ζη dimers. The exact number of subunits setting up one TCR molecule is still not well established, however, several models have been proposed for the composition of the TCR.

a) the heptameric model (10) predicts the presence of a TCR$\alpha\beta$ hetrodimer associated with a CD3$\gamma\delta\varepsilon$ complex and a ζζ or ζη dimer .

b) the octameric model (TCR$\alpha\beta$/CD3$\gamma\varepsilon\delta\varepsilon$/ζζ) has replaced the heptameric model when it was demonstrated that two CD3ε chains are present in the TCR complex (11-13). The model predicts that the $\gamma\varepsilon$ dimer is associated with the β chain and the $\delta\varepsilon$ dimer is associated with the α chain. This model cannot explain how the ζ_2 dimer is associated with the TCR.

c) The decameric model (14) has been recently proposed for TCR based on the finding that the variable domain of the α chain crystallized as a dimer of two Vα homodimers (14, 15). The decameric model predicts that the TCR/CD3/ζ complex is bivalent. The bivalent decamer comprises two TCRαβ heterodimers with a net transmembrane charge (TmC) of +6, a single CD3γε module (TmC= –2), a single CD3δε module (TmC= –2), and a single ζζ or ζη dimer (TmC= –2). Thus organized, the complex has a thermodinamically favourable neutral TmC which is probably a requirement for the stabilization of the TCR complex in the hydrophobic transmembrane region.

2.2. T Cell Antigen Receptor Function

2.2.1. Antigen recognition by TCRs

Most of the αβ T cells recognize antigens as small peptide fragments bound to major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs) (16-22). Recently, it has been shown that αβ T cells can also recognize non-protein antigens such as lipids and lipoglycans of the bacterial cell wall, presented in association with the MHC protein CD1 (22- 24). In contrast, γδ T cells appear to recognize both peptidic and non-peptidic antigens in a TCR-dependent but MHC-independent manner (25, 26).

2.2.1.1. Antigen recognition by αβ-TCR

The primary function of αβ T cells is to recognize the presence of pathogens within the body and to activate their disposal either directly or by recruiting other immune cells (16). Most of the αβ T cells recognize antigens as small peptide fragments bound to major hisocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs). There are two distinct classes of MHC molecules (16). Class I molecules are expressed on almost all nucleated cells and present antigens derived from cytoplasmic peptides like cytoplasmic and nuclear proteins of viral origin or proteins from intracytoplasmic bacteria and class II molecules are expressed on professional APCs and that present peptide antigens derived from extracellular sources (16). Class I molecules

present antigen to cytolytic $CD8^+$ T cells that become activated and directly eliminate the presenting cell *via* apoptosis induction, while the recognition of complexes of antigenic peptides with MHC class II molecules results in the activation of both the APC and of $CD4^+$ T helper cells. Activation of T helper secrete cytokines that stimulate the macrophages to kill phagocytosed micro-organisms, or activate B cells and drive their differentiation into antibody secreting cells, or help cytotoxic T cells to kill target cells (16).

The way how αβ TCR recognizes its peptide/MHC ligand was postulated from the analysis of the CDRs of the antigen binding subunits (17-21). The CDR1 and CDR2 loops are relatively conserved and are restricted in size and conformation suggesting a predominant role in binding to the MHC molecules. In contrast, CDR3 loops are hypervariable and have been predicted to be involved in peptide recognition. Mutagenesis studies supported the previous predictions that CDR 1 and 2 of both α and β chains are involved in binding to the MHC molecule whereas CDRs 3 are critical for the recognition of the peptide. The precise orientation between a MHC- peptide complex and the TCR has been recently established when the high-resolution crystal strucure of a human TCR-MHC-peptide complex was reported (reviewed in 20). In this structure, the CDR1 and CDR3 of both α and β chains interact with the peptide. All three CDRs of the α chain and CDR3 of the β chain interact with the MHC molecule.

The αβ T cells can recognize also nonprotein antigens such as lipids and lipoglycans of the bacterial cell wall, presented in association with other MHC gene products - the CD1 molecules (22-24). Such αβ T cells have either double negative, $CD4^-$ $CD8^-$ or single positive, $CD4^-CD8^+$ phenotype in humans.

2.2.1.2. Antigen recognition by γδ-TCR

The γδ T cells apparently share a propensity with macrophages to recognize nonpeptidic molecules of the kind most commonly associated with micoorganisms, viruses and stressed cells but not by normal cells (25, 26). With few exceptions, recognition of these antigens by γδ T cells involves the TCR but does not require APCs to express MHC gene products or to have a functional antigen processing machinery (26). Functionally, γδ T cells appear to posses intrinsic cytolytic activity against transformed cells of various

origins and to provide signals that can significantly influence the quality of innate as well as aquired immune reponses (26). In other instances, γδ T cells may participate in non-immune functions aimed at preserving or re-establishing tissue integrity (26).

2.2.2. Mechanism of transition from antigen binding and recognition to cell activation

The sensitivity of antigen-specific response by T cells is remarkable. However, *in vitro* the TCR αβ interaction with MHC-antigenic peptide complex is characterized by low affinity and high off rate (27-29). To explain this high-sensitivity-low-affinity paradox several models for T cell activation have been proposed.

The first model predicts that following the engagement, TCR molecules oligomerize at the plane of the cell-cell contact (28-31). Receptor clustering could favour sustained signalling in three ways (32):

1) by increasing the likelihood of contacts between the TCR and the MHC-bound ligand; the increased concentration of TCR molecules in a high-density zone would allow low-affinity receptors to initiate and maintain signals;

2) by increasing the concentration of cytosolic signalling molecules and second messengers at regionally organized focal points in the proximity of TCRs;

3) by excluding negative regulatory molecules such as phosphatases from the zone of antigen receptor signalling.

The degree of assembly of the signalling complex determines the extent and qualitative nature of the transduced signal (33).

The second model predicts that under physiological conditions T cells are triggered by sequential monovalent engagement of many TCRs by a small number of peptide-MHC complexes (34-36). This allows accumulation of TCR-activated signalling molecules over time and cell activation. The model is sustained by time-lapsed microscopy which reveals that the T-cell -APC interaction is a dynamic process, with the T cells "crawling" on the surface of the APC (37).

2.2.3. Signal transduction *via* T cell receptor

Following antigen recognition, T cell receptor signalling is the next decisive factor in the initiation of the specific immune response. The capacity of the TCR to transduce signals across the membrane is mediated by the ITAM sequences that are located in cytoplasmic regions of the TCR invariant chains (3, 7, 38- 40).

The initial membrane proximal event triggered by the TCR is activation of protein tyrosine kinases (PTKs) with the resultant phosphorylation of ITAMs and other cellular proteins (41, 42). The function of the PTKs pathway is obligatory for the T cell activation as proved by the inhibitory effects of tyrosine kinase inhibitors on TCR induced IL-2 production (43). This biochemical response couples the TCR to a divergent array of signal trasduction molecules including Src, Syk and Tec family tyrosine kinases (41, 44), enzymes that regulate lipid metabolism and Ca^{2+} mobilization (45), serine/threonine kinases (45), GTP binding proteins (46), and adaptor molecules (47). Several transcription factors are subsequently activated which ultimately results in modulation of T cell surface phenotype, the regulation of the secretion of critical cytokines and clonal expansion (48).

2.2.3.1. Activation of S*rc* family PTKs

The earliest biochemical response elicited by the TCR is activation of Src family nonreceptor PTKs. The Src family of PTKs consists of nine closely related members, each of which contains an amino-terminal myristilation site (Src homology 4 domain, SH4), an amino-terminal segment specific to the particular member, a proline rich domain -binding region (SH3), a phosphotyrosine-binding domain (SH2), a catalytic domain (SH1) and a carboxy-terminal tail (Fig 2.3.) (49).
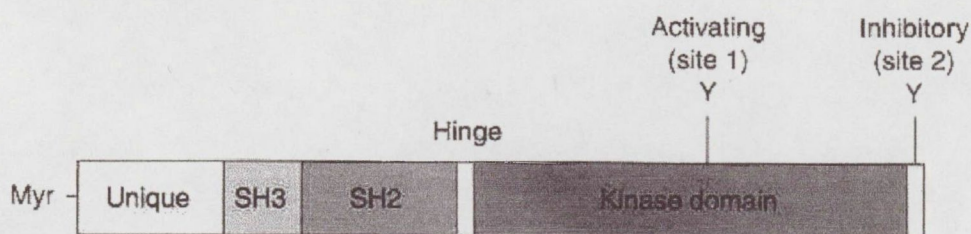
Fig. 2.3. Schematic structure of Src- family tyrosine kinases. Myr: myristilation; SH: Src homology domain; Y: tyrosine residue (*copied from ref. 54).*

Two src tyrosine kinases have been implicated in T-cell activation: p59^{fyn} which associates with the cytoplasmic domain of the ζ chain of the TCR, and p56^{lck} which couples to the CD4 and CD8 molecules and therefore is in close proximity to the TCR during antigen recognition (50-53).

Src kinases have two principal regulatory tyrosine phosphorylation sites: one is located within the SH1 domain and the other is located in the carboxy terminal region (Fig.2.3.). The crystal structure of active Lck indicates that phosphorylation within the kinase domain enhances kinase activity by correctly positioning a loop within the catalytic domain for optimum phosphotransferase activity (Fig.2.4.) (54-56). Phosphorylation of the C-terminal inhibitory site inhibits kinase activity because this phosphotyrosine residue forms an intramolecular association with the SH2 domain. This induces a conformation with low catalytic activity, stabilized by an interaction between the SH3 domain with a segment within the hinge region that joins the kinase domain and the SH2 domain (57).
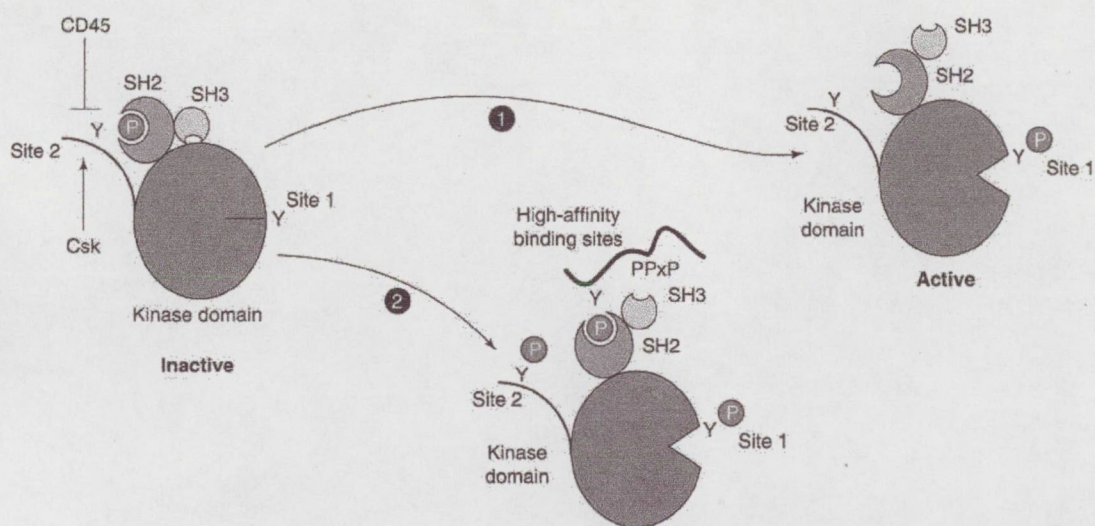
Fig. 2.4. Mechanisms for *Src*-family kinases activation. In resting T cells, CD45 opposes the inhibitory kinase Csk, but simultaneously dephosphorylates the site within the kinase domain, with the net effect of keeping the *Src* kinases inactive. Activation of Src kinases can be achieved by: (1) dephosphorylation of the inhibitory site (site 2) and phosphorylation of the site within the kinase domain (site 1); or (2) interaction of the kinase with high-affinity binding sites for the SH2 and SH3 domains in other proteins (*copied from ref. 54*).

Phosphorylation of the tyrosine within the kinase domain is achieved by intermolecular transphosphorylation (54), while the C-terminal phosphorylation is achieved by the C-terminal Src kinase (Csk) (Fig. 2.4.). This kinase, has a similar structure to *Src*-family members, but it is devoid of sites for lipid modification and tyrosine phosphorylation, and therefore, it is constitutively active (54).

Significantly, the intramolecular interaction between the inhibitory tyrosine phosphate and the SH2 domain of *Src* kinases is of low affinity. Furthermore, investigations on the mechanism of activation of Hck kinase revealed that the SH3 - catalytic domain interaction may be more important than the SH2-tail interaction in maintaining the repressed form, since high-affinity ligands for the SH3 domain are more potent than SH2 ligands in activating kinase (57, 58). Thus, a possible mechanism for activation of *Src*-family kinases is the binding of the SH2 or SH3, or both, domains to high affinity sites in other molecules.

Another model for activation of *Src*-family kinases involves the protein tyrosine phosphatase CD45 (reviewed in ref. 59). According to this model, in resting T lymphocytes CD45 is constitutively active and dephosphorylates both the
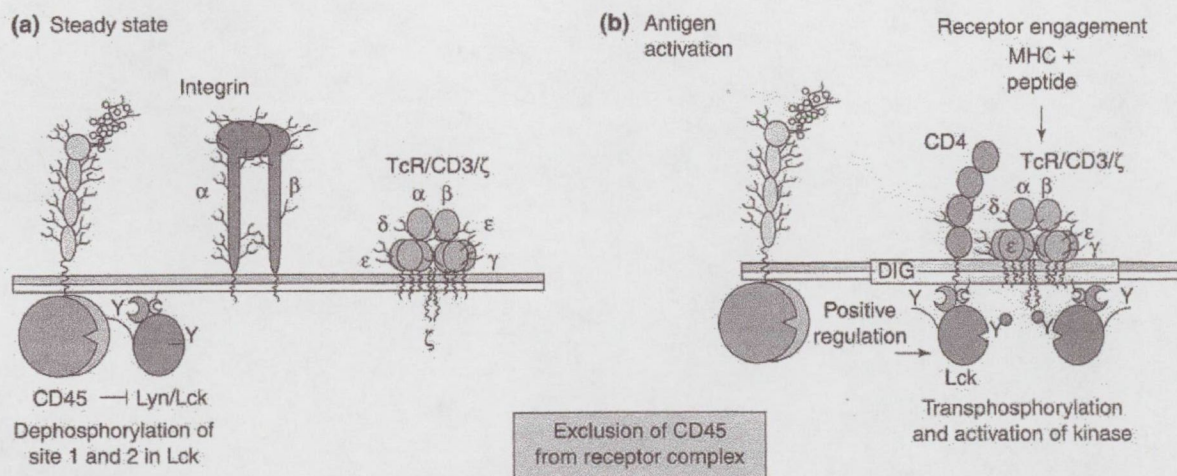
Fig. 2.5. Model for TCR activation. (a) In resting cells (steady state), various molecules are distributed randomly in the cell membrane. Src-familly kinases are under continual regulation by two active enzymes, CD45 and Csk. CD45 simultaneously dephosphorylates the inhibitory site of Lck and/or Fyn (on the left of the molecule) and the autophosphorylation site (on the right of the molecule) with the net effect of keeping Src kinases inactive. (b) Antigen receptor engagement leads to the formation of glycosphingolipid-enriched domain (DIG). This results in the selective recruitement of Src kinases to the TCR and in the exclusion of CD45. Because the inhibitory site within Src kinases is already dephosphorylated, transphosphorylation occurs leading to kinase activation (*copied from ref. 54*).

autophosphorylation and negative regulatory tyrosine residues of the Src family PTKs keeping therefore the Src kinases inactive (60) (Fig.2.5. a). After TCR stimulation, Src-family kinases, CD4, CD8 and LAT are recruited at the apposition of the membranes. CD45 is sequestered from the developing signalling complex and the transphosphorylation and kinase activation is allowed (Fig.2.5. b) (54).

The importance of *Src* family kinases Lck and Fyn in TCR signalling was revealed by studies using mutant T cell lines, or by gene targeting in mice (reviewed in ref. 53). Following activation of *Src* kinases, the CD3 and ζ chain ITAMs are rapidly tyrosine phosphorylated and become docking sites for SH2-domain containing effector and adaptor molecules involved in TCR signalling cascade. Studies of the JCaM 1.6 -a mutant Jurkat leukaemic T cell line deficient in Lck- showed a dramatic decrease in TCR-induced phosphorylation of ζ chain and of ZAP-70, and led to the proposal that TCR signalling is initiated by Lck activation (53). Accordingly, in Lck-deficient mice the basal phosphorylation of ζ and the TCR-induced phosphorylations of CD3 δ and ZAP-70 were greatly reduced, though not completely abrogated suggesting that in the absence of Lck another kinase may partially take over its function (53). The obvious candidate for this is the Fyn kinase which, like Lck is activated upon TCR stimulation. The idea that Fyn and Lck may have overlapping functions in TCR signalling comes from analysis of Lck$^{-/-}$Fyn$^{-/-}$ double mutants. In Lck$^{-/-}$ mice, thymocyte development is partially blocked at the CD4$^-$ CD8$^-$ double negative to CD4$^+$CD8$^+$ double positive transition. In contrast, in Lck$^{-/-}$Fyn$^{-/-}$ mice development is completely arrested at the double negative stage, demonstrating that Fyn can partially substitute for Lck (53).

Recent data demonstrates a functional role for Lck beyond ITAM phosphorylation and activation of ZAP-70 and Syk PTKs (61, 62). The expression of constitutively active Syk family PTKs in the p56lck-deficient JCaM 1.6 cells failed to rescue signalling events downstream of tyrosine phosphorylation, demonstrating a functional role for Lck beyond the activation of ZAP and Syk (61). Furthermore, co-expression of Syk and Lck, or TCR activation (which leads to Lck activation) in cells expressing Syk, results in enhanced phosphorylation of Shc, suggesting a synergism between the two kinases (62).

2.2.3.2. ζ-ITAMs phosphorylation and their role in the formation of the signalling complex

The phosphorylation of the CD3 and ζ ITAMs is mediated by activated $p56^{lck}$ and $p59^{fyn}$ (41, 42, 45, 63).

A specific order of ζ chain phosphorylation has recently been described using site-specific anti-phosphotyrosine antibodies (63). Phosphorylation of the membrane-proximal ITAM precedes phosphorylation of the other two ITAMs (63). The differently tyrosine phosphorylated ζ chains migrates forms in SDS-PAGE as several distinct molecular species with molecular mass of 18, 21 and 23 kDa, which represent different phosphorylation of the six tyrosine residues in the three ITAMs (referred to as A1, A2, B1, B2, C1 and C2) (63, 64). The pp21 phosphorylation is due to A2, B1 and C2

Fig.2.6. Structure of SH2-domain containg adaptor and effector molecules expressed in T cells.

phosphorylation, while pp23 is represented mainly by the fully phosphorylated form but may also contain incompletely phosphorylated ζ species (63). In resting T cells the nonphosphorylated 16 kDa ζ chain (p16) and the partially phosphorylated pp21 forms are found (64). Upon TCR stimulation the phosphorylation of p16 and pp21 rapidly increases and multiple phospho-species of ζ with molecular masses of 18, 21 and 23-kD appear (63, 64). The ratio of pp21 and pp23 can be altered after stimulation of T cells with suboptimal ligands, suggesting a discriminatory role of ζ phosphorylation in T-cell activation (63, 65-67).

The mechanism that conducts the generation of various phosphorylated forms *in vivo* has not been established. Once phosphorylated, ITAMs act as high affinity docking sites for the SH2 domains found in certain intracellular proteins with enzyme activity

(effector molecules) and in proteins that posses no intrinsic enzymatic function, but mediate protein-protein interactions (adaptor molecules), coupling therefore TCR to the intracellular signal transduction pathways (68-79) (fig.2.6.)

2.2.3.3. Specificity and multiplicity of ζITAMs

The requirement for multiple ITAMs within the ζ chain may be explained by two hypothesis:

a) individual ITAMs may function as docking regions for distinct effector molecules in order to activate distinct signalling pathways (the 'specificity model')

b) the presence of multiple ITAMs may provide a mechanism for signal amplification, with different activation thresholds required for distinct functions (the 'threshold model').

There are experimental evidences supporting both alternatives.

The hypotesis that ITAMs are involved in signal amplification (the 'threshold model') is supported by experimental data which prove that:

1) In Jurkat T cells the stimulation of chimeric receptor complexes containing one, two or three ζ ITAM motifs resulted in the induction of identical patterns of tyrosine-phosphorylated proteins. Furthermore, enhanced but not qualitatively distinct responses were seen when the chimera contained multiple ITAMs (3);

2) Expression of truncated ζ chain lacking all ITAMs or of ζ chain containing only the ζ1 or ζ3 ITAM in ζ-/- knock-out mice reconstitutes normal thymic development and activation of TCR (80);

3) Analysis of ζ deficient mice that had been reconstituted with either full lenght or single ITAM containing ζ subunits revealed that similar signalling events occur after TCR ligation regardless the number of ζ-ITAMs present in the TCR complex (81);

4) The induction of cytoskeletal rearrangements by TCR can be mediated by any of the ζ or CD3ϵ ITAMs (82).

The assumption that the presence of three ITAMs in the ζ chain is required for the formation of qualitatively distinct intracellular signals ('the specificity model') is supported by the following experimental data:

1) TCR agonists and antagonists initiate different patterns of ζ chain phosphorylation, resulting either in T cell activation or anergy (63, 65-67);

2) The three ITAMs of the TCR ζ chain differ in their ability to associate with intracellular SH2 domain containing proteins *in vitro* (68). Moreover, the same ITAM may interact with different proteins when it is found in a mono- or bi-phosphorylated form (69-75) (Tab.2.1.);

Table 2.1. Qualitative and quantitative differences in the ability of differently phosphorylated ζ-ITAM peptides to interact with T-cell adaptor and signaling proteins. The relative intensity of the interaction is indicated with "+". Empty cells indicate that no data concerning the interaction were available. The references are indicated in superscript.

Target protein	ITAM1			ITAM2			ITAM3		
	ζ1pyy	ζ1ypy	ζ1pypy	ζ2pyy	ζ2ypy	ζ2pypy	ζ3pyy	ζ3ypy	ζ3pypy
p56lck	++[73]	+[73]			++++[73]		++[73]	++[73]	
p59fyn	-[69]	++[69]	+/-[68,69]			++[68]		-[68]	
ZAP-70	+[69,73,103]	+/-[69,73,103]	+++[69,73,130,132]	+[69,73]	+/-[73]	+++[73,132]	+[73]	+[73]	++[73,132]
Syk			+++[132]		+++[132]				+++[166,132]
PI3K (p85)			+++[168,71,131]			++[168,71,131]		+[67]	+[68,70,71,131]
Shc	+[73]	++[73,130]	+++[71,130]	+[73]	++[73]	-[71]	++[73]	++[71,73]	+[71]
Grb-2			+[71]			+++[71]			-[71]
RasGAP			+++[68,71]			++[68,71]		++[71]	+[68,71]
SHP2			+[131]						

3) There is a heterogeneity in the amino acids adjacent to the phosphotyrosines in the various ζ ITAMs. It has been shown that these aminoacids determine the specificity of binding of SH2 domains, suggesting that individual ζ ITAMs could couple to distinct SH2 domain signalling proteins (83, 84);

4) Different ζ ITAMs have distinct abilities to promote apoptosis. Analysis of apoptosis induced by single ζ ITAM Tac chimeras in T cells revealed that the amino-terminal ζITAM stimulated greater apoptosis than the carboxy-terminal ζITAM, while the central ζITAM was unable to trigger apoptosis (85);

5) Detergent-soluble and cytoskeleton associated TCRs contain differently phosphorylated ζ molecules (86).

The T cell response to extracellular signals may depend on the composition of the developing signalling complex on the cytoplasmic tail of the TCR ζ chain, which is determined by the phosphorylation pattern of the ζ ITAMs. It is also possible that the 'specificity model' and the 'threshold model' are not mutually exclusive, and may be differentially utilised depending on specific conditions of T cell activation.

2.2.3.4. Activation of ZAP-70 and Syk PTKs

Tyrosine phosphorylation of the ITAMs in ζ chain and their sequential SH2-mediated interaction with ZAP-70 tyrosine kinase are essential for the initiation of TCR-mediated signal transduction (65-67, 87, 88). Patients lacking functional ZAP protein display severe defects in TCR signaling as well as an absence of peripheral T cells (87). Similar defects in the Syk gene have not been reported. Furthermore, thymic development of lymphocytes in ZAP-70 $^{-/-}$ mice is completely blocked at the double-positive stage (reviewed in 53).

The binding of ZAP-70/Syk PTKs with the doubly phosphorylated ITAMs (dp-ITAMs) results in the activation of these PTKs (41, 42, 88). The activated kinases then phosphorylates substrates such as Cbl, SLP-76 76 (SH2 domain-containing leukocyte phosphoprotein of 76 kDa), Vav, PLCγ (phospholipase C γ), and LAT (linker for activation of T cells) (88-92, 94). Further signal transduction events lead to the activation of both Ca/calcineurin- and Ras-mediated signaling pathways (see section 2.2.3.5.). Analysis of the recently identified P116 cell line (a variant of Jurkat T cell line) which is deficient in both ZAP-70 and Syk has confirmed two crucial roles for ZAP70/Syk family PTKs: first in the phosphorylation of PLCγ1, SLP-76 and LAT; second in TCR-induced Ca^{2+} mobilization, activation of the transcription factor NFAT (nuclear factor of activated T cells) and IL-2 production (96).

Syk and ZAP-70 are both composed of two tandem amino-terminal SH2 domains separated by an interdomain (interdomain A), followed by an extended interdomain region (interdomain B), the kinase domain and by a short C-terminal tail (reviewed in ref. 88) (Fig. 2.7.). The two kinases share greater than 50% sequence identity, and several conserved tyrosine residues that are important both for the regulation of the enzyme

88) (Fig. 2.7.). The two kinases share greater than 50% sequence identity, and several conserved tyrosine residues that are important both for the regulation of the enzyme activity and for the association of these kinases with other signalling molecules (Fig 2.7.) (88). Most of these conserved tyrosine residues are located in interdomain B. The Y315 and Y319 in ZAP which corresponds to Y348 and Y352 in Syk are implicated in positive regulation of the kinase activity (88). Phosphorylation of these tyrosine residues is also important for the association of ZAP and Syk with the SH2 domain of Vav (Y315/348) and with the SH2 domain of PLCγ1 and/or p56lck (Y319/352). In contrast, Y292 in ZAP and Y323 in Syk are negative regulatory sites. A balance between negative regulation via Y292/Y323 and positive regulation via Y315/348 and Y319/352 may therefore control the activity of ZAP and Syk (88).

The kinase domains of ZAP and Syk contain also conserved regulatory sites that are found within the activation loop of the kinase domain, Y492/493 in ZAP and Y525/526 in Syk. In ZAP-70, Y492 and Y493 are sites for tyrosine phosphorylation by Lck (88). Y492 has been shown to be a negative regulatory site, whereas Y493 is a positive regulatory site for ZAP-70 kinase activity. In Syk, Y525 and Y526 are sites for autophosphorylation and both act as positive regulators of Syk activity (88).

The SH2 domains of Syk and ZAP-70 have 56% sequence homology. The most distinctive aspect of the SH2 domains present in ZAP-70 is that they bind to their targets in the TCR/CD3 complex in a co-operative manner (88, 95). The molecular basis of this co-operativity has been established with the crystalization of its tandem SH2 domains complexed with a phosphorylated ITAM peptide from the TCR ζ chain (95). The N-terminal SH2

Fig. 2.7. Structure of Syk-family PTKs. The Syk family kinases are composed of two SH2 domains (SH2-N and SH2-C) with an intervening interdomain A (IA), an extended interdomain B (IB) and kinase domain (Kinase). Conserved tyrosine phosphorylation sites are indicated. *(Copied from ref. 88).*

domain appears to be incomplete and requires amino acid contributions from the C-terminal SH2 domain to help complete the phosphotyrosine binding (PTB) pocket. In

ITAM peptide revealed that the two SH2 domains of Syk form two independent binding pockets for the phosphotyrosines (96).

Despite the structural similarities there is evidence indicating that Syk and ZAP-70 are differentially regulated both in terms of expression and activity (88). ZAP-70 is expresed in a very restricted manner, present only in T cells and natural killer cells. Syk, however, has a broader pattern of hematopoietic expression, including T and B cells, as well as macrophages, monocytes, mast cells, and platelets (88). However, while ZAP-70 is expressed in all T cells, Syk is present only in thymocytes and intraepithelial γδ T lymphocytes and in naïve but not in proliferating mature αβ T cells (93).

Both SH2 domains of ZAP and Syk have to be occupied with phosphotyrosine residues for full activation of the enzymes. Although both kinases bind dp-ITAMs with similar affinities only Syk kinase activity is directly enhanced by phospho-ITAM binding (41, 70, 88). Phospho-ITAM binding results in an upregulation of Syk activity by facilitating the cross-phosphorylation of two Syk molecules (41, 88). In contrast, binding of ZAP-70 to doubly phosphorylated ITAMs is required but not sufficent for enhancing the catalytic activity of ZAP. Activation of ZAP requires phosphorylation of Tyr493 by Src PTKs. Phosphorylation of this site can be mediated by *Src* PTKs, but not by ZAP-70 autophosphorylation (41, 88).

Furthermore, like *Src* family kinases, Syk but not ZAP-70 has the capacity to directly phosphorylate a ζ-derived peptide *in vitro* (98, 99). Also, Syk but not ZAP-70, can reconstitute signalling in mutant Jurkat T cell deficient in either p56[lck] or CD45 (97). Studies using the ZAP/Syk negative Jurkat mutant P116 transfected with either Syk or ZAP revealed that Syk-expressing cells were partially activated even in the absence of TCR ligands (98, 99) indicating that the activation threshold for Syk is significantly lower than that of ZAP-70.

2.2.3.5. Signal transmission from PTKs to the nucleus

The role of ZAP and Syk is to couple antigen receptors to essential signalling pathways- one mediated by the GTPases p21ras and the other mediated by Ca^{2+}/calcineurin (45, 46). Recent data suggest that connection of TCR to the Ras pathway is mediated by a series of adaptor molecules among which are: Shc, LAT, SLP-76 and Grb2 (growth factor receptor binding protein 2) (47, 94). LAT is also essential for linking

TCR to PLC pathway (47, 94). Some of these proteins - i.e: LAT, SLP-76, and PLCγ – are ZAP/Syk substrates (89, 92, 94). Another substrate of Syk family kinases- the guanine nucleotide exchange factor Vav- ensures the link between TCR and the cytoskeleton (47, 90).

2.2.3.5.1. Regulation of inositol lipid metabolism, Ca^{2+}/calcineurin and PKC pathway byTCR

The first well characterized PTK-controlled TCR signalling pathway is mediated by PLCγ1 (45, 48). PLC γ1 hydolyzes inositol phospholipids and thereby generates inositol polyphosphates which regulate intracellular calcium levels and diacylglycerols which activates the serine/threonine kinase, protein kinase C (PKC) (45). PLC γ is tyrosine phosphorylated by Syk family kinases in TCR-activated cells, a process that is important for its activation. The TCR also induces the formation of a protein complex between the SH2 domains of PLCγ and LAT. Since LAT is a transmembrane protein, the interaction between LAT and PLCγ may be important to recruite PLCγ to its substrates located in the plasma membrane (46). The function of calcium and PKC signals in TCR signalling has been explored mainly in the context of TCR regulation of the production of cytokines. For example, the IL-2 gene induction requires the co-ordinate action of multiple transcription factors that include NFAT, AP-1, NFkB and Oct-1 (45) whose activity is controlled by PLC pathway (Fig.2.8.) (46). Pharmacological agents, ionomycine and phorbolesters, that directly induce the Ca^{2+} elevation and PKC activation, respectively, stimulates T lymphocytes to proliferate and to produce IL-2 (45, 100).

2.2.3.5.2. Ras GTP-ase pathway

The level of active p21ras GTP complex is determined by a balance of the rate of hydrolysis of bound GTP and the exchange rate of bound GDP for cytosolic GTP (45, 47). The GTPase activity of Ras in mammalians is controlled by Ras GTP-ase activating proteins (GAP) p120-GAP and by proteins that regulate guanine nucleotide exchange on Ras, (guanine nucleotide exchange factors, GEFs). Signals via TCR are able to regulate the activation of the guanine nucleotide binding proteins p21ras both via inhibition of GAPs and activation of GEFs (45). The mechanism that allows TCR to inhibit GAP proteins is unclear. The regulation of RasGEFs by TCR involves an adaptor protein- Grb 2

GAPs and activation of GEFs (45). The mechanism that allows TCR to inhibit GAP proteins is unclear. The regulation of RasGEFs by TCR involves an adaptor protein- Grb 2 (47). Grb2 is a small protein comprising a central SH2 domain flanked by two SH3 domains (Fig. 2.6.). The SH2 domain of Grb2 binds to tyrosine phosphorylated molecules from the TCR complex and its SH3 domains bind to the proline-rich domain of Sos (a GEF) bringing therefore Sos to the plasma membrane where it may facilitate Ras activation (45). Grb-2 is inducibly associated with Sos following TCR ligation (47). Grb2/Sos complex may be bound either directly to phosphorylated ITAMs or indirectly through interacting with other adaptors such as Shc, LAT and/or SLP-76 (47) (Fig. 2.8).
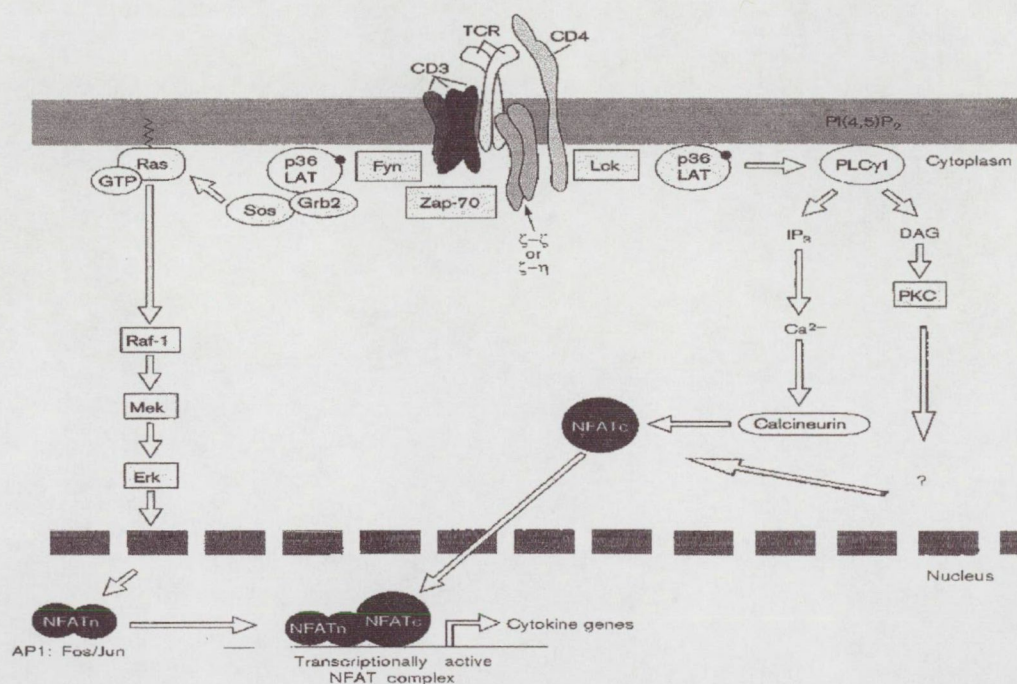
Fig.2.8. Signal transmission from TCR to the nucleus. Engagement of cell-surface TCR complex initiates the activation of membrane proximal PTKs, which leads to tyrosine phosphorylation of LAT and couples the TCR to PLCγ and Ras-regulated signaling pathways. Transcription factors of the NFAT ffamily regulate cytokine gene expression and are activated by signals emerging from Ras which act in synergy with calcium/calcineurin and PKC signals. DAG, diacylglycerol; NFATc, cytosolic NFAT; NFATn, nuclear NFAT; P2 diphosphate; ● phosphorylated. *(Copied from ref.46)*

The transmission of signals from Ras to the nucleus involves the regulation of the MAP kinases (mitogen activated kinases), ERK1 and ERK2 (reviewed in refs. 45, 47). The MAP kinases are activated by a serine/threonine kinase cascade involving a MAP kinase kinase (MAPKK) that phosphorylates and stimulates ERK1 and ERK2 directly. The activity of the MAPKK is itself controlled by phosphorylation, with MAP kinase

domain of Raf-1 can interact directly with GTP-Ras, and it has been suggested the role of Ras is to recruit Raf-1 to the plasma membrane where it is activated. Once activated, ERK2 may translocate to the nucleus where they can directly modulate transcriptional factors and gene expression.

2.2.3.6. The role of cytoskeleton in TCR signalling

T cell receptor activation is associated with changes in cell morphology that are a result of cytoskeleton rearrangement. These changes lead to the organization of focal actin-scaffolded signaling "highways" whose function is to sustain TCR signalling and co-ordinate downstream signalling events such that complete activation is achieved and late events such as proliferation and cytokine secretion can occur (46, 101). The link between TCR and cytoskeleton is insured by Vav. Vav is a GEF with selectivity for Rho family GTPases, Rac 1, CDC42, and RhoA (reviewed in 46). The guanine exchange activity of Vav is directly regulated by tyrosine phophorylation. In activated T cells Vav is a substrate for Syk family kinases (91). In addition, a protein complex beetwen Vav and another Syk PTKs substrate, the adapter protein SLP-76, is formed in response to antigen stimulation in T cells (46, 47). The formation of this complex is proposed to regulate the cellular localization of Vav, thereby promoting its interactions with Rho GTPases and/or tyrosine kinases. The GTPases, Rac, CDC42, and Rho function as molecular switches in cells and orchestrate receptor-mediated responses such as cytoskeletal changes and DNA synthesis (46). Target molecules for Rac, CDC42, and Rho differ widely in their functions and include such molecules as phosphatidylinositol 4-phosphate 5-kinase (PIP5K), CDC-42-associated Wiscott Aldrich syndrome protein (WASP), myosin light chain (MLC) phosphatase, and the p21-activated kinase (PAK). PIP5K phosphorylates PIP, resulting in the production and local accumulation of phosphatidylinositol 4,5 biphosphate (PIP$_2$) (46). High concentrations of PIP$_2$ can promote local actin polymerization and anchoring of the actin cytoskeleton to the cell membrane and to the organization of focal actin-scaffolded signaling "highways" (46, 101).

2.2.3.7. Signal termination

In contrast to T-cell activation, the mechanism of negative regulation of T cells is largely undefined. Negative regulation takes place in two phases- one during the initial

triggering of T cells and the other during latter stages (reviewed in ref. 102). Negative regulation of the initial phase of T cell activation appears to be mediated by two distinct mechanisms:

a) the active induction of the negative signals through stimulation of cell surface molecules such as cytotoxic T lymphocyte antigen 4 (CTLA-4)

b) the induction of unresponsive/anergic or supressive status through various mechanisms including inhibitory cytokines as well as modulation of the TCR complex and signaling molecules by redox regulation (102).

For the late phase of T cell responses, negative fedback is required. This involves induction of growth inhibition, cell cycle arrest and apoptosis which are mediated through various molecules including inhibitory cytokines and the Fas system.

T cells from CTLA4-/- mice revealed hyperphosphorylation of various proteins including TCR ζ chain, and showed that TCR-associated kinases Lck, Fyn and ZAP-70 were constitutively activated, suggesting that CTLA-4 interferes with the activation of these molecules (102). In normal cells CTLA-4 was found to associate with the SH2 domain of SH2 domain containing PTPase 2 (SHP-2) via a phosphorylated tyrosine-containing motif: Tyr-Val-Lys-Met suggesting that phosphorylation of CTLA-4 recruits SHP-2 and that the activated SHP-2 may dephosphorylate TCR-associated kinases and other substrates.

Other PTPs have been suggested to play an important role in the negative regulation of TCR signalling such as SHP-1 and CD45. SHP-1 can act at multiple steps in TCR activation cascade by dephosphorylating TCRζ, p56^{lck} and ZAP-70 in vivo (103, 104). CD45 has been shown to co-distribute with ZAP-70 in intact T cells, and ZAP-70 was dephosphorylated by CD45 in vitro (105).

The mechanism that controls the activity of these phosphatases is still unknown.

2.2.3.8. CD45 structure and its role in TCR signaling

The CD45 (also termed leukocyte common antigen-LCA, Ly5, B220 in B cells and T200 in T lymphocytes) is a family of transmembrane protein tyrosine phosphatases (PTPases) uniquely and abundantly expressed on nucleated hematopoietic cells. CD45 is encoded by a single gene located in a syntenic region found on chromosome 1 both in

(PTPases) uniquely and abundantly expressed on nucleated hematopoietic cells. CD45 is encoded by a single gene located in a syntenic region found on chromosome 1 both in humans and mice (106). It functions as a key regulatory element in TCR signalling by controlling the activity of Src PTKs p56^{lck} and p59^{fyn}.

2.2.3.8. 1. Structure of CD45

CD45 is a transmembrane protein consisting of a large, heavily glycosilated, amino-terminal external domain of approximately 400-500 residues, a single transmembrane region and a large highly conserved carboxy-terminal cytoplasmic domain of 707 residues (106, 107) (Fig.2.9.). CD45 is structurally heterogenous. The different molecular weight isoforms are ranging from approx. 180 kDa to 220 kDa and are expressed in cell-type specific patterns. The B lymphocytes express the highest MW form of 220 kDa, thymocytes express predominantly the lowest MW form of 180 kDa, and different subsets of T cells express multiple MW

Legend:
- ▬ A exon
- ▭ B exon
- ▬ C exon
- 〜 Miristic acid
- ⌐• N-glycosilation
- / O-glycosilation
- [P] PTP domain

Citoplasm

Fig. 2.9. Structure of CD45. CD45 consits of a variable extracellular domain that contains N- and O- linked glycosilation sites, a transmembrane region and an intracellular part that contains the two phosphatase domains. The regions encoded by the alternatively spliced exons A, B and C are shown.

forms (107). The structural heterogeneity of CD45 was found to be the result of differences in the primary structure of the external domain. Six isoforms of the molecule were identified by sequencing mouse, rat and human cDNAs (107). These isoforms are generated by the alternative splicing of exons 4- 6 (also termed exons A, B and C) each encoding approx. 50 amino acids inserted near the amino terminus of the molecule (108, 109). The sequences encoded by the alternaltively-spliced exons are rich in serine and threonine residues and contain multiple potential sites for O-linked oligosaccharides. There is direct biochemical evidence that these regions of the molecule are modified by extensive O-linked glycosylation (110). In addition, it has been shown that human CD45

A variety of anti-CD45 monoclonal antibodies (MAbs) that selectively react with different MW forms of CD45 glycoprotein bind to antigenic determinants localized to one or more of the segments encoded by the alternatively-spliced exons (112). These MAbs are designated as CD45R (restricted) antibodies with CD45RA, CD45RB and CD45RC Mabs reacting with CD45 isoforms containing the segments encoded by exons 4, 5 and 6, respectively. In human, a CD45R0 MAb, UCHL-1, has been obtained that recognizes only the smallest CD45 isoform. CD45R MAbs have been useful in distinguishing between different subpopulations of functional T cells (113, 114). The human T cells can be divided into two subpopulations -naive and memory T cells- based upon their expression of CD45 RA or CD45R0 isoforms (113).

Unlike the extracellular region, the transmembrane region and the intracellular 707-residue C-terminal part of CD45 are identical in all isoforms.

The cytoplasmic domain of CD45 consits of a membrane proximal region, followed by two PTPase domains of approximately 240 residues separated by a short spacer region, and a carboxy-terminal tail (106). The second (membrane distal) PTPase domain appears to be catalytically inactive. CD45 mutants containing the PTPase I domain but not the PTPase II domain have been shown to have less than 14% activity as compared to the wild type CD45 implying that the activity of PTPase domain I was not independent upon domain II (115). Whether the second PTPase domain plays a regulatory role or is required to maintain the active conformation of domain I is unclear.

2.2.3.8.2. The role of CD45 in TCR signaling

CD45 plays an important role in signal transduction and T lymphocyte activation. CD45-deficient lymphocytes are greatly diminished in their capacity to respond to antigen or to receptor cross-linking (116). Many physiological effects of various anti-CD45 MAbs including stimulation of IL-2 production (117), cell-mediated cytotoxicity (118), neutrophyl migration (119) and the stimulation of proliferative responses (117, 120) have been described. CD 45 activity was found to be increased up to 10 times during mitosis both in a pre-B lymphoma and in cytolytic T lymphocyte lines suggesting that CD45 has a role in phosphorylation events during cell division (121). T cells lacking CD45 fail to respond to stimulation by antigen or mitogenic antibodies (116, 122) and responsiveness is restored on introducing CD45 into the cells by transfection (123). The requirement for

CD45 in T cell activation seems to reside at a very early stage in the signal transduction cascade, since, in the absence of CD45, the rapid receptor-triggered tyrosine phosphorylation of cellular proteins is severely reduced (124), resulting in impaired activation of PLCγ (125) and disturbed Ca^{2+} homeostasis (107, 124). It was suggested that CD45 regulates these events is by regulating the Src-family PTKs $p56^{lck}$ and $p59^{fyn}$ (54, 56, 126, 127 and see also chapter 3.2.1.). In CD45-deficient T cells, carboxy-terminal tyrosine phosphorylation of $p56^{lck}$ and $p59^{fyn}$ is increased, resulting in a decrease in their kinase activities (reviewed in ref. 54). As both $p56^{lck}$ and $p59^{fyn}$ have been implicated in signal transduction through the TCR, the inability to activate these enzymes is likely to result in antigen receptor unresponsiveness (128).

Whether CD45 functions to regulate other aspects of activation is unknown. However, CD45 was shown to interact strongly and specifically with phosphorylated TCR ζ chain and to dephosphorylate it (129). Therefore, CD45 may also function in either maintaining the ζ chain in a dephosphorylated form, or in dephosphorylating ζ chain after cellular activation.

2.2.3.8.3. The role of CD45 isoforms in the regulation of TCR signaling

Convincing evidence that different CD45 isoforms vary in their ability to promote TCR signalling has come from studies in which different CD45 isoforms were expressed in a murine thymoma cell line co-transfected with CD4 and a clonotypic TCR (130). Comparison of the isoform-specific subclones revealed that MHC-peptide-TCR interactions induced IL-2 secretion in the order CD45R0> CD45RC> CD45RABC = CD45RBC. Differences in biological responsiveness occurred in the absence of consistent differences in either total tyrosine phosphorylation or Src family PTK activity, suggesting that CD45 may have other substrates than the Src family PTKs.

Furthermore, CD45R0 was found to preferentially co-distribute with the TCR/CD4 when compared with CD45RABC (131). The ability to co-cap with CD4-TCR maps to CD45 ectodomain (131). These results suggest that the expression of distinct CD45 isoforms play a key role in modulating TCR responsiveness to antigenic stimulation.

3. AIMS OF THE STUDY

Despite the extensive investigation, there are still two main controversial topics regarding the ζ chain signalling- one concerning the mechanism that leads to the generation of the differently phosphorylated forms of the TCR ζ chain, the other one regarding to the significance of the presence of three functional units (ITAMs) in the cytoplasmic tail of the ζ chain.

In order to get new data about the mechanism that contributes to the generation of the differently phosphorylated forms of the TCR ζ chain we tried to answer the following questions:

1. Which ITAMs and individual tyrosine residues in the human ζ chain can be phosphorylated *in vitro* by Lck and Fyn protein tyrosine kinases?

2. Does the CD45 PTPase have a role in the formation of the ζ chain phosphorylation pattern?

Our second aim was to determine whether the three ITAMs within the ζ chain are functionally equivalent or they are required to activate distinct signalling pathways. For this purpose we tried to answer the following questions:

1. Which procedure for cell permeabilization is suitable for studying early signalling events such as tyrosine phosphorylation?

2. What is the effect of the various mono- and bi-phosphorylated ζITAM peptides on tyrosine phosphorylation of cellular proteins?

3. What can be the enzymatic components (e.g. PTKs and/or PTPs) involved in the triggering of T cells by ITAM phosphopeptides?

4. Does the phospho-ITAM peptides modulate the adaptor-protein mediated interactions?

4. Materials and Methods

4.1. Cells and antibodies.

The human T cell lines, Jurkat, J6 and HPB-ALL were cultured in RPMI 1640 (GIBCO) tissue culture medium supplemented with 5% heat inactivated FCS (Gen-Inter). The JCaM 1.6., a p56lck negative variant of Jurkat, was cultured in RPMI supplemented with 10% heat inactivated FCS. Human peripheral blood mononuclear cells (PBMC) were obtained from healthy donors by density gradient centrifugation over Ficoll-Hypaque. Polyclonal antisera were generated by injecting rabbits with branched multiple antigenic peptides (MAPs) of the p56[lck] (aminoacids 39-64), p59[fyn] (aminoacids 20-48) and the ZAP-70 (aminoacids 485-499) sequences (140). Immunoglobulin fractions of the antisera were purified by MAbTrap G according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). Anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology. Monoclonal antibodies recognizing CD45, GB3 (103) and T2/53 reactive with CD43, were developed in our laboratory (141, 142). Rabbit immunoglobulin to mouse immunoglobulins (RaMIg) and RaMIg coupled to horseradish peroxidase (HRPO) were purchased from DAKO.

4.2. Synthesis of ζ oligopeptides.

The nonphosphorylated peptide substrates (Table 5.1.) were synthesized by solid phase technique utilizing tBoc chemistry. The peptides were purified by semipreparative reverse phase HPLC and were characterized by amino acid composition and mass spectrometry. The purity of the peptides was above 97% as determined by HPLC.

The phosphorylated peptides (Table 2.) were prepared using Fmoc (106) technique. The phosphate moiety was incorporated with di-tert. butyl-N,N' diethyl phosphoramidite method as described previously (144, 145). The isolation and

characterization were carried out as described for nonphosphorylated peptides except that an additional capillary zone electrophoresis step was included.

4.3. Cell permeabilization

Cells were harvested by centrifugation, washed in RPMI without supplements, resuspended in RPMI and incubated for 5 min. at 37°. In order to find a rapid and efficient permeabilizing agent, the following permeabilization procedures were applied:

a) Streptolysin O permeabilisation. Streptolysin O (Sigma) permeabilization was performed according to the protocol described by Spiller and Tidd (146). Cells (1×10^7/sample) were washed once in permeabilization buffer (Ca^{2+}-Mg^{2+} free PBS supplemented with 10mM DTT), resuspended in 1ml permeabilization buffer containing 0.5-200 I.U/ml of streptolysin O (Sigma) and incubated at 37°C for 5 to 30 minutes.

b) Digitonin permeabilization (159). Cells (2×10^7) were incubated for 5 min at 37°C in 1ml PBS containing 2mM EGTA, 2mM $MgCl_2$, 2% BSA and 10-50 µg digitonin (136).

c) L-a-lysophosphatidyl choline permeabilization (136, 137, 149). Different human leukaemic cell lines were pelleted, washed once with RPMI, resuspended at 4×10^7/ml in ice-cold permeabilization buffer (see ref. 70 and Appendix) and incubated further on ice for 5 min in a cold room. Permeabilization was initiated by adding 1/10 vol. of ice cold LPC stock containing 10- 3000 µg/ml LPC and cells were further incubated on ice for 1 min. Alternatively, all the above mentioned steps were carried out at room temperature or at 37° C.

Permeability was assessed using trypan blue exclusion in each case.

4.4. Cell stimulation and anti-phosphotyrosine blotting.*(134)*

Stimulation of intact or permeable cells (4×10^7/ml) was initiated by adding either anti-CD3 mAb, UCHT1 (10µg/ml) (kindly provided by Dr. P.C.L. Beverly) or 500µM ζ-

ITAM peptides. Cells were further incubated for 2 min at 37°C and activation was stopped with 2x concentrated ice-cold lysis buffer (see Appendix). The cells were incubated on ice for 30 min and nuclear/cytoskeletal components were removed by centrifugation at 12,000×g for 15 min. Cell lysates from 2×10^5cells were diluted with 3x concentrated reducing Laemmli sample buffer (see Appendix.) and samples were boiled for 5 min before SDS-PAGE analysis. Proteins and prestained molecular weight markers (GIBCO-BRL) were separated on 10% SDS-PAGE, then transferred to nitrocellulose membranes (Schleicher & Schuell) using 20% methanol containing transfer buffer (see Appendix). The membranes were blocked using Tris-buffered saline (TBS) (see Appendix). containing 3% cold fish gelatine (Sigma) for 1 h at room temperature and subsequently probed with 4G10 anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology Inc.) diluted 1:10,000 in gelatine-TBS for 1 h at 4°C. Membranes were then washed three times with TBS containing 0.05% Tween -20, and incubated further for 1h at 4°C with rabbit anti-mouse IgG conjugated to horseradish peroxidase1: 7500 (DAKO), and washed again. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) detection system (Amersham).

4.5. Kinase immunoprecipitation and peptide phosphorylation assay (136, 145)

For immunoprecipitation of kinases, postnuclear supernatants from nonstimulated or peptide-stimulated Jurkat T cells (1×10^7/sample) were mixed with antibody-coupled agarose beads (10 µl/sample) and incubated for 1h at 4°C. The immunoprecipitates were washed once in kinase lysis buffer and twice in kinase assay buffer (see Appendix). The enzyme reaction was performed in 20µl kinase assay buffer (see Appendix) containing 1µCi of $[\gamma\text{-}^{32}P]$ATP and 15 nmol oligopeptide substrate for 20 minutes at 37°C and was stopped by cooling the samples to 4°C. The phosphorylated peptides were separated in a 40% alkaline polyacrylamide gel (147). The gel was dried and analyzed by phosphoimager.

4.6. Detection of the ITAM-induced kinase phosphorylation in permeabilized T cells

Permeabilized Jurkat T cells (1×10^7/sample) were stimulated for 3 min at 37°C with 500μM ζ-ITAM peptides or anti CD3 mAb in the presence of 10μCi [γ-^{32}P]ATP or left unstimulated. Cell lysis and immunoprecipitations were performed as described above (see sections 4.4 and 4.5). Immunoprecipitated kinases and were eluted from beads using 10 μl of 2× concentrated Laemmli sample buffer and analysed using 10% SDS-PAGE gel. In order to hydrolyse the radioactive phosphate groups from phospho-serine and phospho-threonine residues, the gels were incubated in 1M KOH at 55°C for 1h, then dried and analyzed by using phospho-imager (Molecular Dynamics 445 SI).

4.7. Purification of Grb-2 associated proteins

After being stimulatd or left untreated, 1×10^7 J6 T cells were lysed in ice cold lysis buffer for 15 min on ice. After centrifugation the supernatants were mixed with 5μg of GST-Grb fusion protein coupled to glutathione beads (kindly provided by Dr László Buday, SOTE Institute of Biochemistry, Budapest) and incubated for 1h at 4°C. The precipitates were washed three times with lysis buffer (see Appendix) and boiled for 5 min in Laemmli sample buffer The electrophoresis and immunobloting of affinity purified proteins were performed as described in section 4.4.

4.8. Immunoprecipitation of CD45 and phosphatase assay.(134, 146)

HPB-ALL cells (10^7 cells/sample) were lysed for 1h in ice cold phosphatase lysis buffer (see Appendix) supplemented with 10μg/ml aprotinin and 1mM PMSF. Postnuclear supernatants were mixed with 10μl protein G-coupled Sepharose beads (Pharmacia) precoated with 20μg affinity purified anti CD45 mAb and incubated for 1h at 4°C. The immunoprecipitates were washed twice in phosphatase lysis buffer and once in phosphatase assay buffer (see Appendix). The beads were incubated in 10μl phosphatase assay buffer supplemented with 1mM DTT and 2.5 nmols phosphopeptide for 1h at 37°C. The amount of the released inorganic phosphatase was determined according to D. H. W.

Ng et. al. (148). Brifely, the supernatants of the reaction mixtures (10 µl) were transferred into the wells of a 96 well microtiter plate (Falcon), mixed with 90 µl of malachite green reagent and incubated further for 15 minutes at room temperature for colour development. Absorbance was determined at 650nm wavelength using an EIA plate reader. For each peptide background absorbance was evaluated using a control sample
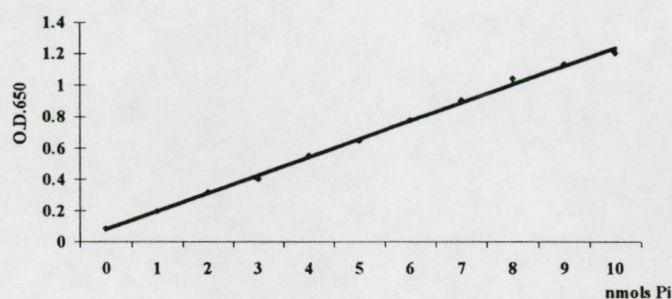
Fig.4.1. Standard curve for the evaluation of inorganic phosphate concentration using malachite green reagent. The standard curve for inorganic phosphate detection was obtained using potassium dihydrogen phosphate (KH_2PO_4) over a range of concentration between 0 and 10 nmol.

which contained 10 µl phosphatase assay buffer supplemented with 1mM DTT, 2.5 nmols phosphopeptide and 90 µl malachite green reagent. The absorbance of the control was substracted from sample absorbance. The value obtained after substraction was used to determine the release of inorganic phosphate from a standard curve for inorganic phosphate detection (Fig. 4.1).

5. Results

5.1. The role of PTKs and CD45 in the generation of different phospho-ζ forms

5.1.1 All the tyrosine residues within the ITAMs of the TCR ζ chain are phosphorylated by Src kinases

The TCR ζ chain is phosphorylated on multiple tyrosine residues upon T cell stimulation and *Src* family tyrosine kinases, p56^{lck} and p59^{fyn} have been implicated in this process (135). To compare the specificity of different kinases in the phosphorylation of particular tyrosines of the ζ chain we have carried out *in vitro* phosphorylation experiments using immunoprecipitated enzymes as the source of kinase activity and substrates representing different parts of the ζ chain. Table 5.1. shows the aminoacid sequence of ζ chain and the oligopeptides used in this study: 1. synthetic ITAM fragments (underlined), 2. one-tyrosine residue containing short oligopeptides (printed with blank characters).

A.

```
              ζ0              ζ(1)a              ζ(1)b
RVKFSRSAEPPAYQQGQNQLYNELNLGRREEYDVLDKRRGRD
                            ζ(1)

              ζ(2)a                ζ(2)b
PEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK
                            ζ(2)

     ζ(3)a              ζ(3)b
GHDGLYQGLSTATKDTYDALHMQALPPR
              ζ(3)
```

B.

ζ0	ζ[61–67]
ζ(1)	ζ[69–86]
ζ(1)a	ζ[69–75]
ζ(1)b	ζ[80–86]
ζ(2)	ζ[106–126]
ζ(2)a	ζ[106–113]
ζ(2)b	ζ[119–126]
ζ(3)	ζ[138–155]
ζ(3)a	ζ[138–144]
ζ(3)b	ζ[149–155]

Table 5.1. A. The aminoacid sequence of the ζ chain intracellular part and the localization of the oligopeptide substrates. The three ITAMs are underlined and marked as ζ(1), ζ(2), ζ(3). The seven synthetic oligopeptides representing the individual tyrosines using blank characters and marked as ζ(0), ζ(1)a, ζ(1)b, ζ(2)a, ζ(2)b, ζ(3)a, ζ(3)b. B. The position of the oligopeptide substrates within the ζ chain is shown.

First, we investigated the phosphorylation of the oligopeptides corresponding to the ITAMs of the ζ chain. All ITAM-s ($\zeta(1)$, $\zeta(2)$, $\zeta(3)$) were phosphorylated by the immunoprecipitates of p56^{lck} and p59^{fyn} (Fig.5.1.A) as compared to the controls, ZAP 70 and rabbit anti-mouse immunoglobulins (RaMIg) (Fig.5.1.B.). Similar patterns of phosphorylation of the ITAMs were obtained when immunoprecipitates of p56^{lck} and p59^{fyn} were used as kinase source (Fig.5.1. A). The $\zeta(1)$ ITAM was poorly phosphorylated by both p56^{lck} and p59^{fyn} as compared to the $\zeta(2)$ and $\zeta(3)$. This may be a consequence of the solubility problems we encounterd in the case of $\zeta(1)$ peptide. The p56^{lck} phosphorylated all the ITAMs more strongly than p59^{fyn} did.
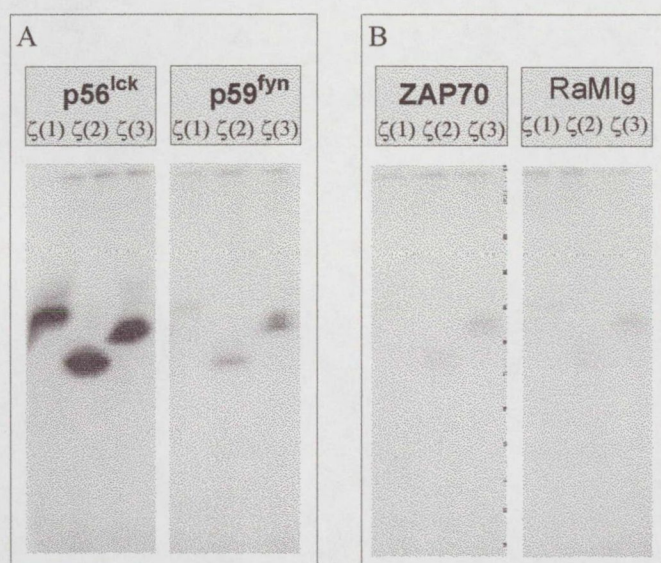
Fig. 5.1. In vitro phosphorylation of the oligopeptides representing the ITAMs of the ζ chain by p56lck and p59fyn kinases. The phosphorylation of the $\zeta(1)$, $\zeta(2)$ and $\zeta(3)$ oligopeptides was analyzed in the presence of immunoprecipitated p56lck, p59fyn (A) and ZAP-70 (B) kinases. RaMIg coated beads were used for controlling the nonspecific background of the kinase reaction (B). The phosphorylated peptide substrates were separated in 40% alkalic acrylamide gels.

To determine exactly which tyrosine residues of the ζ chain were phosphorylated by the different tyrosine kinases, a panel of short oligopeptides containing the individual intracellular tyrosines with the neighbouring protein sequences were used as substrates. As shown in Fig.5.2., six of seven oligopeptides designated as $\zeta(1)a$, $\zeta(1)b$, $\zeta(2)a$, $\zeta(2)b$, $\zeta(3)a$ and $\zeta(3)b$ (Table 5.1.) were phosphorylated by Src family member kinases, p56^{lck} and p59^{fyn}, while the $\zeta(0)$ control oligopeptide which contains a tyrosine residue located outside the ITAMs remained unphosphorylated. The intensity of the phosphorylation obtained by the various enzymes was different, as p56^{lck} gave a much stronger signal than p59^{fyn}. When the ZAP70 immunoprecipitate was used, none of the oligopeptides were phosphorylated.
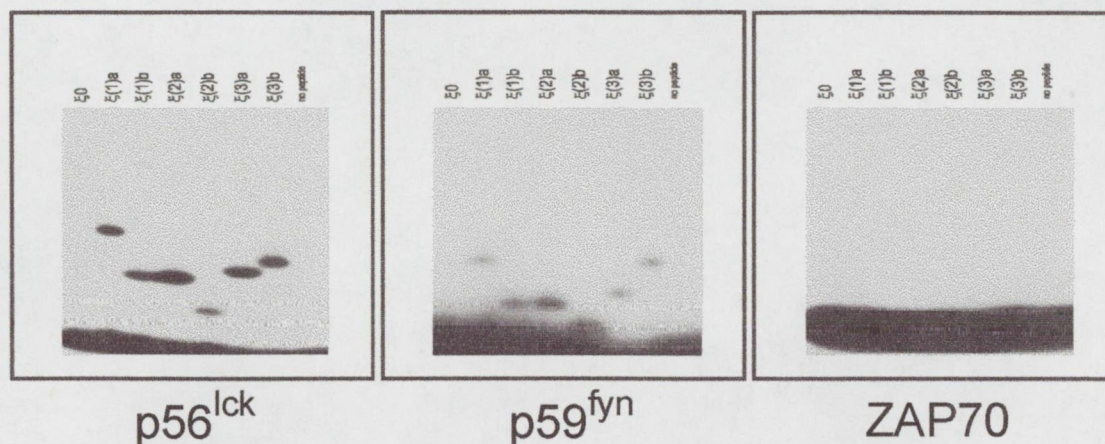
Fig. 5.2. In vitro phosphorylation of the short ζ oligopeptides by p56^{lck} and p59^{fyn} kinases. The phosphorylation of the ζ(1)a, ζ(1)b, ζ(2)a, ζ(2)b, ζ(3)a, ζ(3)b and the ζ0 oligopeptides were analyzed in the presence of immunoprecipitaed kinases. The phosphorylated peptide substrates were separated in 40% alkalic acrylamide gels.

5.1.2. CD 45 discriminates between individual phospho tyrosine residues of the ζ chain ITAMs.

Table 5.2. Sequence and designation of the synthetic phosphopeptide substrates. The designation of the phospho-ζ ITAMs peptides is indicated in the left panel. The six oligopeptides representing individual phosphotyrosines of the ζ chain are underlined and their designation is shown under the sequence.

Differently phosphorylated forms of oligopeptides corresponding to the ζ ITAMs were synthetized and used as substrates for CD45. The sequence and the designation of the phospho-ITAMs are indicated in Table 5.2. The ζ(1)y^{P}y^P ζ(1)yy^P, ζ(2)y^{P}y^P and ζ(2)yy^P peptides were the optimal substrates since they were completely dephosphorylated by CD45 (Fig. 5.3.).

The ζ(2)y^{P}y and ζ(3)y^{P}y^P ITAMs were poorer substrates for CD45 and the ζ(1)y^{P}y, ζ(3)y^{P}y and ζ(3)yy^P were the least efficiently dephosphorylated phosphopeptides (Fig.5.3.A). Using the short peptides corresponding to half-ITAM sequences we did not found significant differences between the degree of dephosphorylation with the exception of the Cζ(2)py that was dephosphorylated by the highest degree (Fig.5.3.B.). This finding was according to the results obtained with the whole ITAM oligopeptides since Cζ(2)py corresponded to the ζ(2) C terminal phosphorylated ITAM.

A preferential hydrolysis of the C-terminal phosphotyrosine by CD45 has been observed in the case of both whole and half ITAM derived phosphopeptides. Moreover, the hydrolysis of the N-terminal phosphotyrosine in the ITAMs peptides was enhanced when the C-terminal tyrosine was in phosphorylated form (Fig.5.3.A.). Using CD43 immunoprecipitate in the phosphatase assay as a negative control, no inorganic phosphate release was detected (data not shown). The double phosphorylated peptides were dephosphorylated better as it was expected from the results obtained with sigle phosphorylated peptides. The best example was the ζ(3) peptide, where the N terminal phosphopeptide was hardly dephosphorylated (4%) and the C terminal phosphopeptide was dephosphorylated by a low rate (21%). In contrast, the double phosphorylated peptide was dephosphorylated much better (67%).

A

B
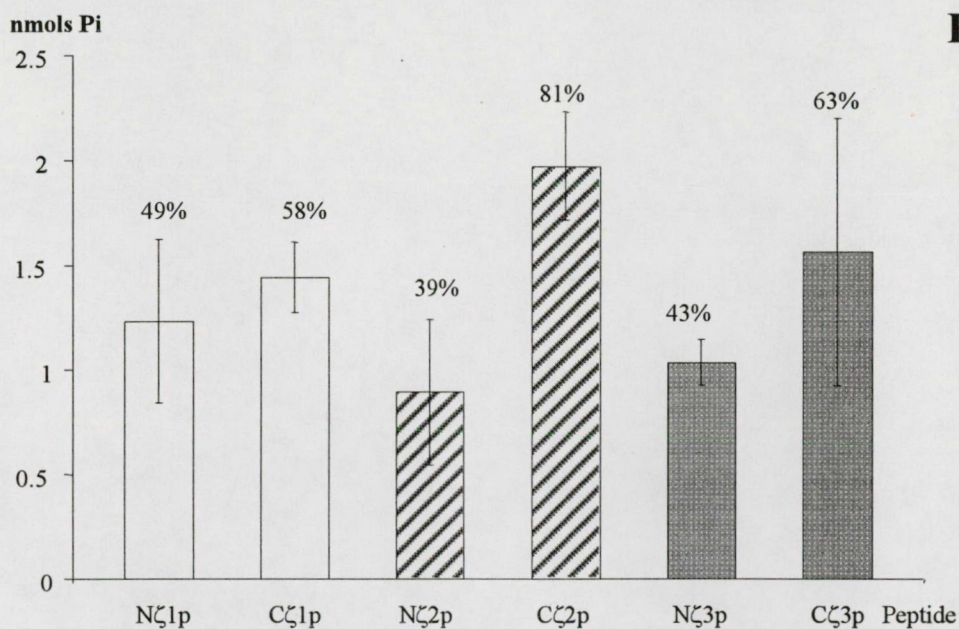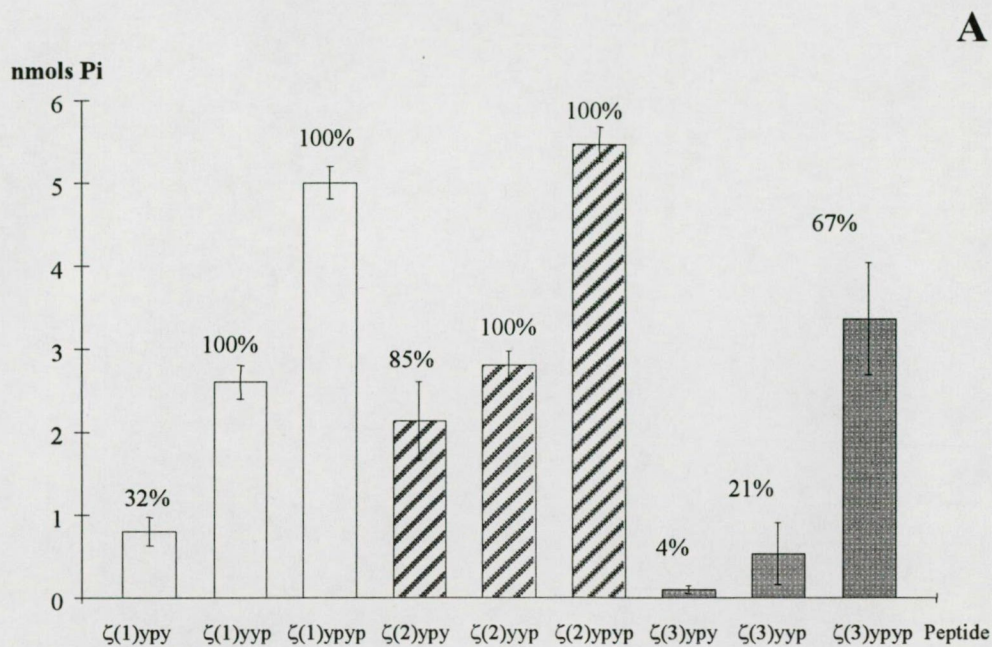
Fig.5.3. *In vitro* **dephosphorylation of the phosphopeptide substrates representing the differently phosphorylated ITAMs (A) or the individual phosphotyrosine residues (B) of the ζ chain by CD45.** CD45 immunoprecipitates were used for dephosphorylation of the phosphopeptide substrates listed in Table 5.2. The amount of released inorganic phosphate was measured as described under Section 3. The mean data and the standard deviation from three independent experiments are presented. Above the bars the numerical data represent the released phosphate as the percentage of the starting phosphate content of phosphopeptide substrates.

5.2.Study on the role of different phosphorylated forms of the ζ-ITAMs in the induction of the early biochemical events of T- cell activation

One of the earliest biochemical events during T cell activation is the tyrosine phosphorylation of the ITAMs in the TCR ζ chain and CD3 chains. The pattern of the phosphorylation may essentially regulate the outcome of the cell response to the extracellular signals. Our aim was to analyze the role of the phosphorylation of the individual ζ phospho-tyrosine residues in the induction of the early signaling events during T cell activation. To examine this question we analyzed the effect of synthetic oligopeptides representing the differently phosphorylated forms (N- or C-terminal, or both tyrosines) of the three ζ chain ITAMs on the early signaling events in permeabilized lymphocytes. The first step was to establish the proper conditions for permeabilization of cells used for intracellular tyrosine phosphorylation studies (136, 137).

5.2.1. Conditions for permeabilization of cells used for intracellular tyrosine phosphorylation

5.2.1.1.Selection of an optimal reagent for cell permeabilization

We compared different reagents, namely streptolysin O (SLO), digitonin and L-α lysphosphatidylcholine (LPC) for their ability to efficiently permeabilize leukaemic T cells and/or peripheral blood mononuclear cells (PBMC).

Streptolysin-O mediated permeabilization required a high concentration ($\geq$ 200 I.U/ml) and long incubation (30 minutes) to fully permeabilize 10^7 J6 cells or PBMC at 37^0C (Fig. 5.4.). Permeabilization with digitonin also required 10-30 min and it was not well reproducible (136).
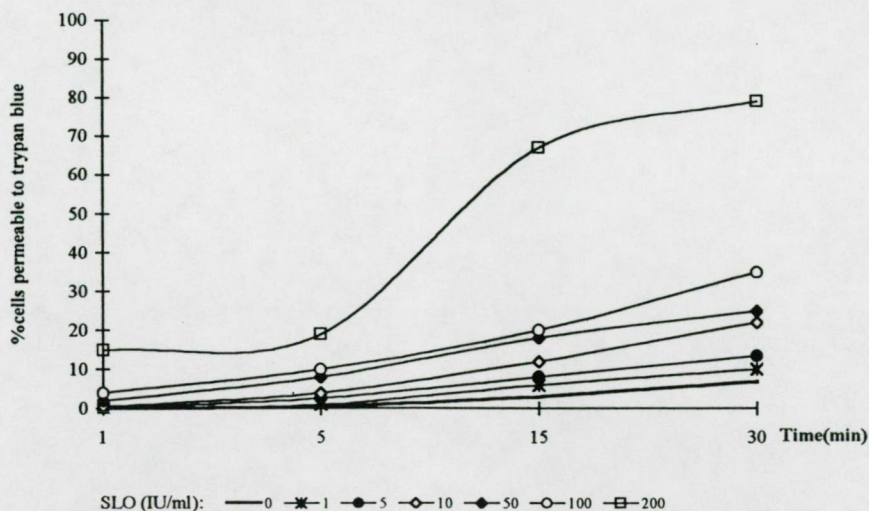
Fig.5.4 Kinetics and concentration-dependence of cell permeabilization with streptolysin-O (SLO). Jurkat T cells (10^7/ml) were incubated for the indicated time points with different amounts of SLO. Cell permeability was assesed using trypan blue exclusion assay.

The LPC mediated permeabilization has been the most reliable, because it was highly reproducible in the case of different types of cells (different leukaemic cell lines, PBMC) and the permeabilization was completed in a short time (less than 1 min). The amount of LPC, the appropriate time and the optimal temperature required for pemeabilization have been established in a serial of experiments. We found that LPC at 50 μg/ml permeabilized a wide variety of cell lines at 10^7-10^8 cell/ml concentration within 1 min (Fig. 5.5.). Permeabilization carried out at 4°C or 37°C was equally efficient (data not shown).
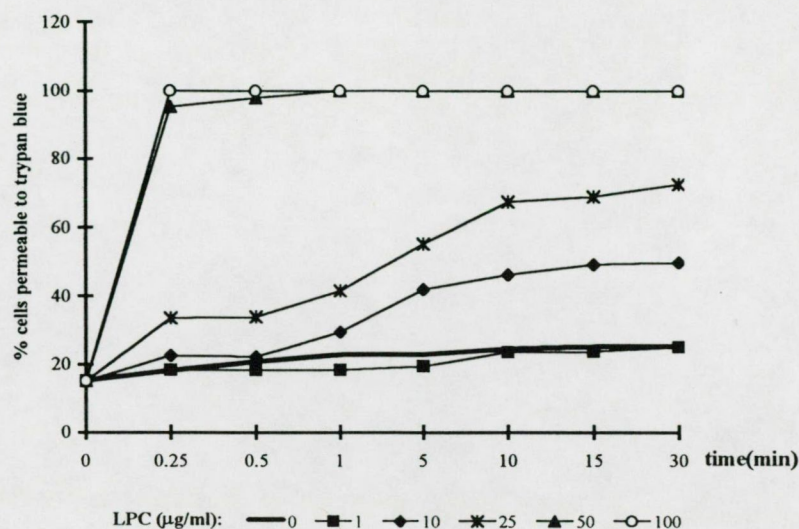
Fig. 5.5. Kinetics and concentration-dependence of cell permeabilization with lysophosphatidyl choline (LPC). Jurkat T cells (4×10^7/ml) were incubated for the indicated time points with different amounts of LPC. Cell permeability was assesed using trypan blue exclusion assay.

5.2.1.2. Conditions for low background protein tyrosine phosphorylation in permeabilized leukaemic cells

A common feature of the most frequently used permeabilizing reagents is that they induce a significant tyrosine phosphorylation in most of the leukaemic T cell lines, which may obscure the regulatory effect of the biomolecules which are tested (Chitu et. al. unpublished observation and 70, 72). Therefore, we aimed to establish a permeabilization procedure that resolves this problem.

First, we determined the temperature conditions and the composition of the permeabilization buffer that produce a low background protein tyrosine phosphorylation and a good signal to noise ratio in LPC permeabilized cells. We found that the temperature conditions during permeabilization dramatically influenced the overall tyrosine phosphorylation. Cells permeabilized at 37^0 C gave a high background signal

(Fig. 5.6. C. lane 1) while the cells permeabilized on ice showed a much lower basal tyrosine phosphorylation (Fig.5.6.B lane 1) (137).
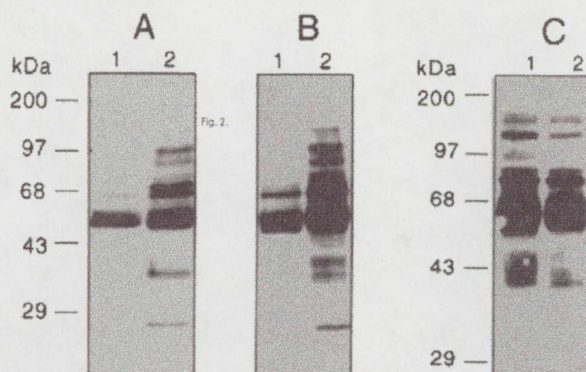
Fig. 5.6. Tyrosine phosphorylation induced by TCR in intact and permeabilized cells. Intact J6 cells (A) or J6 cells permeabilized with LPC on ice (B) or at 37°C (C) were stimulated with 10 µg/ml UCHT-1 for 2 min at 37°C (lanes 2) or were left unstimulated (lanes 1). The induction of protein tyrosine phosphorylation was analyzed by immunobloting using anti-phosphotyrosine 4G10.

When the low temperature was not strictly controlled during the permeabilization, namely the procedure was not done in a cold room, keeping the samples and reagents on ice, the LPC induced tyrosine phosphorylation in unstimulated cells was always observed (data not shown). We also assessed whether the induction of the background at higher
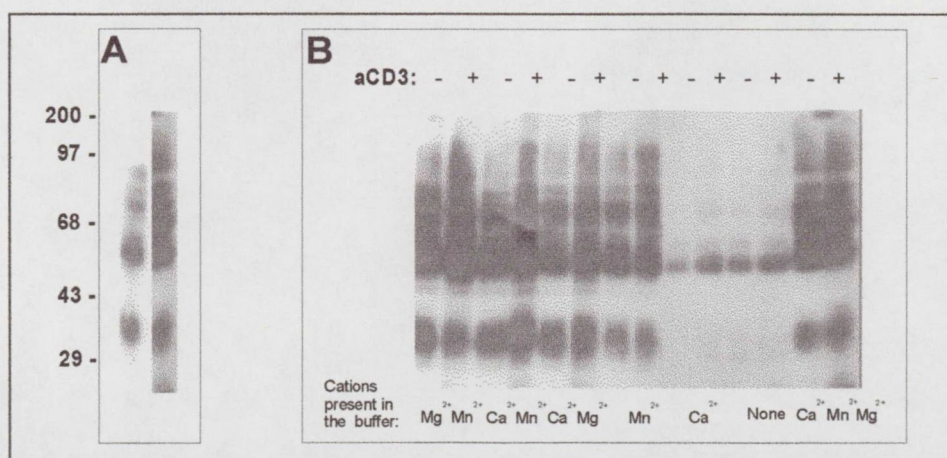
Fig. 5.7. Effect of the elimination of bivalent cations from the permeabilisation buffer on permeabilization-induced background. A. Non-permeabilized Jurkat T cells. non-stimulated or stimulated with 10µg/ml of anti-CD3 MAb UCHT-1. B. Cells were permeabilized with LPC for 1 min. at 37°C in 40 mM HEPES supplemented with the indicated cations at the concentrations specified in Section 4 (*Materials and Methods*) and stimulated with UCHT-1 or left unstimulated. The induction of protein tyrosine phosphorylation was analyzed by immunoblotting of SDS-PAGE fractionated whole cell lysates, with anti-phosphotyrosine Mab, 4G10.

temperatures than 4^0C can be avoided by elimination of bivalent cations like Ca^{2+}, Mg^{2+} and Mn^{2+} from the permeabilization buffer. We found that omitting any of the three ions from the permeabilizing buffer did not affect either the background or the induced phosphorylation when permeabilization was carried out at higher temperature (Fig5.7.). When Mg^{2+} and Mn^{2+} or all three cations were eliminated from the permeabilizing buffer, both the background and the TCR-induced tyrosine phosphorylation decreased dramatically (137). This result shows that kinase activity requires the presence of either Mg^{2+} or Mn^{2+} while Ca^{2+} presence is not obligatory for the activity of tyrosine kinases.

5.2.1.3 Responsiveness of permeabilized cells to anti TCR stimulation
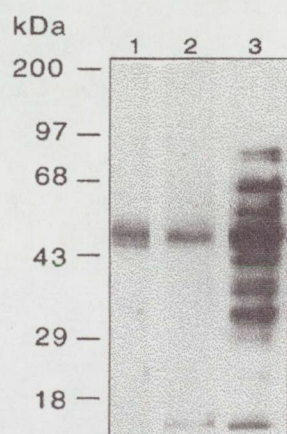
Fig.5.8. Stimulation of tyrosine phosphorylation by TCR ζ peptides in LPC-permeabilized J6 cells. Cells, permeabilized with LPC were untreated (lane1) or were treated with synthetic peptides derived from the second ITAM of TCR ζ chain in unphosphorylated (lane 2) or biphosphorylated (lane 3) forms.

We determined whether LPC permeabilized T cells were able to respond to TCR stimulation by analyzing the TCR induced tyrosine phosphorylation and whether this response was comparable to that of intact cells. As Figure 5.6. A and B shows, the background tyrosine phosphorylation in unstimulated cells (lanes 1) and the induced tyrosine phosphorylation in TCR activated cells (lanes 2) are comparable in intact (panel A) and permeabilized (panel B) cells when permeabilization occurred on ice. In contrast, permeabilization at 37°C increased the tyrosine phosphorylation in non-stimulated cells and concealed the anti-TCR induced tyrosine phosphorylation (Fig. 5.6. C.) Next we analyzed whether the cells, permeabilized in the above-described manner, were suitable for studying the tyrosine phosphorylation induced by a membrane impermeable synthetic peptide. A biphosphorylated peptide representing the second ITAM of the TCR ζ subunit (138, 139) and its non-phosphorylated counterpart were introduced into LPC permeabilized J6 cells. As the Fig. 5.8. shows, the phosphopeptide induced a high degree of phosphorylation (lane 3) compared to the unstimulated cells or cells stimulated with non-phosphorylated peptide (lanes 1 and 2 respectively) (137).

5.2.2 Effect of differently phosphorylated forms of the ζ-ITAMs on tyrosine phosphorylation in permeabilized T cells

5.2.2.1. Differently phosphorylated ζ-ITAM peptides induce partially distinct patterns of tyrosine phosphorylation in permeabilized Jurkat T cells.

Our aim was to determine whether the differently phosphorylated forms of the ITAMs found in the TCR ζ-chain may trigger qualitatively or quantitatively distinct early biochemical events of T- cell activation. For this purpose, a panel of mono-, bi- and non-phosphorylated oligopeptides corresponding to each ITAM in the ζ-chain (Table 5.2.) were introduced into permeabilized Jurkat T cells and the induction of protein tyrosine phosphorylation was analyzed by anti-phosphotyrosine blotting. As Fig.5.9.shows, the effect of ITAM peptides on the pattern of the tyrosine phosphorylation of intracellular substrates was dependent on the primary structure and phosphorylation pattern of the phospho-peptides. Permeabilizing buffer alone or the non-phosphorylated peptides had essentially no effect (Fig.5.9.). Both qualitative and quantitative differences were observed in the ability of phospho-ITAMs to induce tyrosine phosphorylation (Fig 5.9
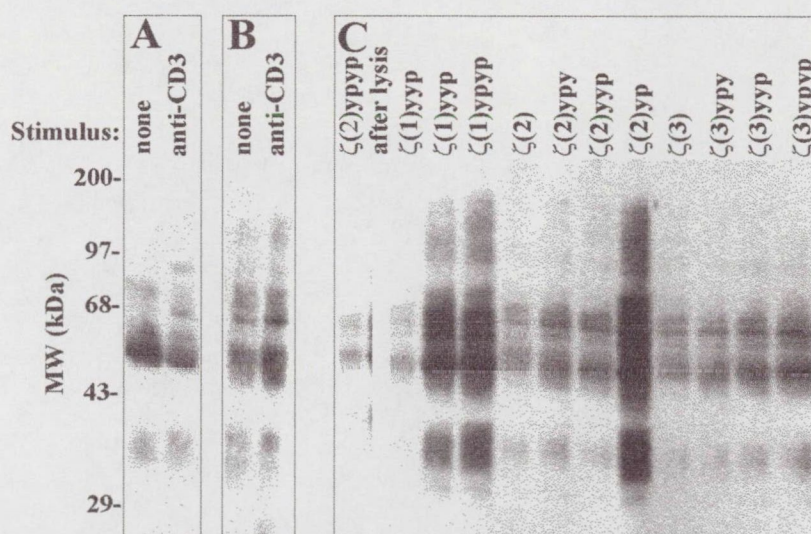
Fig. 5.9. Whole-cell protein tyrosine phosphorylation in Jurkat T cells stimulated with anti-CD3 mAb or differently phosphorylated ζ–ITAM peptides. *A.* Intact cells were stimulated for 3 min with anti-CD3 mAb UCHT-1 or left unstimulated. *B* and *C.* L-α lysophosphatidylcholine permeabilized cells were stimulated for 2 min with either anti-CD3 mAb or ζ-ITAM peptides. Whole-cell lysates were resolved by 10% SDS-PAGE and immunoblotted with anti-phosphotyrosine mAb 4G10.

and Table 5.3.). For example, the phosphorylation of proteins with high molecular weights (69- 150kDa) was induced only by $\zeta(1)yy^P$, $\zeta(1)y^Py^P$ and $\zeta(2)y^Py^P$. Moreover, the increase in the phosphorylation of several proteins such as p51-53, p46-48, p42-43 was induced by all phospho- ζITAM peptides except for the N-terminus phosphorylated forms although in variable extent. Similarly, the increased phosphorylation of proteins with molecular masses 33-35 and 31-32 kDa was induced by all phospho- ζITAM peptides except for the N-terminus phosphorylated forms of the first and third ITAM ($\zeta(1)y^Py$ and $\zeta(3)y^Py$, respectively).

Tyrosine phosphorylation of p29 was induced only by cell stimulation with anti-CD3 mAb or $\zeta(1)yy^P$, $\zeta(1)y^Py^P$ and $\zeta(2)y^Py^P$ (Table 5.3.). As shown in Fig. 5.9. and in Table 3, there are quantitative differences in the ability of phospho-ITAMs to induce tyrosine phosphorylation of intracellular proteins. The most potent inductors of tyrosine phosphorylation were the peptides corresponding to the biphosphorylated forms of ITAM1 and ITAM2 ($\zeta(1)y^Py^P$ and $\zeta(2)y^Py^P$), followed by $\zeta(1)yy^P$ and $\zeta(3)y^Py^P$. In contrast, $\zeta(1)y^Py$ and $\zeta(3)y^Py$ showed the poorest capacity to induce tyrosine phosphorylation of intracellular proteins. Similar patterns of tyrosine phosphorylation were found when PBMC were used (not shown). Effective induction of protein phosphorylation was dependent on cellular architecture, since addition of $\zeta(2)y^Py^P$ (one of the most potent

Fig. 5.10. Dose-dependence of TCR $\zeta(2)y^Py^P$ ITAM-induced tyrosine phosphorylation. Permeabilized J6 cells were stimulated for 3 min with different concentrations of $\zeta(2)y^Py^P$ peptide as indicated. The induction of protein tyrosine phosphorylation was analyzed by SDS-PAGE of whole cell lysates followed by imunoblotting with anti-phosphotyrosine mAb, 4G10.

inductor of tyrosine phosphorylation) following cell lysis did not induce any response (Fig5.9.).

The phospho-ITAM stimulation was time and concentration dependent. Protein tyrosine phosphorylation was detected using $\zeta(2)y^P y^P$ peptide at a concentration higher than 100 μM (Fig5.10.). The requirement for such a high concentration of peptide was consistent with the reported binding affinities (nM-μM) of biphosphorylated ITAMs for the SH2 domains of ZAP-70 (70). Another reason for the requirement of this high concentration of peptides might be that the phospho-ITAM peptides were not protected and therefore they could be rapidly dephosphorylated by intracellular protein tyrosine phosphatases. Under these conditions, only a small portion of phospho-ITAM peptide may reach its site of action.
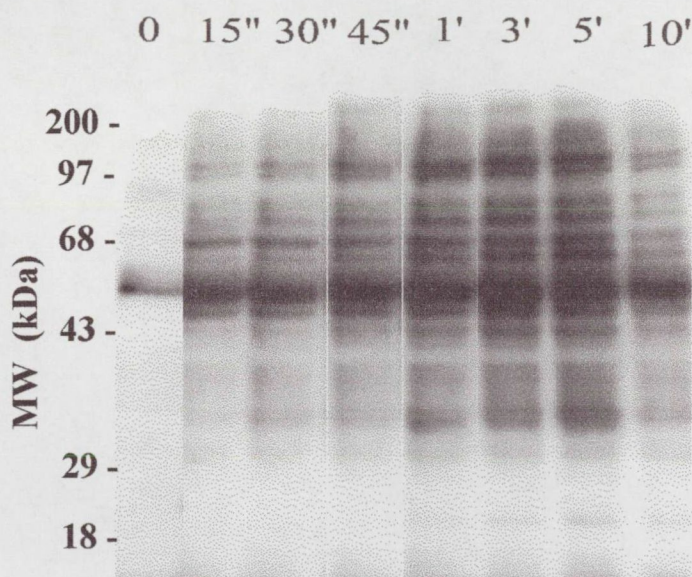
Fig. 5.11. Kinetics of $\zeta(2)y^P y^P$ ITAM (500 μM)- induced protein tyrosine phosphorylation. Permeabilized J6 cells were stimulated with 500 μM $\zeta(2)y^P y^P$ peptide for the indicated time points. Detergent-soluble proteins were separated by 10% SDS-PAGE, followed by immunoblotting with antiphosphotyrosine mAb. The numbers on the left indicate the migration of molecular weight markers in kilodaltons.

Induction of protein tyrosine phosphorylation occurred as fast as 15 sec following stimulation, and maximal phosphorylation was observed between 1 and 5 min (Fig. 5.11.). High dose (500μM) of non-phosphorylated ζ peptides, used as controls, did not induce tyrosine phosphorylation of intracellular substrates within 3 min of stimulation (Fig. 5.9.), indicating that the tyrosine residues were not phosphorylated in permeabilized cells (Fig.5.9.). Therefore, the data obtained using single phosphorylated or non-phosphorylated peptides reflect the function of the particular peptide and do not show the function of a peptide modified by the cellular signal transduction machinery.

5.2.2.2. Analysis of the role of different tyrosine kinases in the ITAM-induced tyrosine phosphorylation

In order to determine the role of p56^{lck} and ZAP-70 – two tyrosine kinases essential for T cell activation- in the induction of tyrosine phosphorylation by phospho-ζITAMs, we compared the effect of ζ–ITAM peptides in Jurkat T line, the p56^{lck} deficient JCaM 1.6. and the P116 Syk/ZAP deficient Jurkat variants. As shown in Figure5.12. and Table 5.3. the pattern of phospho- ITAM induced tyrosine phospho-proteins in J CaM 1.6 cells was partially different from that of Jurkat T cells. In JCaM1.6. cells the ζ(1)ITAM peptides did not induce phosphorylation of proteins with molecular mass of 140-150 and 103-107 kDa and the phosphorylation of proteins with molecular mass 78-80, 61-63, 56-57 and 51-53 kDa was much weaker compared to that of Jurkat. In the case of ζ(2) phospho-peptides, the N-terminus phosphorylated form of the ITAM2 peptide, ζ2y^{P}y was unable to induce tyrosine phosphorylation in JCaM 1.6.
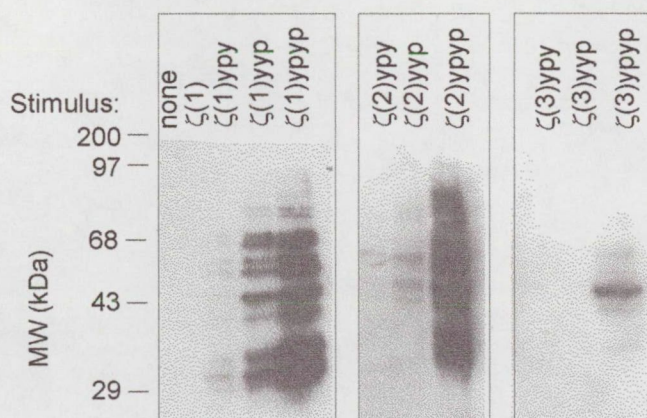
Fig. 5.12. Whole cell protein tyrosine phosphorylation in JCaM 1.6. T cells stimulated with phosphorylated and non-phosphorylated -ITAM peptides. Permeabilzed cells were either left unstimulated or were stimulated for 3 min with 500 µM peptides corresponding to the different phospho-forms of TCR ζ-chain ITAMs. Whole cell lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine mAb 4G10. The migration of molecular weight markers is indicated at the left in kDa.

The peptide corresponding to the C-terminus phosphorylated form of the ζ(2) failed to induce tyrosine phosphorylation of proteins with molecular mass of 61-63, 56-57, 35-37 and 31-33 kDa, in JCaM 1.6. as compared to Jurkat cells (Table 5.3.). The tyrosine phospho-proteins of molecular mass 61-63, 56-57, 33-35 and 31-33 kDa were induced by ζ(2)y^{P}y only in Jurkat. Moreover, the stimulation of Jurkat but not JCam 1.6. cells with the ζ(2)y^{P}y^{P} peptide resulted in the phosphorylation of proteins

with molecular masses of 140-150 and 103-107 kDa. The effect of monophosphorylated $\zeta(3)$ peptides seemed to be totally dependent on p56^{lck} activity, since they did not induce any signal in JCaM 1.6. The stimulation of JCaM 1.6. T cells with biphosphorylated $\zeta(3)$ peptide ($\zeta(3)y^Py^P$) resulted in phosphorylation of a single band with a relative molecular mass of 46-48 kDa.

In order to establish the contribution of *Syk* family kinases in the induction of tyrosine phosphorylation by phospho-ITAM peptides, we used P116 cells - a recently characterized Syk/ZAP deficient variant of Jurkat (96). As previously reported by
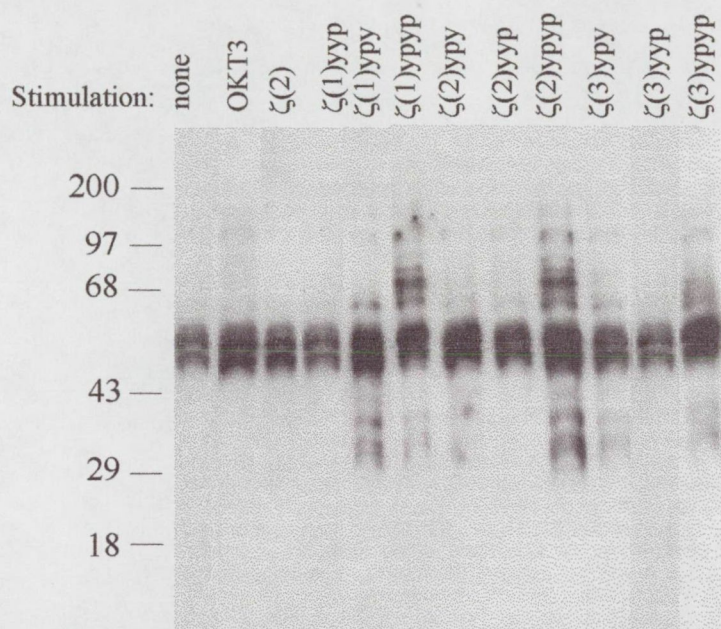
Fig. 5.13. Whole cell protein tyrosine phosphorylation in P116 cells stimulated with phosphorylated and non-phosphorylated ζ-ITAM peptides. Permeabilized cells were either left unstimulated or stimulated for 3 min with 10μg/ml anti CD3 mAb OKT3 or with 500 μM ζ-peptides. Whole cell lysates were resolved by SDS-Page and immunobloted with anti-phosphotyrosim\ne mAb 4G10. The migration of molecular weight markers is indicated on the left in kilodaltons.

Williams et al., (96) P116 cells showed no consistent increase in protein tyrosine phosphorylation in response to anti CD3 antibodies (Fig. 5.13 lanes none and OKT3), while in wild type Jurkat T cells TCR stimulation triggered rapid increase in tyrosine phosphorylation of several proteins (Fig. 5.9. and Table 5.3.). The first and second ITAM peptides, when biphosphorylated, induced a reasonable tyrosine phosphorylation in P116 cells (Fig. 5.13 and Table 5.3.).

49

Table 5.3. Qualitative and quantitative differences in the effect of differentially phosphorylated ζ-ITAM peptides on intracellular protein tyrosine phosphorylation in permeabilized T cell lines (wild type Jurkat and the p56^{lck}-negative JCaM 1.6. and ZAP/Syk negative P116 Jurkat variants). The table contains the evaluation of tyrosine phosphorylated bands that reproducibly appeared in two or three independent experiments. Empty cells indicate the absence of the band, shadowed cells indicate increased phosphorylation as compared to the nonstimulated control; ND: not determined.

Possible identity (based on the MW)		PLCγ Fyb	Vav	SLP-76	ZAP-70 Syk EMT Tec	Phospho-fyn/ phospho-lck Tec	p56^{lck} tubulin Rlk	Shc Rlk	Shc	?	LAT	?	?
MW (kDa)		140-150	103-107	78-80	69-71	61-63	56-57	51-53	46-48	42-43	35-37	31-33	29-30
Nonstimulated control	Jurkat					+/-	+/-	+/-	+	+/-	+/-	+/-	
	JCam												
	P116								2+	2+			
Anti-CD3	Jurkat				+	+	+/-	+	+	+	+	+	+
	JCam					ND							
	P116								3+	3+			
Non-phosphorylated ITAM	Jurkat					+/-	+/-	+/-	+	+/-	+/-	+/-	
	JCam												
	P116								2+	2+			
$\zeta(1)y^p y$	Jurkat					+	+	+/-	+	+/-	+/-	+/-	
	JCam												
	P116								2+	2+			
$\zeta(1)yy^p$	Jurkat	+	+/-	+	+	4+	4+	4+	3+	2+	2+	2+	+
	JCam			+/-	+	+	+	+	2+	2+	2+	2+	+
	P116						+		2+	2+	+	+	+
$\zeta(1)y^p y^p$	Jurkat	2+	+	+	2+	4+	4+	4+	3+	2+	3+	4+	+
	JCam			+/-	2+	2+	2+	2+	2+	2+	3+	3+	+
	P116			+		2+	+		3+	3+	+	+	+
$\zeta(2)y^p y$	Jurkat					2+	3+	+/-			+	+	
	JCam												
	P116						+/-		3+	3+			
$\zeta(2)yy^p$	Jurkat					2+	3+	+/-	+/-		+	+	
	JCam						+/-	+/-	+/-				
	P116								3+	3+			
$\zeta(2)y^p y^p$	Jurkat	3+	3+	2+	3+	4+	4+	4+	3+	3+	3+	3+	+
	JCam			3+	3+	3+	3+	3+	3+	3+	3+	3+	+
	P116		+	+		2+	+		3+	3+	+	+	+
$\zeta(3)y^p y$	Jurkat					+/-	+	+/-	+	+/-	+/-	+/-	
	JCam												
	P116						+		2+	2+			
$\zeta(3)yy^p$	Jurkat					+	+	+	2+	+	+	+	
	JCam												
	P116								2+	2+			
$\zeta(3)y^p y^p$	Jurkat					2+	2+	2+	2+	+	2+	2+	
	JCam								2+				
	P116					+	+		3+	3+	+/-	+/-	

However, the intensity of ITAM-induced phosphorylation was lower in P116 compared to Jurkat and, tyrosine phosphorylation of proteins with molecular mass of 150, 69-71 and 51-53 kDa could not be induced in P116.

5.2.2.3. Phospho-ITAM peptides induce phosphorylation of p56lck and ZAP-70 protein tyrosine kinases.

The earliest steps in TCR-mediated signal transduction are the phosphorylation and activation of *Src* and S*yk* family tyrosine kinases. To determine whether these events were triggered by phosphorylated ζ ITAMs, phospho-ITAM peptides were introduced into permeabilized Jurkat cells in the presence of γ^{32}P-ATP and kinase phosphorylation was assessed.
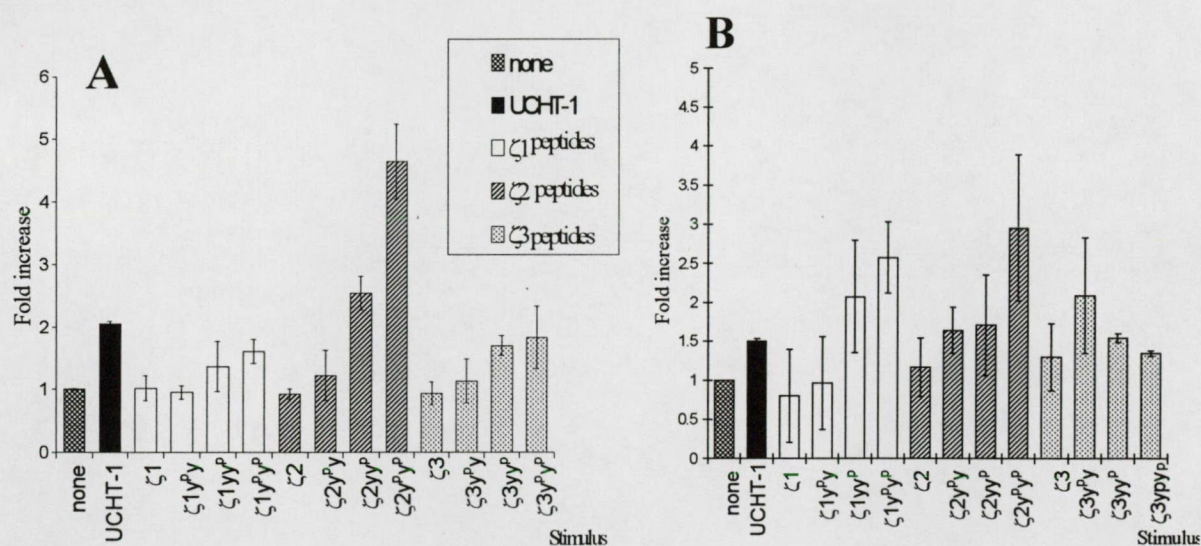
Fig.5.14. Incorporation of ^{32}P into p56^{lck} (A) and ZAP-70 (B) after stimulation of Jurkat T cells with ζ-ITAM phosphopeptides. Permeabilized Jurkat T cells were left unstimulated (none) or stimulated either with 500 µM of ζ peptide or with 10 µg/ml anti CD3 mAb UCHT-1 in the presence of [γ-^{32}P] ATP as described under section *Materials and Methods*. The increase in phosphorylation upon stimulation is expressed as the ratio between ^{32}P incorporated in p56^{lck} and ZAP kinases after stimulation and the amount of ^{32}P incorporated without stimulation.

Fig. 5.14. shows the changes in Lck and ZAP-70 phosphorylation induced by different ζ ITAM peptides. The effect of phospho-ITAM peptides on tyrosine phosphorylation of Lck (Fig.5.14. A) and ZAP-70 (Fig. 5.14. B) was dependent on the primary structure and phosphorylation pattern of the phospho-peptides. The phosphorylated ζ-ITAM peptides

displayed the following hierarchy of increasing Lck phosphorylation: $\zeta(2)y^Py^P>>\zeta(2)yy^P>\zeta(3)y^Py^P\geq\zeta(3)yy^P\geq$ $\zeta(1)y^Py^P>\zeta(1)yy^P>\zeta(2)y^Py$ $>\zeta(3)y^Py\geq\zeta(1)y^Py$. For ZAP phosphorylation the hierarchy was different: $\zeta(2)y^Py^P>\zeta(1)y^Py^P>$ $\zeta(1)yy^P\geq\zeta(3)y^Py$ $>\zeta(2)yy^P\geq\zeta(2)y^Py\geq\zeta(3)yy^P>\zeta(3)y^Py^P=\zeta(1)y^Py$.

5.2.2.4. Protein tyrosine phosphatase inhibitor, PAO modifies the phosphorylation pattern of intracellular proteins induced by ζ-ITAM phosphopeptides.

In order to determine the contribution of protein tyrosine phosphatases (PTPs) to the tyrosine phosphorylation induced by the ζ-ITAM phosphopeptides, we performed experiments where permeabilized T cells were stimulated with peptides in the presence or absence of a PTP inhibitor, phenylarsine oxide (PAO). As Fig.5.15. shows, ζ-ITAM phosphopeptides induced a stronger tyrosine phosphorylation in Jurkat T cells in the absence of 15mM PAO as they did in the presence of PAO. Treatment of permeabilized cells with PAO alone induced phosphorylation of proteins of molecular masses 63, 56, 46, 42 and 29 kDa as compared to the untreated cells (Fig.5.15). Phosphopeptide – induced phosphorylation of proteins with molecular masses of 150, 120, 102 and 75 kDa was completely inhibited, while peptide-induced phosphorylation of proteins with molecular masses of 46, 43, 36, 30 and 29 kDa was essentially not affected by PAO (Fig 5.15 and Table 5.4.). The peptide-induced phosphorylation of proteins with molecular masses 63, 56, 53, 34 and 28 kDa was decreased in the presence of PAO.
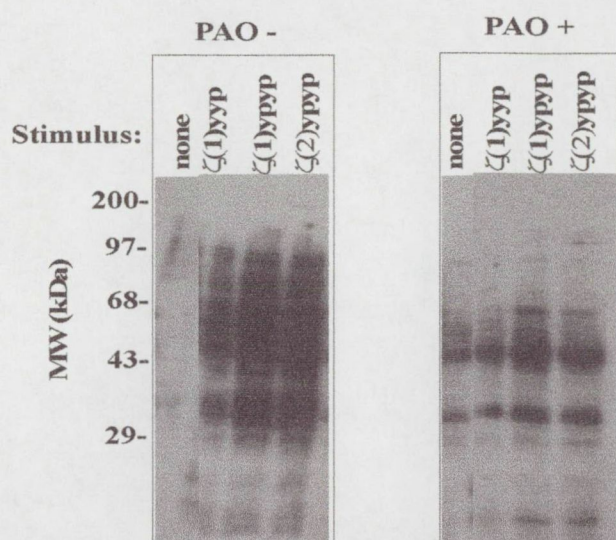
Fig. 5.15. Effect of phenylarsine oxide (PAO) on ζ-ITAM induced tyrosine phosphorylation in Jurkat T cells. Permeabilized Jurkat T cells were left unstimulated (none) or were stimulated for 3 min with 500 μM phospho-ITAM peptide in the presence (PAO+) or the absence (PAO-) of 15 mM PAO. Tyrosine phosphoproteins from whole cell lysates were detected by anti-phosphotyrosine blotting. The migration of molecular weight markers is indicated on the left in kilodaltons.

Table 5.4. Effect of tyrosine phosphatase inhibitor PAO on the ζ-ITAM peptides induced protein tyrosine phosphorylation in Jurkat T cells. Empty cells indicate the absence of the band, shadowed cells indicate increased phosphorylation as compared to the nonstimulated control.

Possible identity (based on MW)		PLCγ	P120/130 (Fyb)	Vav	SLP-76	pfyn/ p60lck Tec	p56^{lck} tubulin Rlk	Shc Rlk	Shc	?	LAT	?	?	?	?
MW (kDa)		150	120	102	75	63	56	53	46	43	36	34	30	29	28
Nonstimu -lated control	PAO -					+/-	+/-	+/-	+/-	+/-				+	+/-	
	PAO+					+	+	+	+					+	+	
ζ(1)yy^p	PAO -	++	++	++	++	+++	++	++	++	+++	++	+	++	++	+
	PAO+					+	+	+	++	++	++	+/-	+	++	+/-
ζ(1)y^{p}y^p	PAO -	+++	+++	+++	+++	++++	+++	+++	+++	++++	+++	++	+++	+++	++
	PAO+					++	+	++	+++	+++	+++	+	+++	+++	+
ζ(2)y^{p}y^p	PAO -	+++	+++	+++	+++	++++	+++	+++	+++	++++	+++	++	+++	+++	++
	PAO+					++	+	++	+++	+++	+++	+	+++	+++	+

5.2.3. ζ-ITAM1 and ζ-ITAM2 but not ζ-ITAM3 phosphopeptides induce association of Grb-2 with tyrosine phosphorylated proteins

In order to determine whether phospho-ζ peptides regulate T cell response only by modulating tyrosine phosphorylation or by affecting adaptor protein mediated molecular interactions as well, we examined the changes in the pattern of growth factor receptor binding protein 2 (Grb-2) associated phosphoproteins in ζ peptide stimulated T cells. Grb 2 is an adaptor protein which ensures the link between TCR and Ras activation pathway.

We have used GST-Grb2 fusion protein to affinity purify proteins that associate with Grb-2 from lysates prepared from non-stimulated or phospho-peptide stimulated J6 T cells. The data on Figure.5.16.show that the Grb-2 fusion protein precipitates multiple proteins that are tyrosine phosphorylated in peptide-stimulated cells.
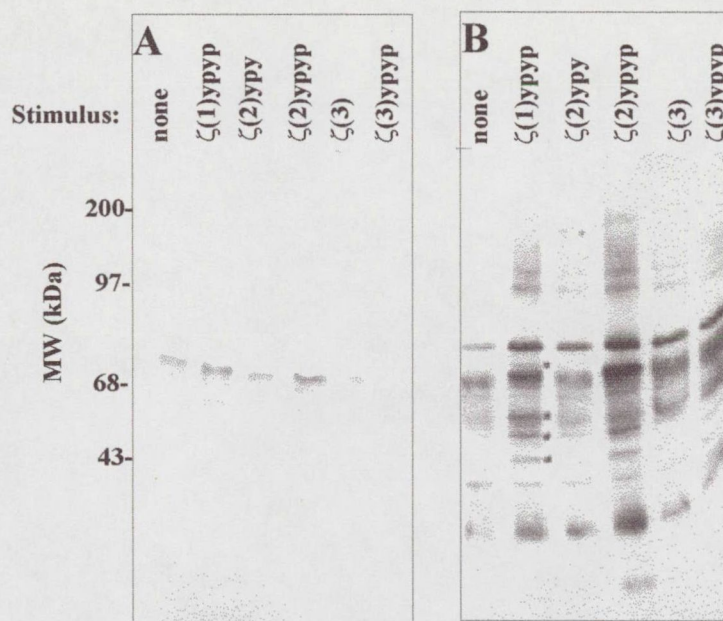
Fig. 5.16. Analysis of the association of tyrosine posphorylated proteins isolated from ITAM-stimulated T cells with GST-Grb-2 fusion protein. J6 cells were stimulated with anti-CD3 mAb UCHT-1 or with the indicated ζ-ITAM peptides. Cellular proteins were precipitated using either glutathione-agarose beads (A) or glutathione-agarose beads preloaded with GST-Grb2 fusion protein (B). Affinity-purified proteins were subjected to 10% SDS-PAGE and analyzed by immunobloting with the anti-phosphotyrosine mAb 4G10. The migration of the molecular mass standards is indicated to the left in kilodaltons.

The pattern of peptide-induced associations is different depending on the primary structure and the phosphorylation site of the peptides. Biphosphorylated $\zeta(1)$ITAM peptide ($\zeta(1)y^P y^P$) induced Grb-2 association with tyrosine phosphoproteins of 127, 113, 52, 46, 36 and 27 kDa and increased Grb-2 association with proteins of 85, 71 and 55 kDa as compared to the non-stimulated control. Biphosphorylated $\zeta(2)$ ($\zeta(2)y^P y^P$) induced the association of Grb-2 with identical proteins as $\zeta(1)y^P y^P$ did and with three additional proteins with molecular mass of 175, 153 and 20 kDa. The N-terminus phosphorylated $\zeta(2)$ITAM ($\zeta(2)ypy$) induced only a single new association with p36. In contrast, biphosphorylated $\zeta(3)$ITAM ($\zeta(3)ypyp$) as well as the nonphosphorylated $\zeta(3)$ITAM ($\zeta(3)$) peptides did not induce neither new association nor the increase in Grb-2 association with the constitutively associated proteins.

5.3. Phospho-ζ ITAM peptides induce tyrosine phosphorylation in B cells
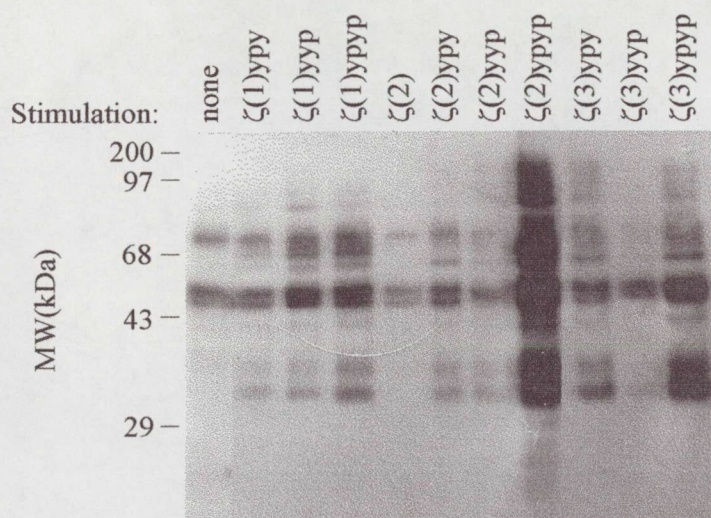
Fig. 5.17. Effect of ζ-ITSAM peptides in B cell line BL-41. Cells were stimulated for 3 min with 500 µM ζ-ITAM peptides or left unstimulated. Whole cell lysates were resolved by SDS-PAGE and immunobloted with antiphosphotyrosine mAb 4G10.

The well documented structural and functional conservation of the ITAMs in lypmhocyte signalling, prompted us to determine the effect of ζ-ITAM phospho-peptides on tyrosine phospho-rylation in B cells. As Fig. 5.17. shows, different ζ chain ITAMs are able to induce distinct patterns of tyrosine phosphorylation in B cells. Similarly to T cells, the effect of ITAMs was dependent on their primary structure and the site of phosphorylation. The $\zeta(2)y^P y^P$ peptide was the most potent inductor of tyrosine phosphorylation both in T and B cells. However, unlike T cells where $\zeta(1)$ peptides $\zeta(1)yy^P$ and $\zeta(1)y^P y^P$ were very

effective in stimulation of tyrosine phosphorylation, in B cells these peptides were much less active. Furthermore, the $\zeta(3)$ peptides $\zeta(3)y^Py$ and $\zeta(3)y^Py^P$ that were almost inactive in T cells, were very efficient in inducing tyrosine phosphorylation in B cells.

6. Discussion

6.1. Src-family PTKs and CD45 act together in the generation of TCR ζ chain phosphorylation

Phosphorylation of multiple tyrosine residues within the ζ polypeptide is crucial for the recruitment of effector molecules into the TCR complex. Differently phosphorylated forms of the ζ chain subunit can be found in T cells at distinct differentiation stages or activated under different conditions (150). The different ζ ITAM sequences may be responsible for connecting the TCR to various effector pathways. Several important signalling and adaptor proteins, such as ZAP70, Shc, Grb2, $p59^{fyn}$ and the p85 subunit of PI3K bind to the phosphorylated TCR ITAM sequences *via* their SH2 domains *in vitro* (83, 153, 154) on a sequence and phosphorylation dependent fashion (68-74). Therefore, the composition of the signalling complex formed on the internal face of the TCR may depend on the phosphorylation pattern of the ζ and CD3 chains and may regulate the eventual cell response. However, the mechanism of formation of the distinct phosphorylation patterns and the contribution of the kinases and phosphatases to this process have not been yet revealed. Therefore, first we have investigated the phosphorylation of a substrate panel derived from the human ζ chain in the presence of different *Src* family kinases.

Our results showed that the *Src* kinases, $p56^{lck}$ and $p59^{fyn}$, that have been implicated in TCR signalling (135), phosphorylated the ζ chain-related oligopeptides. A higher intensity of overall phosphorylation was detected using $p56^{lck}$ compared to $p59^{fyn}$. These results were consistent with previous findings that under physiological conditions $p56^{lck}$ likely represented the relevant kinase acting in ζ chain phosphorylation (151). However, it can not be excluded that the detected difference was due to the distinct affinity of the antibodies used for the isolation of the enzymes. The experiments concerning the phosphorylation of the seven individual tyrosines located in the

intracellular part of the human ζ chain, showed that six of them were phosphorylated by Src family tyrosine kinases p56^{lck} and p59^{fyn}, but not by ZAP-70. All tyrosine residues susceptible to phosphorylation were located in the functionally important conserved ITAMs. The non-conserved, membrane proximal tyrosine was not phosphorylated by either src or syk kinases or by cell free extract prepared from HPB-ALL cells (138), suggesting that this tyrosine did not have a phosphorylation dependent function. The patterns of the peptide phosphorylation generated by different Src kinases, including those which were irrelevant in TCR signalling (138), such as p59^{fyn} and p60^{src} were similar. Similarity in the patterns meant that the various Src kinases did not show different specificity toward the peptides. These results suggested that the Src kinases were not at all or might not be the only components regulating the generation of different phosphorylation patterns in the ζ chain.

The phosphorylation stage of the proteins is determined by the balance between the kinases and phosphatases. The main tyrosine phosphatase that may control the phosphorylation pattern of the ζ chain is CD45. Therefore we have examined the role of, CD45, in this process. The results showed that each ITAMs phosphorylated on the N-terminal tyrosine were poorer substrates for CD45 compared to the identical peptide phosphorylated on C-terminal tyrosine residues. It was observed that the dephosphorylation of the C-terminal phosphotyrosine in $\zeta(1)$ and $\zeta(2)$ ITAMs was complete, independently of the phosphorylation state of the N-terminal tyrosine residue. In contrast, the hydrolysis of the N-terminal phosphotyrosine in these peptides was enhanced when the C-terminal tyrosine was in phosphorylated form. When doubly phosphorylated peptides of any ITAMs were used as substrates, the amount of released Pi was greater than the sum of Pi released from the N- and C-terminal monophosphorylated peptides. This suggested that the interaction of CD45 with one of the phosphotyrosine residues increased its activity toward the second phospho-tyrosine. Substrate binding may alter catalytic activity and/or affinity of CD45 PTPase domain 1 or may induce the cryptic PTPase activity of domain 2. Although, the $\zeta(3)$ phospho-peptides were the weakest substrates, the dephosphorylation efficiency of both the C and N-terminal tyrosine was dramatically influenced by the phosphorylation state of the neighbouring tyrosine residue.

Despite the observation that CD45 showed a preference toward the C terminal phospho-tyrosines in either the short or the full length ITAM peptides, there was a much less pronounced difference in the dephosphorylation rate of the different short phosphopeptides compared to the whole ITAM sequences. A possible explanation for this may be that the short peptides did not have the proper conformation and/or did not contain aminoacids important for the substrate specificity. This hypothesis was supported by data published previously by Zhang et al. (152) which showed that the *Yersinia* PTPase and rat PTP-1 substrate specificity for EGF receptor derived phosphopeptides was primarily dictated by residues located to the N-terminal side of the phosphotyrosine residue.

The ability of CD45 to discriminate between the different ITAM-derived phosphopeptides suggests that CD45 may play a role in formation of a functional pattern of the phosphorylated ζ chain.

We suggest that the *Src* family kinases and the CD45 tyrosine phosphatase act together in controlling the ζ chain phosphorylation. The ability of the CD45 to distinguish between the various tyrosine residues may play an essential role in the formation of a functional phosphorylation pattern 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.

6.2. L-α lysophosphatidyl choline permeabilized cells are suitable tools for intracellular tyrosine phosphorylation studies

Permeabilized cells are widely applied for studying the effect of membrane impermeant biomolecules on signalling pathways. After permeabilization with reagents commonly used for this purpose, the architecture of the cells remains essentially intact and hence they retain their ability to respond to cell surface receptor stimulation as far as early cellular responses are concerned. Early signalling events, such as tyrosine and serine phosphorylation of cellular proteins (70, 72, 149), generation of cAMP (156) or inositol-phosphate hydrolysis (157, 158) can be investigated after the stimulation of permeabilized cells. However, a common feature of most of the permeabilizing reagents is that they

induce significant tyrosine phosphorylation in most of the leukaemic cell lines, which may obscure the regulatory effect of the biomolecules which are tested (70, 72, 137). We selected L-α lysphosphatidylcholine as permeabilizing reagent, because it effectively permeabilized many types of cells within 1 min and generated pore size that allowed molecules as large as 150 kDa to enter the cells (137). The results show that the well-controlled, low temperature during permeabilization is a key factor for successful usage of permeabilized cells for signal transduction studies. Furthermore, the procedure described by us allows a fast and efficient permeabilization of a variety of leukaemic cell lines without affecting the responsiveness of the cells to external stimuli (137).

6.3. Different ζ-ITAMs play distinct role in early biochemical events of T- cell activation

The presence of multiple of ITAMs within the ζ chain raises the question whether the individual ITAMs and their differently phosphorylated forms may function as docking regions for distinct effector molecules in order to activate distinct signalling pathways or it may simply provide a mechanism for signal amplification. To answer this question, we analyzed the effect of synthetic oligopeptides representing the differently phosphorylated forms (N-, C-terminal, or both tyrosines) of the three ζ chain ITAMs on the early signalling events in permeabilized leukaemic T cells.

Our findings showed that synthetic phosphopeptides representing the differently phosphorylated forms of the ζ-ITAMs were capable of inducing protein tyrosine phosphorylation of partially distinct sets of substrates, when introduced into T cells. Effective induction of protein phosphorylation was dependent on cellular architecture, since addition of $\zeta(2)y^p y^p$ (one of the most potent inductor of tyrosine phosphorylation) following cell lysis did not induce any response. 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 (54, 88), one possible explanation for the ITAM-induced increase in tyrosine phosphorylation of intracellular substrates is the modulation of the activity of these kinases. Further evidence that the phospho-ITAM induced increase in tyrosine phosphorylation was

mediated at least partially by tyrosine kinases involved in TCR signalling comes from the experiments using Jurkat variants, deficient in kinases. When the kinase deficient cell lines were stimulated with phospho-ITAM peptides different patterns of tyrosine phosphorylation were found as compared to Jurkat. As it is summarised in Table 6.1., kinase requirements for induction of phosphorylation by the different forms of the ζ ITAMs are considerably different. All N-terminal phosphorylated ITAMs ($\zeta(1)y^Py$, $\zeta(2)y^Py$ and $\zeta(3)y^Py$) and the C-terminal phosphorylated ITAM 3 ($\zeta(3)yy^P$) need the presence of Lck and $\zeta(1)y^Py$ and $\zeta(3)yy^P$ phosphopeptides demanded for active ZAP, as well. In contrast, function of $\zeta(3)y^Py$ peptide was not affected by the absence of ZAP. The effect of biphosphorylated ITAM peptides and of $\zeta(1)yy^P$ was dependent on the presence of enzymatically active Lck or Syk/ZAP kinases in a variable extent. Some peptides failed to induce the phosphorylation of specific proteins, for example the proteins with molecular masses of 140-150 and 103-107 kDa could not be induced by $\zeta(2)y^Py^P$ in JCaM while phosphorylation of other substrates was unaffected.

Table 6.1. Requirement for Lck and ZAP/SYk PTKs for phospho –ITAMs induced tyrosine phosphorylation. The evaluation was done by comparing the pattern and the intensity of peptide-induced tyrosine phosphorylation of cell proteins in wild-type Jurkat and kinase deficient cell lines, P116 and JCaM 1.6. „yes": absolutely required, „mostly" indicates that in kinase deficient cells only the increased phosphorylation of constitutively phosphorylated bands was observed, „partial" indicates differences in the pattern of tyrosine phosphorylation, „no"-not required.

PTK	Requirement for PTK for phospho –ITAMs induced tyrosine phosphorylation								
	$\zeta(1)y^Py$	$\zeta(1)yy^P$	$\zeta(1)y^Py^P$	$\zeta(2)y^Py$	$\zeta(2)yy^P$	$\zeta(2)y^Py^P$	$\zeta(3)y^Py$	$\zeta(3)yy^P$	$\zeta(3)y^Py^P$
p56^{lck}	yes	partial	partial	yes	partial	partial	yes	yes	most
ZAP/Syk	yes	partial	partial	most	most	partial	no	yes	partial

Recent data have demonstrated a functional role for Lck beyond ITAM phosphorylation and the activation of ZAP-70 and Syk PTKs (61, 62). The expression of constitutively active Syk or Zap-70 family PTKs in the p56lck-deficient JCaM 1.6 cells failed to rescue signalling events downstream of tyrosine phosphorylation, demonstrating a functional role for Lck beyond the activation of ZAP and Syk (61). Additionally, phosphorylation of

Shc, a downstream signalling component in TCR activation is synergisticly phosphorylated by Syk and Lck (62). These data may explain the differences that we found both in the pattern of induced tyrosine phosphoproteins and the decreased intensity of phosphorylation of several intracellular proteins when phospho-ITAM peptides were introduced into JCaM 1.6., (Lck deficient cells) and in wild type Jurkat T cells. A good example is the decrease in the phosphorylation of the 51-53 kDa molecular mass protein in JCaM induced by the ζ(1) peptides compared to Jurkat. This 51-53 kDa band may represent tyrosine phosphorylated Shc, that is a substrate for both Syk and Lck (62).

The induction of tyrosine phosphorylated bands in the absence of p56^{lck} may be attributed to the alternative activation of p59^{fyn} and/or Syk/ZAP, or other, unidentified tyrosine kinases, or to the involvement of tyrosine phosphatases. Gene targeting in mice has revealed that p59^{fyn} can partially substitute for Lck in TCR signalling (53, see also section 2.2.3.1.). Furthermore, unlike ZAP-70, Syk may be activated by direct binding of phosphorylated ITAMs and it does not require the phosphorylation by Lck to become fully activated (41, 88). Further evidence for the involvement of Syk-family kinases in ζITAM-induced tyrosine phosphorylation comes from experiments where Syk/ZAP deficient Jurkat variant P116 was stimulated with phospho-ζ peptides. In the absence of active Syk-family kinases, the overall peptide-induced tyrosine phosphorylation of cellular proteins was weaker as compared to wild type Jurkat. Significantly, the phosphorylation of several proteins such as the one with molecular mass of 150 kDa, could not be induced neither in JCaM 1.6. nor in P116 cells. The molecular weight of this protein coincided with that of PLCγ that is known a major substrate for Syk and ZAP (92). Moreover, Williams and co-workers demonstrated that both Lck and Syk or ZAP-70 kinases are required for TCR-induced tyrosine phosphorylation of PLCγ (98).

Recent data show that beside the activation of *Src* and *Syk* family tyrosine kinases, TCR engagement results also in the activation of three members of the Tec family tyrosine kinases: EMT/Itk (inducible T cell kinase), Tec and Rlk/Txk (44, 161-163). Tec family members are SH2 domain containing proteins and they have been suggested to function downstream of *Src* family kinases (163). Therefore, we cannot rule out the possibility that other tyrosine kinase(s) than the *Src* and *Syk* family kinases can mediate the

induction of tyrosine phosphorylation by phospho-ITAMs both in wild-type Jurkat and kinase-deficient Jurkat variants P116 and JCaM 1.6.

The phosphorylation status of intracellular proteins is determined by the concerted function of kinases and phosphatases. Contribution of tyrosine phosphatases to the ζ-peptides induced phosphorylation pattern was determined using a protein tyrosine phosphatase inhibitor, PAO. Although the pattern of peptide-induced phosphorylation in the presence or absence of PAO was similar, yet 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.

The main consequence of the TCR-induced tyrosine phosphorylation is the initiation of downstream signalling cascade by promoting phosphotyrosine-mediated protein-protein interactions. Following T cell stimulation, Grb-2 adaptor protein is associated with phosphorylated signalling proteins via its SH2 domain and hence activating the Ras/MAP kinase pathway. Using biphosphorylated ITAM peptides the following results were obtained: the $\zeta(1)$ and $\zeta(2)$ but not the $\zeta(3)$ phosphopeptides induce association between Grb-2 and tyrosine phosphorylated proteins. The pattern of Grb-2 associated phosphoproteins was different when $\zeta(1)$ ITAM or $\zeta(2)$ ITAM peptides were used. Hence, the three ζ-ITAMs differ not only in the induction of tyrosine phosphorylation but also in their capacity to couple TCR to downstream Grb-2 mediated signalling pathways.

6.4. TCR ζ chain ITAM phosphopeptides induce different patterns of tyrosine phosphorylation in B and T cell lines

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).

7. Concluding Remarks

Multiple signaling pathways can be initiated by activation of the TCR. The TCR must provide a mechanism(s) by which the activation machinery is driven into one or the other pathway. The generation of various phosphorylated forms of the ζ chain is most likely one of those mechanisms. The sequence of events at the ζ chain is probably co-ordinated through various levels of regulation. One level of regulation would be the control of the generation of various phosphorylated forms of the ζ chain. This may be mediated through the temporal activation of specific kinases (63) and/or the balance between kinase(s) and phosphatase(s) activities (138). Another level of control would be the binding of distinct signalling proteins at distinct ITAM sequences that may allow for multiple, well controlled signalling pathways to be activated through the TCR complex (68-74). Our data indicate that the three ζ-ITAMs differ in their capacity to induce tyrosine phosphorylation of intracellular proteins and of individual tyrosine kinases (p56^{lck} and ZAP-70) in permeabilized T cells, according to their phosphorylation site and their primary sequence. The first and second ITAM sequences of the ζ chain may have similar but not totally overlapping functions. This conclusion results from their similar but not identical abilities to induce tyrosine phosphorylation and association of Grb-2 with intracellular phosphoproteins. 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 events and they are unable to induce either new association or the increase in the amount of Grb-2 associated phosphoproteins. We also found that from a panel of peptides representing the mono- and biphosphorylated forms of the three ITAM sequences present in the ζ chain, the phospho-peptides corresponding to the third ITAM were the weakest substrates for CD45 in vitro (138, see also section 5.1.). These findings suggest that ζ(3) ITAM have a special function in TCR signalling which may be reflected by its poor dephosphorylation by CD45 and by its

weak ability to induce tyrosine phosphorylation and association of cellular proteins with Grb-2. Other investigations also have suggested that the C terminal ITAM ($\zeta(3)$) may be unique among the TCR ITAMs. It shows the poorest capacity to bind to ZAP and Syk (153), however it exerts a preferential binding to the actin cytoskeleton (160). It was also indicated, that the most significant change from α-helix to β-sheet conformation occurs in the $\zeta(3)$ ITAM upon phosphorylation (139).

Taken toghether, our findings support the idea that the role of the three ITAMs from the ζ chain is to activate multiple signalling pathways through the TCR complex. This function is dependent both on their pattern of phosphorylation and on their primary sequence.

8. Conclusions

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

➡ CD45 PTPase can discriminate between individulal phospho tyrosine 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 lysophosphatidyl choline 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.

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10. Abbreviations

Ap-1: activating protein 1

APC: antigen presenting cell

ATP: adenosine triphosphate

BCR: B-cell antigen receptor

BSA: bovine serum albumin

CD: cluster of differentiation

CDR: complementarity-determining regions

Csk: C-terminal Src kinase

CTLA-4: cytotoxic T lymphocyte antigen 4

DTT: dithiotreithol

ECL: enhanced chemiluminiscence

EDTA: ethylenediaminetetraacetic acid

EGTA: ethylnglycol-bis-N, N, N' ,N'-tetraacetic acid

ERK: extracellular signal-regulated kinase

FcR: Fc receptors

FCS: fetal calf serum

GAP: Ras GTP-ase activating protein

GEF: guanine nucleotide exchange factor

Grb-2: growth factor receptor binding protein 2

GST: Glutathione-S transferase

GTP: guanosine triphosphate

HPLC: high pressure liquid chromatography

HRPO: horseradish peroxidase

Ig: immunoglobulin

IL-2: interleukin 2

ITAM: Immunoreceptor Tyrosine-based Activation Motif

LAT: linker for activation of T cells

LPC: L-α lysophospatidyl choline

MAbs: monoclonal antibodies

MAP: multiple antigenic peptide

MAPK: mitogen activated kinase

MAPKK: MAPK kinase

MAPKKK: MAPK kinase kinase

MHC: major histocompatibility complex

MLC: myosin light chain

NEPHGE: non-equilibrium pH-gradient gel electrophoresis

NFAT: nuclear factor of activated T cells

NFkB: nuclear factor kB

PAGE: polyacrylamide gel electrophoresis

PAK: p21-activated kinse

PAO: phenylarsine oxide

PBMC: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PI3K: phosphatidylinositol 3-kinase

PIP5K: phosphatidylinositol 4-phosphate 5-kinase

PLC γ: phospholipase Cγ

PMSF: phenylmethhylsulfonyl fluoride

PTK: protein tyrosine kinase

PTP: protein tyrosine phosphatase

RaMIg: rabbit immunoglobulin to mouse immunoglobulins

SDS: sodium dodecyl sulfate

SH: *Src* homology domain

SHP: SH2 domain containing protein tyrosine phosphatase

SLO: streptolysin O

SLP-76: SH2 domain-containing leukocyte phosphoprotein of 76 kDa

TCR: T-cell antigen receptor

TmC: transmembrane charge

WASP: CDC-42 associated Wiscott Aldrich syndrome protein

ZAP-70: zeta associated protein of 70 kDa

11. List of Publications and Abstracts

PUBLICATIONS

Publications connected to the thesis

1. V. Chiţu, Z. Hegedűs, 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. Hegedűs, 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. Hegedűs, 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).

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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).

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ABSTRACTS

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. **Chitu 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. Chitu,** 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

1. Diaconu C. C., Magureanu C., **Chitu 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.

2. **Chitu, V.**, Veliceasa, D., Diaconu, C.C, Tardei, G., Ruta 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.

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4. Diaconu C. C., **Chitu V.**, Tardei G., Bleotu C., Ruta 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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13. Summary in English

The mechanisms of ITAM-mediated signal transduction have been extensively studied. It is well documented that differently phosphorylated forms of the ζ 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 and the exact role of the presence of multiple ITAMs in TCR is not well documented.

Our *aim* was to study 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?

The *results* obtained in this work are as follows:

1. The ζ chain ITAMs and individual tyrosine residues except the non-conserved membrane proximal one were phosphorylated by $p56^{lck}$ and $p59^{fyn}$ CD45 discriminated between the individual phosphotyrosine residues of the ζ chain ITAMs. These findings suggested that CD45 may be involved in the regulation of the phosphorylation pattern of the ζ chain.

2. Cell permeabilization with L-α lysophosphatidylcholine was established and was proven to be efficient and reproducible and did not affect the cell responsiveness to anti-TCR stimulation.

3. Different ζITAMs phosphorylated at distinct sites induced phosphorylation of partially distinct substrate sets in Jurkat T cells

4. Kinase requirements for induction of phosphorylation by the different forms of the ζ ITAMs were considerably different. Effect of certain ITAMs was totally dependent on the presence of enzymatically active Lck or Syk/ZAP kinases and other's effect depended on these kinases in a variable extent.

5. Contribution of tyrosine phosphatases to the ζ-peptides-induced phosphorylation pattern was determined using a protein tyrosine phosphatase inhibitor.

6. Phospho-ITAMs regulated the association of specific proteins with the adaptor protein, Grb2.

The two main *conclusion* drawn from this study are:

1. Kinases of *Src* family and CD45 tyrosine phosphatase may act together on the formation of the ζ chain phosphorylation pattern.

2. The individual ITAMs and their differently phosphorylated forms play partially distinct role in T cell activation and contribute to signal diversification.

14. Magyar nyelvű összefoglaló

A T sejt receptor ζ alegységének ITAM motívumai által közvetített jelátviteli utakról nagyszámú tanulmány jelent már meg. A ζ láncnak különböző mintázatban foszforilált formái vannak jelen az egyes differenciálódási stádiumokban és a különböző feltételek mellett aktivált sejtekben. Nem ismert azonban, hogy mi az egyes ITAM motívumok pontos szerepe, valamint hogy mely molekulák vesznek részt a különbözően foszforilált formák kialakításában.

A munkánk során a következő kérdésekre kerestük a választ:

1. Hozzájárulnak-e a *Src* kinázok és a CD45 protein tirozin foszfatáz a különbözően foszforilált formák létrehozásához?
2. A ζ lánc három ITAM szekvenciája különböző szignál transzdukciós utakat indít-e el?

Eredményeink:

1. A ζ lánc ITAM motívumokat és az egyedi tirozin aminosavakat a $p56^{lck}$ és $p59^{fzn}$ kinázok foszforilálják, a nem konzervált membrán proximális tirozin kivételével. A ζ lánc ITAM motívumainak egyes foszfo-tirozinjait a CD45 különböző affinitással defoszforilálja, s ez arra utal, hogy a CD45 szerepet játszhat a ζ lánc foszforilációs mintázatának kialakításában.
2. A sejtmembrán L-α-lizofoszfatidilkolinnal történő permeabilizálására új, hatékony és megbízható módszert dolgoztunk ki, ami nem befolyásoltja a sejtek anti-TCR stimulusra adott válaszképességét.

3. A ζ lánc egyes ITAM motívumainak különbözően foszforilált formái a Jurkat T sejtekben részben eltérő molekulák foszforilálódását indukálták.

4. A ζ lánc különböző ITAM motívumai által indukált foszforilációhoz eltérő kinázok szükségesek. Egyes ITAM szekvenciák hatása teljes mértékben, míg másoké különböző mértékben függ az Lck illetve Syk/ZAP kinázok enzimaktivitásától.

5. Protein tirozin foszfatáz inhibitor segítségével meghatároztuk a tirozin foszfatázok részvételét a ζ lánc ITAM motívumok által indukált foszforilációs mintázatban.

6. A foszforilált ITAM motívumok szabályozzák a Grb2 adaptor fehérje szignálfehérjékkel való asszociációját.

Következtetések:

1. A *Src* kinázok és a CD45 protein tirozin foszfatáz együttesen alakítják ki a ζ lánc foszforilációs mintázatát.

2. Az egyes ITAM szekvenciák és azok különbözően foszforilált formái eltérő szerepet játszanak a T sejt aktivációban és különböző jelátviteli utak megindításához járulnak hozzá.

APPENDIX 1.: BUFFERS COMPOSITION

PBS
10 mM Na_2HPO_4
1.8 mM NaH_2PO_4
137 mM NaCl
2.6 mM KCl

TBS
10 mM Tris pH 7.5
154 mM NaCl

Permeabilization buffer (Cambier's buffer, see ref. 70)
10mM $MnCl_2$
10mM $Mg(OAc)_2$
296μM $CaCl_2$
2mM EGTA
40mM HEPES pH 7.4

1x LYSIS BUFFER (anti-PY blotting)
50mM HEPES pH 7.4
1% Triton X-100
150mM NaCl
20mM NaF
5mM Na_3VO_4
10mM Na pyrophosphate
2mM EGTA
1mM PMSF, 1μg/ml each of aprotinin and leupeptin- should be added just before use

2x Laemmli sample buffer
125 mM TrisCl
20% glycerol
4.1 % SDS
2% 2-ME or 3.1% DTT
0.001% Bromphenol Blue

10% SDS-PAGE gel
Stacking gel: 5% acrylamide
0.1% bis-acrylamide
0.125 M Tris-HCl pH 6.8
0.1% SDS
0.086% TEMED (N,N,N',N'-tetramethylethylendiamine)
0.043% ammonium persulfate

Resolving gel: 10% acrylamide
0.266% bis-acrylamide
0.375 M Tris-HCl pH 6.8
0.1% SDS

0.066% TEMED (N,N,N',N'-tetramethylethylendiamine)
0.033% ammonium persulfate

SDS-PAGE Running Buffer
25 mM Tris
192 mM glycine
0.1% SDS

Western Blot Transfer Buffer
25 mM Tris
192 mM Glycine
20% methanol

Kinase lysis buffer
50 mM HEPES pH 7.4
150mM NaCl
20mM NaF
200mM Na orthovanadate
1% Triton X-100
1mM PMSF, 1µg/ml each of aprotinin and leupeptin- should be added just before use

Kinase assay buffer.
25 mM HEPES pH 7.4
100mM NaCl
10mM $MgCl_2$
5mM $MnCl_2$
100µM Na orthovanadate

40% alkaline polyacrylamide gel
Stacking gel: 3.3% acrylamide
0.16% bis-acrylamide
0.125 M Tris-HCl pH 6.8
0.05% TEMED (N,N,N',N'-tetramethylethylendiamine)
6M urea
0.1% ammonium persulfate

Resolving gel: 40% acrylamide
0.037% bis-acrylamide
0.75 M Tris-HCl pH 8.8
0.035% TEMED (N,N,N',N'-tetramethylethylendiamine)
0.1% ammonium persulfate

Peptide running buffer:
0.05 M Tris base
0.4 M glycine

Peptide sample buffer:
250 mM Tris pH 6.8
12 M urea
0.001% Bromphenol Blue

Phosphatase lysis buffer*
1% Triton X-100
20mM Tris pH 7.5
150mM NaCl
1.0 mM EDTA
1mM PMSF and 10µg/ml aprotinin- should be added just before use

Phosphatase assay buffer*
50 mM Hepes pH 7.0
KCl 100mM
0.1% Triton X-100
1mM EDTA

Malachite green reagent*
Solution1: 0.135% malachite green oxalat
Solution 2: 4.2% ammonium molibdate in 4M HCl
Malachite green reagent: 1part solution 1
                           1 part solution 2
                           2 parts tridistilled water
                           0.01% Tween 20

*All reagents and buffers used in the phosphatase assay must be high purity to prevent sample contamination with phosphate.