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Functional and structural analysis of the T cell antigen receptor [zeta] chain

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FUNCTIONAL AND STRUCTURAL ANALYSIS OF THE
T CELL ANTIGEN RECEPTOR ζ CHAIN

by

BRYAN ALLEN IRVING

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of
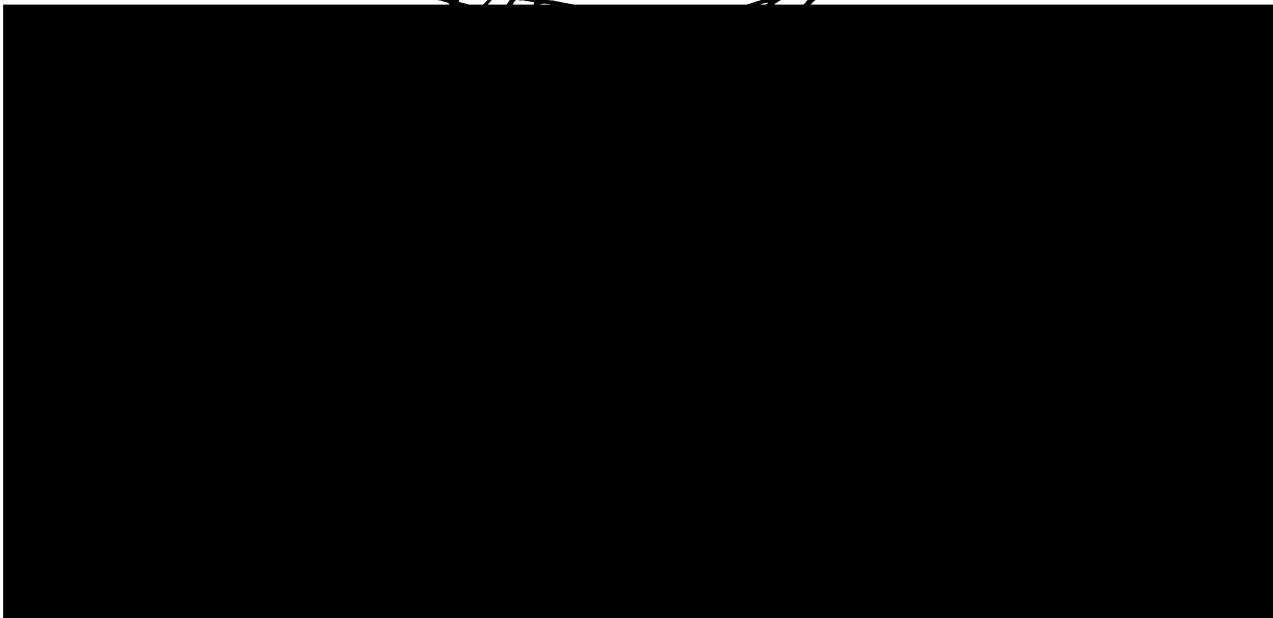
DOCTOR OF PHILOSOPHY

in

PHYSIOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

DEDICATION

To my father, Dr. Theodore E. Irving (November 20, 1932-February 20, 1993), whose sudden passing is very deeply felt.

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Chapters II and III are taken from the following published papers:

Chapter II: Irving, B.A. and Weiss, A. 1991. The cytoplasmic domain of the T cell receptor ζ chain is sufficient to couple to receptor-associated signal transduction pathways. Cell. 64:891-901.

Chapter III: Irving, B.A., Chan, A.C., and Weiss, A. 1993. Functional characterization of a signal transducing motif present in the T cell antigen receptor ζ chain. J. Exp. Med. 177:1093-1103.

v.

Computer art work was kindly provided by Art.

Technical assistance on the Macintosh was graciously provided by my wife,

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ABSTRACT

Functional and structural analysis of the T cell antigen receptor ζ chain.

by Bryan A. Irving.

The function of the T cell antigen receptor invariant chains, CD3 γ, δ, ε, and ζ has been poorly understood. In these studies a direct role for the cytoplasmic domain of the ζ chain in coupling the TCR to intracellular signal transduction pathways is demonstrated. A chimeric protein consisting of the extracellular and transmembrane domains of CD8 linked to the cytoplasmic domain of ζ could be expressed independently of the TCR and is capable of transducing signals which, by criteria of early and late activation, are indistinguishable from those generated by the intact receptor. Analysis of C-terminal truncations of this chimera has identified a functional motif, xxYxxLxxxxxxxYxxL, which is triplicated in ζ and singly represented in a number of signalling subunits associated with hematopoietic antigen receptors. Stimulation of chimeric receptors containing the isolated motif results in the induction of both early and late events associated with T cell activation. Tandem triplication of a single motif results in enhanced signal tranduction, consistent with a role in signal amplification for the presence of three motifs in ζ. Moreover, we demonstrate the association of a recently identified protein tyrosine kinase (PTK) ZAP-70 with this motif, and provide evidence supporting its involvement in ζ function. Preliminary structural analysis by circular dichroism and 2D NMR of peptides encompassing the activation motifs demonstrates a primarily helical secondary structure which can be disrupted by phosphorylation on critical tyrosine residues, supporting a role for ζ phosphorylation in regulating motif conformation. Finally, we demonstrate the

association of ZAP-70 specifically with a doubly phosphorylated peptide containing the motif, consistent with a requirement for phosphorylation of both conserved tyrosines within a given motif for ζ function. The disruption of the motif's helical structure observed with two phosphorylation events, suggests an unwound or open conformation for the active motif. In summary, these studies demonstrate an important function for the TCR ζ chain in signal transduction and localize this function to a sequence motif that interacts with tyrosine kinases.

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CHAPTER I: INTRODUCTION

T cells play a critical role in the mounting of most cellular and humoral immune responses. Central in determining the specificity, diversity, and functional integrity of the T cell repertoire, is the T cell antigen receptor, TCR, an oligomeric complex consisting of a least six distinct transmembrane proteins [1-3]. The clonally variable α and β subunits (Ti$\alpha\beta$) are expressed on the surface of T cells in an obligate, noncovalent association with several invariant subunits: CD3 γ, δ, ϵ, and a dimer composed of members of the ζ-family (discussed below). The TCR must perform two independent functions. First it must recognize a specific peptide antigen displayed in the binding cleft of the appropriate major histocompatiblilty complex (MHC) molecule. This function of the TCR determines which T cells will be selected to participate in a given immune response. The TCR must then translate this recognition event into a cascade of biochemical changes within the cell which ultimately culminates in cellular proliferation, differentiation, and effector function. This introduction will review the current understanding of the proximal signal transduction events initiated by stimulation of the TCR and discuss in more detail the structural complexity of the receptor. In addition, it will describe the rationale for undertaking the course of experimentation described herein to determine the role of the ζ chain in coupling the TCR to intracellular signal transduction pathways.

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Signal transduction events initiated by the TCR:

Role of protein tyrosine kinases

Much progress has been made in defining the nature of the biochemical events which occur following engagement of the TCR by either antigen/MHC or anti-TCR specific monoclonal antibodies (mAbs). The most rapid event detected, occurring within seconds of TCR stimulation, is the induced phosphorylation of a number of cellular proteins on tyrosine residues [4, 5]. Among these tyrosine phosphoproteins are the CD3 and ζ chains of the TCR [6, 7], the tyrosine kinase ZAP-70 [8, 9], phospholipase Cγ1 (PLCγ1) [10-12], and the proto-oncogene, vav [13]. The importance of tyrosine phosphorylation in regulation of cellular growth has been well documented in studies of tyrosine kinase growth factor receptors such as the platelet-derived growth factor receptor, (PDGF-R) or epidermal growth factor receptor (EGF-R) [14, 15]. However, unlike these receptors, the TCR does not contain intrinsic tyrosine kinase activity and, thus, is thought to couple to at least one cytoplasmic tyrosine kinase. Treatment of T cells with tyrosine kinase inhibitors, such as herbimycin A or genestein completely abrogates both proximal and distal events induced by TCR stimulation, underscoring the obligatory nature of this TCR-mediated PTK activation [16, 17].

To date, three cytoplasmic tyrosine kinases have been implicated in TCR function. One candidate, fyn, a 59 kD member of the src family of PTKs [18] is expressed in two distinct forms through tissue-restricted, alternative splicing of its mRNA; one isoform is predominantly expressed in hematopoietic cells, the other in brain [19]. A link between the TCR and fyn was first proposed based on their ability to co-immunoprecipitate under mild,

non-ionic detergent lysis conditions [20]. Notably, the stoichiometry of this association was low, requiring a sensitive *in vitro* kinase assay for its detection. Further support for an association between the TCR and fyn comes from a study in which immunofluorescence microscopy detects an increased co-localization of the two following TCR engagement in intact human T lymphocytes [21]. These data indicate a proximity between fyn and the TCR but suggest the association may be indirect or very weak.

Evidence suggesting a functional role for fyn in TCR signal transduction comes from studies in which various forms of fyn are overexpressed in T cell hybridomas or thymocytes of transgenic mice. Overexpression of the hematapoietic form of fyn in a T cell hybridoma augments TCR specific antibody-mediated induction of tyrosine phosphoproteins and antigen-mediated induction of IL-2 secretion [22]. Moreover, TCR signalling is similarly enhanced in thymocytes which overexpress fyn, as determined by analysis of PTK activation, intracellular calcium mobilization, IL-2 production and proliferation [23]. Perhaps the most compelling evidence implicating fyn in TCR function is the ability of a mutant form of fyn defective in kinase function to inhibit TCR-induced thymocyte proliferation [23]. These data in conjunction with the physical association described between fyn and the TCR support a role for fyn in signal transduction by the TCR.

However, recent genetic evidence suggests its role may be restricted to a small subset of developing thymocytes [24]. Mice in which the *fyn* gene has been disrupted by homologous recombination exhibit normal numbers of T cells in both the periphery and the various thymocyte populations, suggesting

fyn is not required for the process of T cell selection. Furthermore, no impairment in TCR signalling was detected in either mature peripheral T cells or immature (CD4$^+$CD8$^+$) thymocytes. However, loss of fyn resulted in defective signalling through the TCR in mature, single positive (CD4$^+$,CD8$^-$ or CD4$^-$,CD8$^+$) thymocytes. These findings demonstrate that fyn is involved in TCR-mediated signal transduction but that its role is restricted to a subpopulation of mature thymocytes which has not yet exited the thymus.

lck, a lymphocyte-specific member of the src family of PTKs was originally identified in a T cell lymphoma [25]. Insight into a potential role for lck in TCR signalling came with the discovery of its stable association with the cytoplasmic tails of the T cell glycoproteins CD4 and CD8 [26, 27]. The extracellular domains of CD4 and CD8 recognize the non-polymorphic residues of class II and class I MHC molecules, respectively, and, thus, are brought into close proximity with the TCR during antigen recognition [28]. This proximity between the TCR and lck during TCR engagement naturally made lck an attractive candidate for a PTK utilized by the TCR.

Substantial evidence supports an involvement of lck in TCR-mediated signal transduction, though the exact nature of its role is still unclear. Co-engagement of a class II-restricted TCR and CD4 enhances both proximal and distal signalling events associated with stimulation of the TCR alone [29, 30]. Emphasizing the importance of the CD4/lck association are studies utilizing a CD4-dependent hybridoma expressing mutants of CD4 which are unable to associate with lck [31]. Without a functional interaction between CD4 and lck, this cell is completely unresponsive to TCR stimulation by antigen/MHC. Reconstitution with CD4 molecules which associate with lck restores TCR

signalling, further supporting a contribution by lck. However, despite the increase in lck kinase activity observed following engagement of CD4 alone, this itself is insufficient for T cell activation [32]. This finding suggests that while lck is likely involved in TCR signal transduction, it alone does not account for all the TCR-induced kinase activity.

Perhaps the strongest evidence demonstrating a critical function for lck in TCR signal transduction comes from genetic studies performed in both T cell lines and intact mice. The genetic defect has recently been identified in a mutant of the Jurkat leukemic T cell line selected for its inability to activate phospholipase C (see below) following TCR stimulation [33]. Absence of a critical exon in the lck mRNA results in expression of a non-functional lck protein and a concomitant unresponsiveness of the TCR to particular anti-receptor mAbs. Normal TCR function is completely restored upon reconstitution of the clone with an intact lck protein. Similarly, loss of lck in a cytotoxic T cell clone results in impaired TCR signalling [34]. Finally, unlike the genetic disruption of the fyn gene, disruption of lck in the mouse has a profound effect on T cell selection [35]. In these mice, only an extremely small fraction of normal thymocytes is able to mature and populate the periphery suggesting a critical role for lck in TCR signalling during thymic selection. Interestingly, the peripheral T cells which do survive are capable of responding to anti-TCR antibodies suggesting that another kinase can substitute for lck under these circumstances.

The third PTK implicated in TCR-mediated signal transduction is ZAP-70, a 70 kD tyrosine kinase shown to associate with at least two of the TCR invariant chains following receptor engagement [8, 9]. ZAP-70, a novel

tyrosine kinase distinct from those of the src family, is expressed in T cells and natural killer (NK) cells exclusively. This kinase will be discussed further in Chapters III and IV of the dissertation.

While the initiation of signal transduction through the TCR is often described as the activation of one or more tyrosine kinases, this may be an oversimplification. Somatic T cell mutants deficient in surface expression of CD45, a transmembrane protein tyrosine phosphatase, are completely unresponsive to TCR stimulation; no increases in tyrosine phosphoproteins or $[Ca^{+2}]_i$ are observed upon receptor stimulation [36-38]. This finding emphasizes the importance of dephosphorylation in the signalling process. In these mutants, lck is hyperphosphorylated at Tyr505, a site which is homologous to a negative regulatory site in the src PTK [39]. It is hypothesized that CD45 may maintain lck in a dephosphorylated state that is permissive for activation or utilization by the TCR. Thus inhibition or loss of CD45 phosphatase activity would have an inhibitory influence on TCR activity, perhaps through its effect on the negative regulatory site of lck. Interestingly, incubation of T cells with pervanadate, an inhibitor of tyrosine phosphatases has the opposing effect, eliciting activation independently of TCR stimulation [40]. This induced activation occurs independently of CD45 expression [41] but is still dependent on lck (Desai, D.M. and van Oers, N.S.C., unpublished data), suggesting the presence of at least one additional tyrosine phosphatase which acts at a very proximal step and whose function is important for suppressing TCR signalling. Thus, initiation of signalling by the TCR is likely to invoke a fine balance of regulation between both tyrosine-specific kinases and phosphatases.

Activation of the phosphatidylinositol signal transduction pathway

Of the several TCR-induced tyrosine phosphoproteins whose identities are known, PLCγ1 is perhaps the best characterized [10, 11]. PLCγ1 catalyzes the hydrolysis of the phospholipid, phosphatidylinositol-4,5-bis-phosphate into inositol 1,4,5,-tris-phosphate (IP$_3$) and diacylglyerol (DG), two second messengers responsible for increasing intracellular calcium concentrations ([Ca^{+2}]$_i$) and activating protein kinase C (PKC), respectively [42]. Compelling evidence exists supporting a role for activation of the phosphatidylinositol (PI) signalling pathway in T cell activation (reviewed in [43]). Examples include: 1) the liberation of IP$_3$ and DG observed following stimulation of the TCR in human peripheral blood T cells, as well as in T cell clones, hybridomas, and leukemic lines [44]; 2) the ability of calcium ionophores and phorbol esters, pharmacological agents which mimic the effects of IP$_3$ and DG, to activate T cells independently of receptor stimulation [45]; 3) the ability of agents which block sustained increases in intracellular calcium and PKC activation to inhibit distal events associated with T cell activation [46, 47]; and 4) the finding that stimulation of a heterologous receptor, which activates the PI pathway by a mechanism distinct from that utilized by the TCR, results in T cell activation when introduced into a T cell line [48].

Recent studies have revealed that phosphorylaton of PLCγ1 on two critical tyrosine residues is associated with its increased catalytic activity [49, 50], suggesting the mechanism by which TCR stimulation leads to its activation. The ability of tyrosine kinase inhibitors to prevent both TCR-induced phosphorylation and activation of PLCγ1 is consistent with a functional importance for its tyrosine phosphorylation [16].

Whereas the TCR-mediated mobilization of $[Ca^{+2}]_i$ and activation of PKC have long been documented, the impact these intracellular events have on subsequent cellular responses is still largely unknown. One cellular consequence linked to the increased $[Ca^{+2}]_i$ flux is the activation of calcineurin, a calmodulin-activated, serine/threonine phosphatase recently identified as a target of the immunosuppressive drug, cyclosporin A [51]. Cyclosporin A appears to inhibit the activity of calcineurin and, as a likely consequence, prevents the induction of the lymphokine, interleukin-2 (IL-2). Consistent with a role for calcineurin in T cell activation is the finding that a constitutively activated form of calcineurin can synergize with phorbol esters alone to induce IL-2, bypassing the requirement for increases in $[Ca^{+2}]_i$ [52]. Identification of additional cellular proteins or processes effected by the TCR-induced increase in $[Ca^{+2}]_i$ will require further investigation.

Activation of protein kinase C via stimulation of the TCR or treatment of T cells with phorbol esters leads to the phosphorylation of a number of proteins on serine and/or threonine residues. Among these cellular subtrates are the CD3 γ and δ chains [53], the CD4 co-receptor [54], mitogen-activated protein kinase II (MAP-2 kinase) [47, 55], and the serine/threonine kinase, raf-1 [56]. Phosphorylation of CD3 γ and CD4 has been linked to the down-modulation or internalization of the TCR and CD4 [53, 54], respectively, while the phosphorylation of MAP-2 kinase and raf-1 has been implicated in the activation of these kinases [56, 57]. Whether these proteins are phosphorylated directly by PKC, or rather, become phosphorylated as an indirect consequence of PKC activation remains to be determined. Activation of T cells will likely involve a complex network of serine/threonine

phosphorylation events which have yet to be elucidated. Figure 1 summarizes signalling events which occur upon TCR stimulation, demonstrating schematically how they converge to induce the transcriptional activation of the IL-2 gene.

Relating the structure of the TCR to its function.

The TCR was originally identified with the aid of antibodies which reacted against clonotypic determinants on the surface of T cell clones, determinants which appeared to be important for dictating the antigen specificity of the clones [58, 59]. Biochemical characterization of these immunoreactive surface proteins identified a disulfide-linked heterodimer, termed Tiα/β, whose glycosylated components ranged from 40-55 kD in size [60]. Subsequent isolation of the genes encoding Tiα/β revealed that each of these TCR subunits is composed of two extracellular immunoglobulin-like domains, a single transmembrane domain, and a short cytoplasmic domain of only 5 amino acids [61, 62]. Similar to immunoglobulins, these proteins arise through recombination of distinct variable and constant gene segments during thymic ontogeny, accounting for the clonotypic nature of the TCR. Furthermore, the myriad of possible recombinations (> 10^{15}) in addition to the added complexity given the heterodimeric form of Ti explains the enormous diversity of antigen reactivities comprising the T cell repertoire [63]. Gene transfer experiments have unequivocally demonstrated that the Ti heterodimer contains all of the information necessary for antigen-specificity of the TCR [64, 65], with the variable immunoglobulin domains of Tiα/β recognizing determinants in both the antigenic peptide and the antigen-

Figure 1. Summary of signals generated by TCR stimulation.

TCR-mediated activation of at least one protein tyrosine kinase results in tyrosine phosphorylation of the CD3 and ζ chains, PLCγ1, and vav, among other cellular substrates. Phosphorylation of PLCγ1 has been shown to increase its catalytic activity, resulting in the activation of the phosphatidylinositol pathway. The putative interaction between ras and vav is depicted, though no data to date has demonstrated such an association.
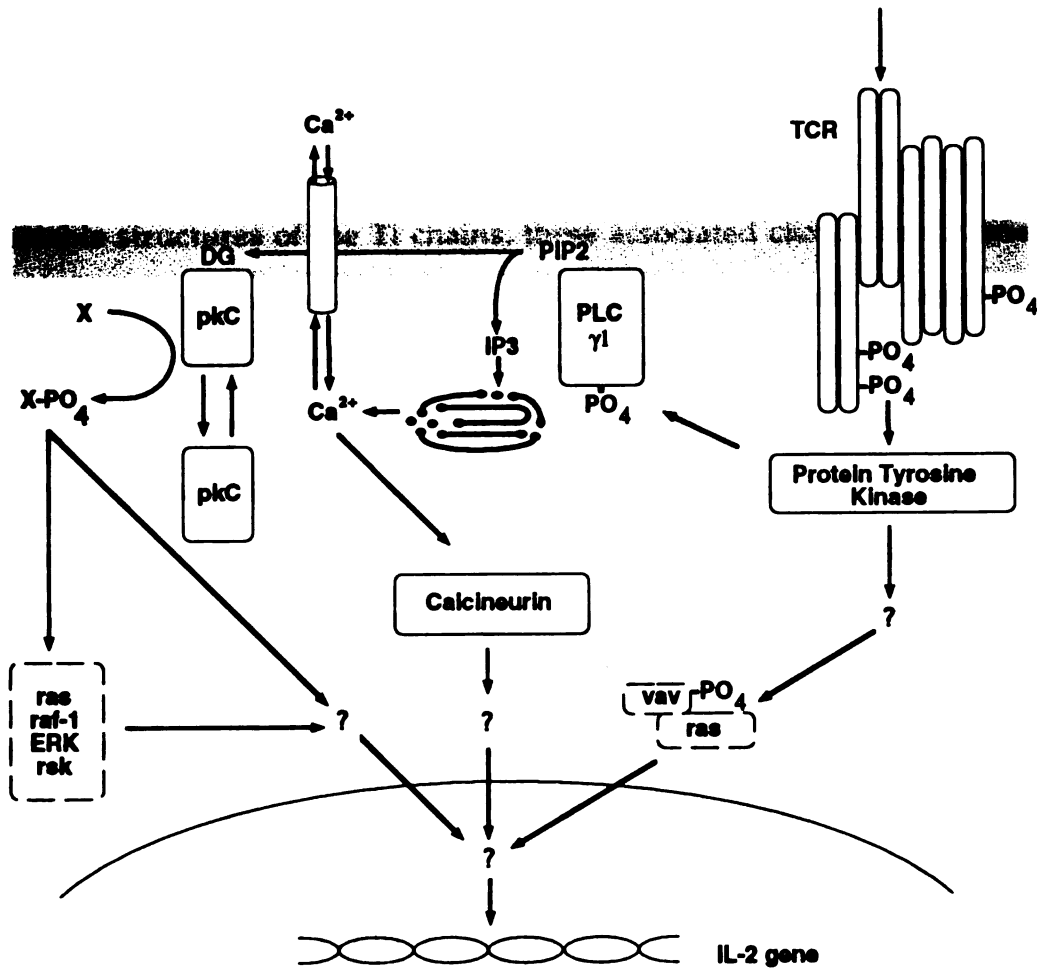
binding pocket of the MHC molecule.

The Ti subunits are expressed on the surface of T cells in a non-covalent association with several transmembrane proteins comprising the CD3 complex and the ζ-family dimer [1] (see Table 1). Somatic T cell variants demonstrate that expression of each of these chains is required for efficient assembly and surface expression of the T cell receptor [66]. In contrast to the variable structures of the Ti chains, these associated chains are invariant. The CD3 chains, γ, δ, and ε are structurally homologous proteins derived from three closely linked genes on chromosome 11 and are believed to have arisen though gene duplication [67]. All three proteins contain a single immunoglobulin-like domain and substantial cytoplasmic domains of 40-55 residues. Although the precise stoichiometry of the TCR has not been established, each receptor is thought to contain two CD3 dimers, each composed of CD3ε linked to either γ or δ; to date, no direct interaction between CD3γ and δ has been detected [68-70]. Evidence obtained utilizing CD3 γ or δ-specific antibodies suggests that receptors can be composed of CD3εγ or εδ dimers exclusively [71], although the prevailing model incorporates both dimers as depicted in Figure 2.

Increasing further the structural complexity of the TCR is the association of the ζ dimer with the CD3 and Ti subunits. The 16kD ζ chain exists in the TCR primarily as a disulfide-linked homodimer or, in a minority of receptors, as a heterodimer linked to the highly homologous protein, η [74, 75]. The 22kD η chain is derived from the ζ gene by alternative splicing of transcripts, differing from ζ only in its most C-terminal exon [73]. Like the CD3 chains, ζ has an extensive cytoplasmic tail (112 amino acids) although its

Table 1. Proteins comprising the T cell antigen receptor. Structural
characteristics of the TCR subunits are illustrated. Information is compiled
from a number of sources. [1, 66, 72, 73]

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Subunit	Structural features	M_r (core M_r)	Cytoplasmic tail length	Oligosaccharides	Phosphorylation	Chromosomal Location
Tiα	two positive charges in transmembrane domain; 2 immunoglobulin domains (1 variable, 1 constant)	45-55kD (32)	5	6 N-linked	—	14
Tiβ	single positive charge in transmembrane domain; 2 immunoglobulin domains (1 variable, 1 constant)	40-50kD (32)	5	2 N-linked	—	7
CD3γ	single neg.-charged TM; 50% homology to CD3δ in cytoplasmic tail	25kD (16)	45	2 N-linked	PO_4-serine/ PO_4-tyrosine	11
CD3δ	single neg.-charged TM	22kD (14)	45	2 N-linked	PO_4-serine/ PO_4-tyrosine	11
CD3ε	single neg.-charged TM	21kD (21)	55	—	PO_4-tyrosine	11
ζ family						
ζ	single neg.-charged TM; disulfide-linked to ζ,η or FcεRIγ; increased M_r if PO4-tyrosine	16kD	113	—	PO_4-tyrosine	1
η (murine)	single neg.-charged TM; linked to ζ or FcεRIγ	22kD	155	—	not detected	1
γ (rat)	single neg.-charged TM: homodimer, ϰ or ζη	7kD	42	—	PO_4-tyrosine	1

Figure 2. Schematic representation of the T cell antigen receptor.
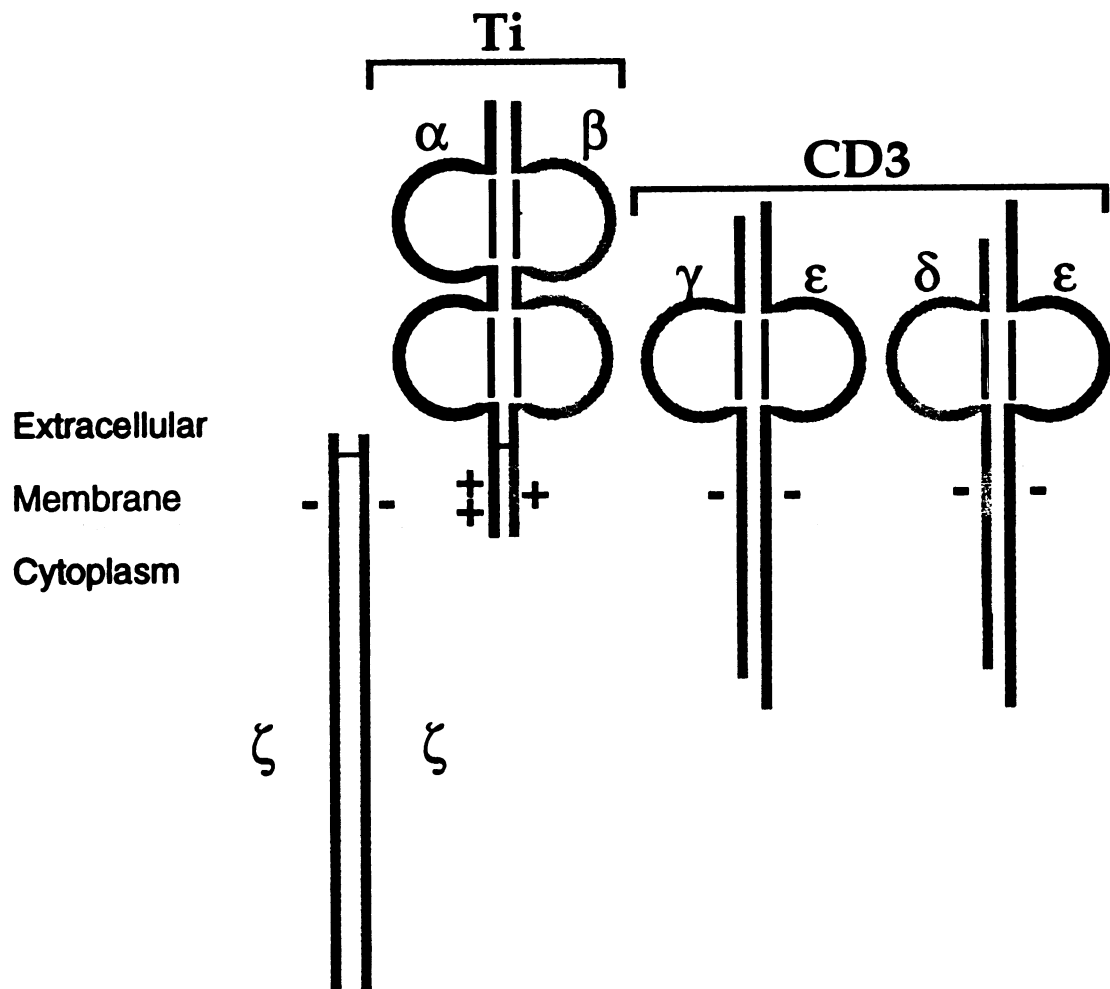
extracellular domain of only 9 amino acids is notably shorter. Expression of ζ is not restricted to T cells, as it has been found expressed in natural killer (NK) cells in association with CD16, the ligand binding subunit of the FcγRIII receptor [76, 77]. In addition, a ζ homologue, the γ subunit of FcϵRI, has also been detected as a component of the TCR, either replacing ζ in a homodimeric form or as a heterodimer complexed with either ζ or η [78, 79]. Thus, ζ might be viewed as the prototypical member of a growing family of receptor-associated proteins.

Several lines of evidence suggest the relevance of the association between the Ti, CD3 and ζ chains. First, antibodies against the Ti subunits coprecipitate the invariant chains, and vice versa [80]. Second, supporting the proximity of the TCR chains is the ability of bifunctional cross-linking agents to physically link the Tiβ and CD3γ subunits [81]. Third, incubation of T cells with antibodies directed against either Ti or the invariant subunits, results in the simultaneous modulation or internalization of both structures [82]. Fourth, and most compelling, T cell variants lacking, either Tiα, Tiβ, CD3γ, δ, or the ζ chain, fail to express significant levels of the remaining chains on the cell surface [83-87]. The evolution of a receptor with such complex structure and stringent requirements for assembly and surface expression suggests the functional importance of its oligomeric state.

Attributing a function to the TCR invariant chains

At the initiation of the work described in this dissertation, a major effort was underway to relate the complex receptor structure to its function as

a signal transducing molecule. Since the discovery of the invariant chains associated with the Ti heterodimer, it had been hypothesized that CD3 and ζ functioned to couple antigen recognition to intracellular signal transduction. This hypothesis was based on the following reasoning: As previously described, the co-requirement for Ti and CD3/ζ expression suggested an importance for the invariant chains in TCR function. In addition, the cytoplasmic tails of the CD3 and ζ chains are sufficiently large to support interactions with intracellular signalling molecules. In contrast, the minimal tails of the Ti subunits are composed primarily of positively charged residues required for proper anchoring of transmembrane proteins in the lipid bilayer. Perhaps providing the best support for a role of the invariant chains in signal transduction was the finding that mAbs against extracellular epitopes of CD3 could elicit intracellular signals similar to those induced by engagement of Ti with antibody or antigen/MHC [88, 89]. However, despite the feasibility of the notion that the CD3 and ζ chains served to transduce signals across the plasma membrane, the sequences of these chains offered no clues to the mechanism of this function.

At the time, attention was particularly drawn to the ζ chain as an attractive candidate for a signal transducing molecule. ζ was a member of a growing family of receptor-associated proteins. Interestingly, each of the receptors with which this family associates, - the TCR, CD16 in NK cells, and FcϵRI in mast cells and basophils-, couples to both a protein tyrosine kinase and the phosphatidylinositol signalling pathway [90, 91], suggesting a possible link between this family of proteins and the receptor's capacity to activate these pathways. Providing further evidence implicating ζ in signal

transduction was a study which suggested a link between the degree of $\zeta\eta$ expression in the TCR and the receptor's ability to activate PLC upon antigen stimulation [92]. It was hypothesized that heterogeneity in receptor composition enabled the TCR to couple differentially to the cell's signalling machinery, with the η chain being required for coupling to the PI pathway. Finally, ζ had been shown to be phosphorylated on multiple tyrosine residues following TCR stimulation [7, 93]. While the function of this phosphorylation was unknown, there was precedence for the importance of tyrosine phosphorylation in the function of several growth factor receptors including those for PDGF, EGF, and insulin [94]. Together, these data suggested an important role for the ζ chain in receptor-mediated signal transduction. However, direct testing of this hypothesis was difficult since the requirement for co-expression of the TCR subunits prohibited expression of individual TCR chains for analysis of their function.

CHAPTER II. THE CYTOPLASMIC DOMAIN OF THE T CELL RECEPTOR ζ CHAIN IS SUFFICIENT TO COUPLE TO RECEPTOR-ASSOCIATED SIGNAL TRANSDUCTION PATHWAYS

Preface

The stringent requirements for surface expression of the TCR had prevented testing the hypothesis that the TCR invariant chains served to couple antigen recognition to intracellular signal transduction. To directly address the role of ζ in TCR-mediated signal transduction, a strategy was devised to circumvent the requirement for expression of its companion chains. This strategy was based on accumulating evidence implicating the transmembrane domains of the various TCR components in proper receptor assembly. One notable feature in all subunits is the presence of at least one charged residue within the otherwise hydrophobic transmembrane domain of each of these molecules [1, 74]. It was thought that these charges, positive in the Ti chains and negative in the CD3 chains (see Figure 2) facilitated proper assembly of the complex [95]. Site-directed mutagenesis of the charged residue within the transmembrane domain of Tiβ had prevented surface TCR expression in two independent T cell lines [96, 97]. Similarly, mutation of the negatively-charged residue in the transmembrane domain of ζ impaired its ability to associate with CD16 [98]. Finally, the transmembrane regions of the Ti chains had been demonstrated to be sufficient for a functional coupling between Ti and the CD3 complex [99]. Based on the reasoning that replacement of ζ's transmembrane domain with that of another molecule might result in the expression of ζ independently of its companion TCR chains, a chimeric protein was constructed consisting of the extracellular and

transmembrane domains of the T cell glycoprotein CD8, linked to the 113 cytoplasmic residues of the human ζ chain. The α chain of CD8 was chosen since it exists as a disulfide-linked dimer and thus, would preserve the dimeric state of ζ in its chimeric form. This CD8/ζ chimeric protein was transfected into the Jurkat T cell leukemic cell line, in addition to a variant deficient in endogenous TCR expression. The results of this study are described below and serve as the basis for the experiments discussed in subsequent chapters.

Summary

The function of the T cell antigen receptor (TCR) invariant chains, CD3 γ, δ, ϵ, and ζ, is poorly understood. Evidence suggests that CD3 couples receptor ligand binding to intracellular signalling events. To examine the role of the CD3 ζ chain in TCR-mediated signal transduction, a chimeric protein linking the extracellular and transmembrane domains of CD8 to the cytoplasmic domain of the ζ chain was constructed. The CD8/ζ chimera is expressed independently of the TCR and is capable of transducing signals which, by criteria of early and late activation, are indistinguishable from those generated by the intact T cell receptor. These data indicate that CD8/ζ can activate the appropriate signal transduction pathways in the absence of CD3 γ, δ and ϵ and suggest that the role of CD3 ζ is to couple the T cell receptor to intracellular signal transduction mechanisms.

Characterization of the CD8/ζ chimera in T cell receptor-positive and -negative Jurkat cells

The CD8/ζ chimeric construct depicted in Figure 3, was transfected via electroporation into both the Jurkat human T cell leukemic line, yielding clone JCD8/ζ 2, and a Jurkat-derived mutant, JRT3.T3.5 deficient in full length Ti β chain transcripts and protein, yielding Jβ⁻CD8/ζ 14. Though JRT3.T3.5 expresses normal levels of Ti α and the CD3 subunits, its deficiency in Ti β expression results in the absence of TCR expression on the cell surface [100]. Transfection of the chimera into this cell would enable us to assess ζ's signalling phenotype without the complication of the additional TCR chains. Levels of surface expression of the chimera and TCR in stably transfected clones were quantitated by flow cytometry using mAbs which recognize either CD8 (OKT8) or the CD3 ε subunit of the TCR (Leu 4). Figure 4 shows fluorescence histograms of these clones which both express high levels of CD8/ζ. In addition, the histograms of a Jurkat clone transfected with wild-type CD8, Jurkat CD8, are presented; this cell was used as a control in all of our experiments. The three clones express comparable levels of CD8 epitopes (Fig. 4; panels b,d,f) and T cell receptors (a,c) with the exception of Jβ⁻CD8/ζ 14, which fails to express surface TCR (panel e). Thus the CD8/ζ chimera can be expressed on the cell surface in the absence of the TCR chains.

To characterize the structure of the CD8/ζ chimeric protein, cells were surface radioiodinated, lysed in 1% NP40, and subjected to immunoprecipitation with OKT8 or a rabbit antiserum raised against a cytoplasmic peptide sequence of murine ζ (Fig. 5). Under reducing conditions, antibodies against either CD8 or ζ precipitate a single

Figure 3. Construction of the CD8/ζ Chimera.

A schematic of the wild type CD8 and ζ proteins with the CD8/ζ chimera. Drawings are roughly to scale, demarcating the extracellular, transmembrane and cytoplasmic domains of each protein. Numbers correspond to amino acids in the mature proteins after signal peptide cleavage.

CD8

| Extracellular | TM | Cytoplasmic |
1   161 188   214

ZETA

| TM | Cytoplasmic |
1 10 31   142

CD8/ζ

| Extracellular | TM | Cytoplasmic |
1   187/31   142
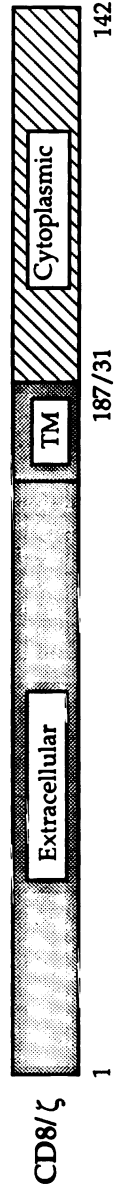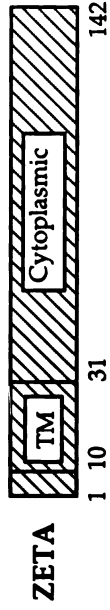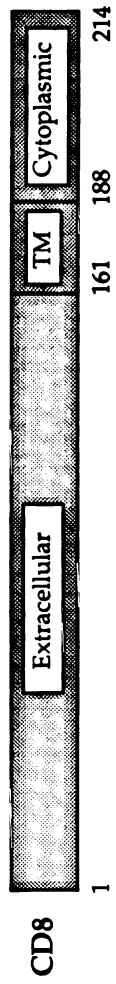
Figure 4. Surface Expression of the T Cell Receptor and CD8 epitope in Jurkat CD8, JCD8/ζ 2, and Jβ-CD8/ζ 14 as assessed by flow cytometry.

The indicated clones were stained with either Leu 4 (anti-CD3ε) or OKT8 (anti-CD8) followed by labeling with fluorescein-conjugated goat anti-mouse Ab (solid lines). Non-specific staining was assessed using a non-reactive mouse IgG (MOPC 195) followed by staining with fluorescein-labeled goat anti-mouse (dotted lines).
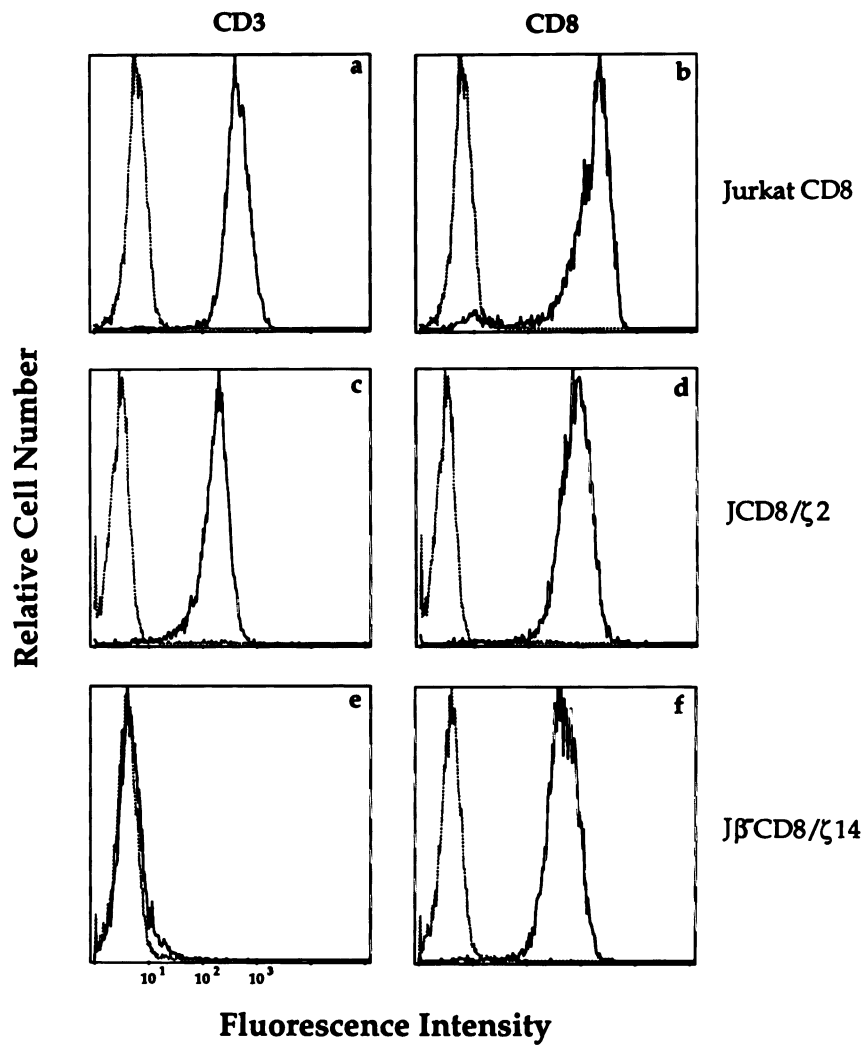
Figure 5. Immunoprecipitations of surface-iodinated Jurkat CD8 and JCD8/ζ 2.

Surface radio-iodinated cells were lysed in 1% NP40 and immunoprecipitated

with normal mouse serum (NMS), normal rabbit serum (NRS), anti-CD8

(OKT8), or an anti-ζ peptide antiserum (#387). Immunoprecipitates were

analyzed under reducing (A) and nonreducing (B) conditions by gel

electrophoresis followed by autoradiography. Each lane represents

approximately 10^7 cell equivalents. The relative positions of the standards are

indicated (kD).

protein of 34-35kD from the chimera-transfected cell (5A, lanes 4 and 6), while

OKT8 precipitates a 29kD protein representing wild-type CD8 from Jurkat CD8

(5A lane 2). Although CD8 in its normal environment has an apparent

molecular weight of 32-34kD, [101], preliminary experiments comparing CD8

in Jurkat and a CD8-positive line, HPB.ALL, suggest that the reduction in size

of CD8 observed here results from a distinct pattern of glycosylation in the

Jurkat host. Under non-reducing conditions a more complex pattern of

proteins is seen in immunoprecipitates of both CD8 and the CD8/ζ chimera

(Figure 5B). This complexity is characteristic of CD8 immunoprecipitates since

homomultimers and heteromultimers have been previously observed [101].

The two prominent species immunoprecipitated from JCD8/ζ 2 migrating at

approximately 70 and 100kD are likely to represent homodimers and

homotrimers of the chimera. As there are no cysteine residues for the

formation of disulfide linkages within the ζ portion of the chimera, any

disulfide bonds formed in the chimera must occur through CD8. Therefore,

any protein forming a heterodimer with CD8/ζ is likely to form one with the

wild type CD8 and thus, should not account for any signalling events

specifically attributable to the CD8/ζ chimera.

Non-covalent association of the chimera with endogenous CD3 γ, δ,

and ε may complicate the interpretation of signals transduced by the chimera.

To determine whether removal of the extracellular and transmembrane

domains of ζ is sufficient to result in its expression independent of the CD3

chains, cells were surface iodinated and lysed in digitonin, a detergent known

to preserve the integrity of the TCR complex. Immunoprecipitates of the TCR

in both Jurkat CD8 and the TCR-expressing chimera-transfectant JCD8/ζ 2,

show identical patterns characteristic of a CD3 (Leu 4) immunoprecipitate

(Fig. 6A, lanes 2 and 5). Though TCR-associated ζ is not well iodinated, as its

extracellular domain contains no tyrosine residues for labeling, ζ

immunoblots of Leu 4 immunoprecipitates confirm that the ζ chain is

efficiently coimmunoprecipitated with CD3 under these lysis conditions (data

not shown). A small quantity of labeled CD3 ε is seen in the Leu 4

immunoprecipitate of the TCR deficient cell (lane 8) despite the fact that this

same mAb failed to stain this cell (Fig. 4, e). The small amount of

immunoprecipitated protein seen may be due to radiolabeling of internal CD3

ε in a small number of permeablized or non-viable cells during the labeling

procedure. Importantly, no CD3 chains are detectable in precipitates of the

CD8/ζ chimera in either TCR-positive or -negative cells (Figure 6A, lanes 6

and 9), nor is any chimera apparent in the Leu 4 precipitate of JCD8/ζ 2 (lane

5). Intentional overexposure of the autoradiogram also fails to reveal TCR

chains coprecipitating with the chimera (data not shown). To further address

the question of co-association of the chimera and TCR chains, we assessed the

effect of antibody-induced down modulation of the TCR on chimera

expression. Whereas overnight incubation of JCD8/ζ 2 with saturating

amounts of C305, a mAb against an epitope of the Jurkat Ti β chain, resulted

in internalization of 94% of the TCR, surface expression of the CD8/ζ chimera

was unaffected (data not shown). By these two independent criteria, no

discernable association exists between CD8/ζ and the CD3 γ, δ, and ε chains.

To determine whether a covalent link exists between endogenous ζ

and the CD8/ζ chimera, ζ immunoblot analysis was performed comparing ζ

and OKT8 immunoprecipitates in Jurkat CD8 and JCD8/ζ 2. (Figure 6B) The

Figure 6. Assessment of association between the CD8/ζ chimera and the CD3
chains.

(A) Immunoprecipitations of digitonin lysates. Cells were surface radio-
iodinated and lysed in 1% digitonin, which preserves the Ti/CD3 association.
Cell lysates were immunoprecipitated with normal mouse serum (NMS),
anti-CD3ε (Leu 4), or anti-CD8 (OKT8). Immunoprecipitates were analyzed
under reducing conditions by SDS/PAGE, followed by autoradiography. The
positions of the chimera and various TCR chains are indicated by arrows at
the left margin of the gel. An asterisk is used (lane 3) to indicate the position
of wild type CD8 protein, as its apparent molecular weight is similar to that of
CD3 γ. Clones and immunoprecipitating antibodies are indicated.

Figure 6B) ζ immunoblot of OKT8 and ζ immunoprecipitates from JCD8/ζ 2
and Jurkat CD8. NP40 lysates were immunoprecipitated with the indicated
antibodies, subjected to polyacrylamide gel electrophoresis under reducing
conditions and transferred to nitrocellulose for blotting with the anti-ζ rabbit
antiserum (#387). Antibodies used are described in figure legend of Fig. 5. ζ is
seen migrating at approximately 16kD, the CD8/ζ chimera, at 35kD.

Figure 6A.
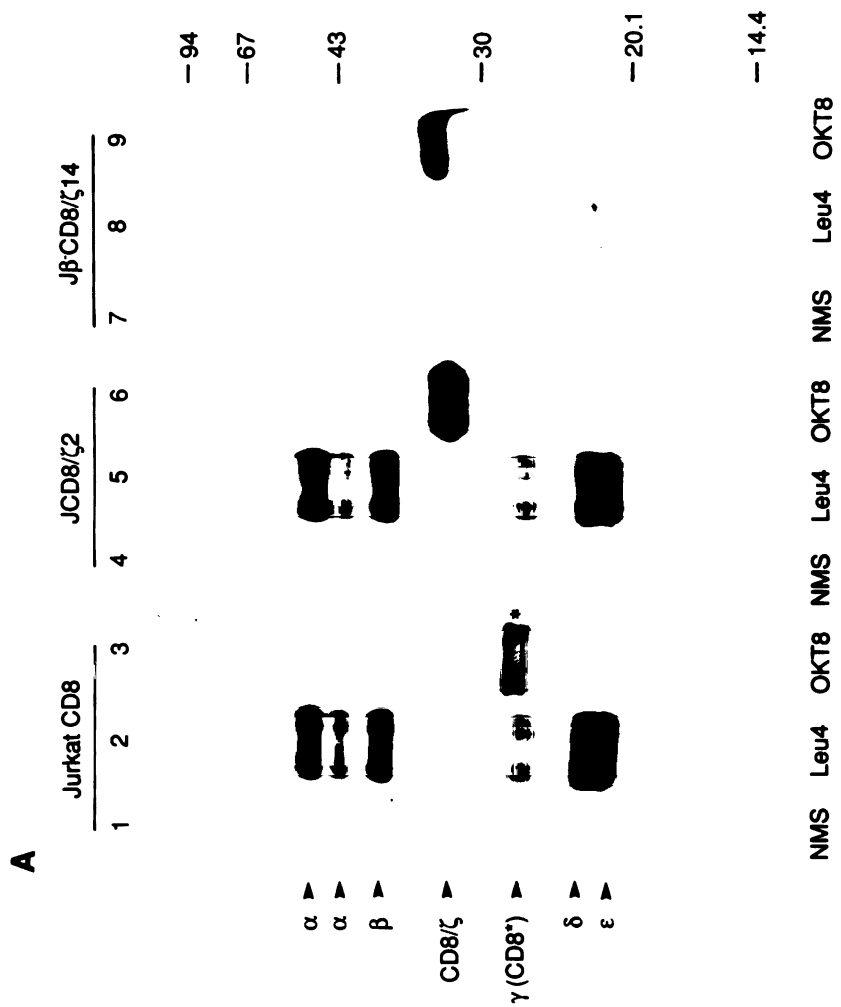
Figure 6B
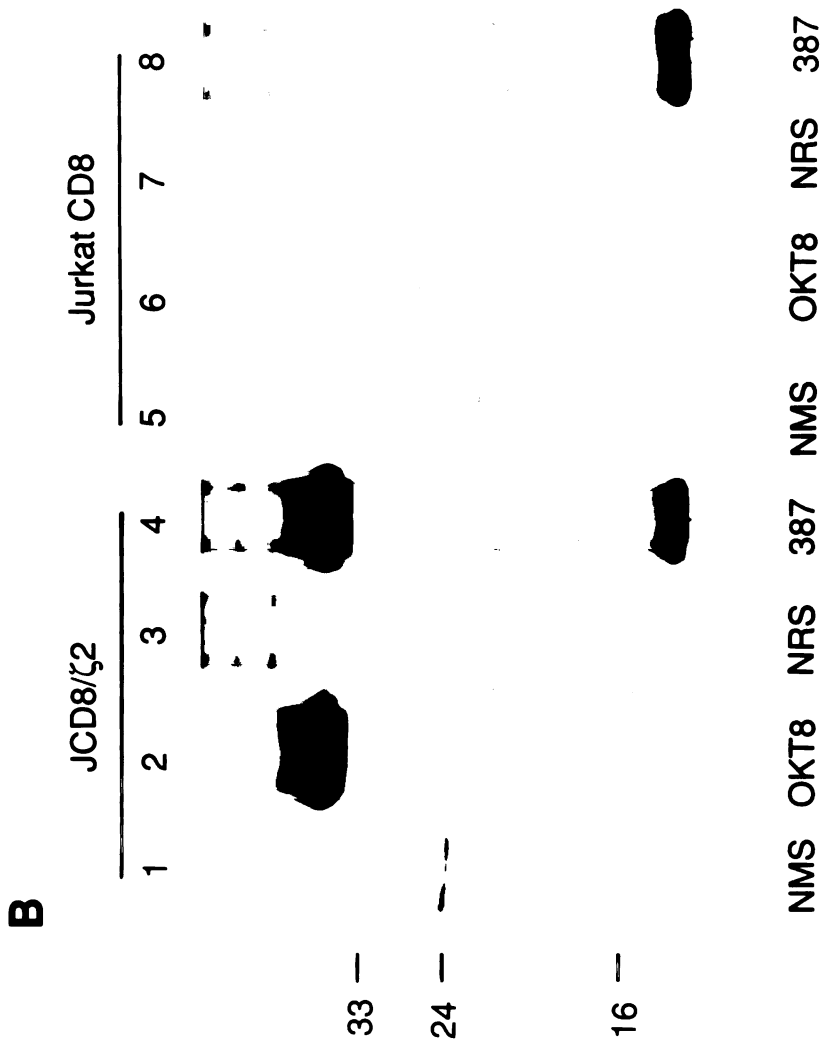
anti-ζ antiserum immunoprecipitates both the chimera and ζ from JCD8/ζ 2 (lane 4), but only endogenous ζ from the Jurkat CD8 control (lane 8). In contrast to the anti-ζ antiserum, OKT8 immunoprecipitates the chimera but not ζ in JCD8/ζ 2 (lane 2), while neither species is detected in Jurkat CD8 (lane 6). Collectively, the results from these experiments and those described above, argue against an interaction between the chimera and endogenous T cell receptor subunits.

Stimulation of CD8/ζ results in activation of the phosphatidylinositol and tyrosine kinase pathways.

To determine whether stimulation of the extracellular domain of CD8/ζ would result in intracellular signalling events, we examined the ability of OKT8 to elicit an increase in cytoplasmic free calcium ($[Ca^{+2}]_i$) in chimera-transfected cells. Figure 7A depicts a typical fluorimetry tracing obtained with JCD8/ζ 2 upon stimulation of its TCR with the anti-Ti β monoclonal antibody C305. Notably, with the addition of soluble OKT8 (Fig. 7B), a substantial increase in $[Ca^{+2}]_i$ is seen, suggesting that the cytoplasmic domain of ζ is capable of coupling to signalling machinery which results in the activation of phospholipase C. We next examined the ability of the chimera to transduce a signal in cells lacking surface expression of the TCR chains. As expected, stimulation of the TCR-negative Jβ^-CD8/ζ 14 with C305 results in no detectable increase in $[Ca^{+2}]_i$; however, OKT8 is still able to elicit a large calcium response (Fig. 7C). The lack of significant increase in $[Ca+2]_i$ with OKT8 stimulation in Jurkat CD8 (Fig. 7D) demonstrates that the ζ portion of the chimera is required for the elicited $[Ca^{+2}]_i$ response.

Figure 7. Calcium mobilization by CD8/ζ in TCR-positive (JCD8/ζ 2) and TCR-negative (Jβ⁻CD8/ζ 14) cells.

Stimulation of JCD8/ζ 2 with anti-Ti β mAb, C305 (A) or anti-CD8 mAb, OKT8 (B). C305 and OKT8 stimulations in Jβ⁻CD8/ζ 14 (C) and OKT8 and C305 stimulations of Jurkat CD8 (D). Cells loaded with Indo-1 were stimulated with soluble C305 or OKT8 ascites (1:1000 dilution) and analyzed for changes in $[Ca^{+2}]$ by fluorimetry as described in Materials and Methods. Stimuli were added at times indicated (arrows).

A. JCD8/ζ2

B. JCD8/ζ2

C. Jβ⁻CD8/ζ14

D. Jurkat CD8

TIME (min)
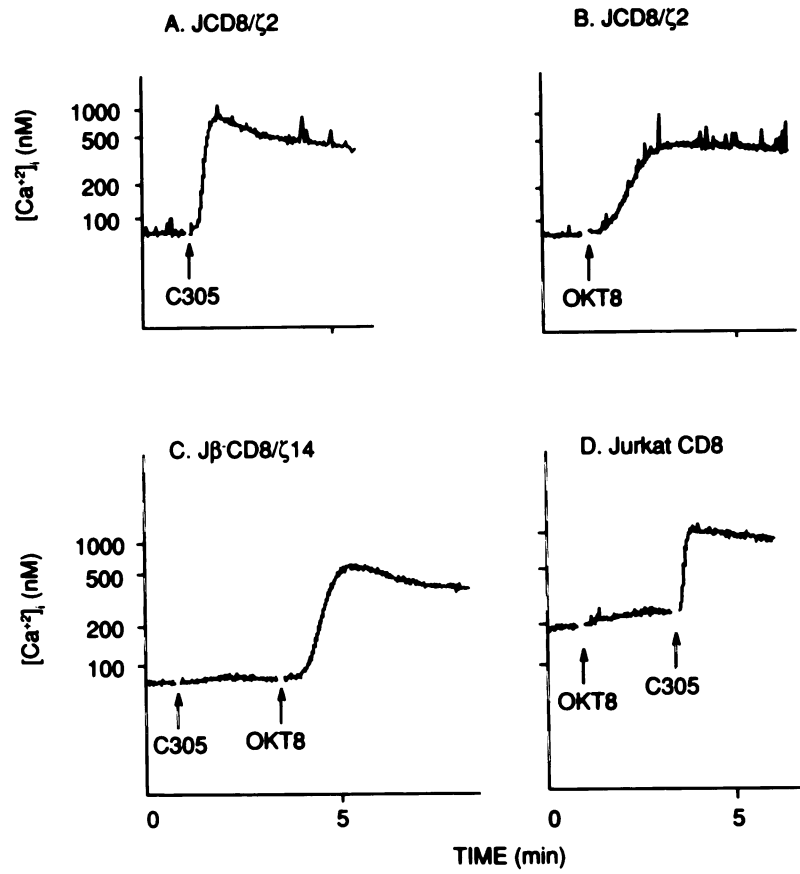
Since the increases in $[Ca^{+2}]_i$ which occur with TCR stimulation are attributed to increases in inositol phosphates, we tested the ability of CD8/ζ to induce PIP_2 hydrolysis by assessing changes in total soluble inositol phosphates following stimulation with OKT8 (Fig. 8). Stimulation of CD8/ζ with OKT8 resulted in the generation of inositol phosphates in both chimera-expressing cells. In contrast, no inositol phosphates were noted with stimulation of the wild-type CD8 protein in Jurkat CD8. Stimulation of the TCR in Jurkat CD8 and JCD8/ζ 2 induced increases in inositol phosphates, whereas in the TCR-deficient transfectant, Jβ⁻CD8/ζ 14, no such increase was observed upon TCR stimulation. These results are consistent with the calcium fluorimetry data and confirm the chimera's ability to activate phospholipase C even in the absence of endogenous cell surface TCR chains.

As stimulation of the T cell receptor activates a tyrosine kinase pathway in addition to the inositol phospholipid pathway, it was important to determine whether chimera stimulation would result in tyrosine kinase activation. Western blots reveal a small number of tyrosine-phosphorylated proteins existing in all three clones prior to stimulation. (Fig. 9, lanes 1,4,7). Upon stimulation of Jurkat CD8 and JCD8/ζ 2 with C305 (anti-Ti β), the tyrosine kinase pathway is activated as demonstrated by the induction of tyrosine phosphorylation of a number of proteins (lanes 2, 5). As expected, C305 has no effect in the TCR-negative transfectant, Jβ⁻CD8/ζ 14 (lane 8). Stimulation of the chimera on both JCD8/ζ 2 and Jβ⁻CD8/ζ 14 with OKT8 (lanes 6 and 9) results in the appearance of a pattern of tyrosine-phosphorylated bands indistinguishable from that seen with TCR stimulation. In contrast, stimulation through wild type CD8 in Jurkat CD8

Figure 8. Generation of soluble inositol phosphates in Jurkat CD8, JCD8/ζ2, and Jβ-CD8/ζ 14.

Cells loaded with ^{3}H-myo-inositol were stimulated for 15 minutes at 37° C with the following antibodies at 1:1000 dilution of ascities: unstimulated (solid); w6/32, an anti-HLA class 1 mAb (cross-hatched); C305, anti-Ti β (light stippling); and OKT8, anti-CD8 (dark stippling). 10 mM LiCl was present during stimulation in order to block the conversion of IP_1 into free inositol and phosphate. Total soluble inositol phosphates were assayed by anion exchange chromatography as previously described [44]. Error bars indicate standard deviations of the mean (n=3).
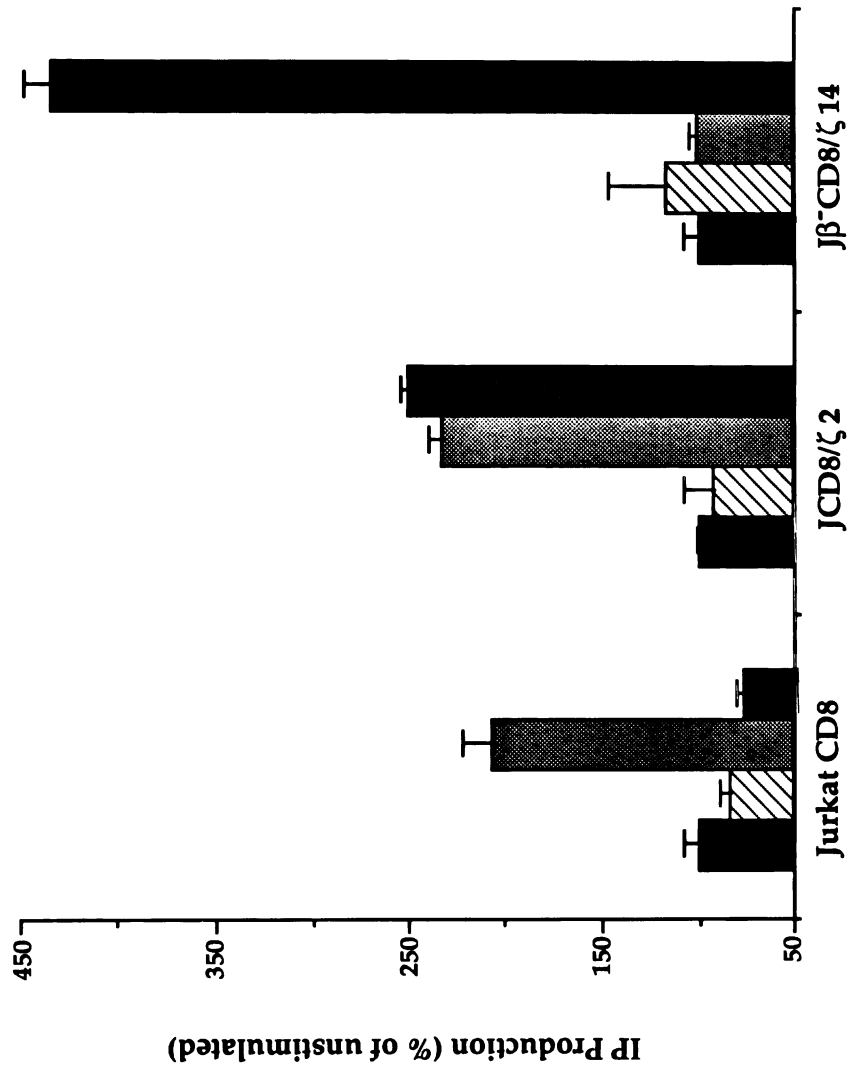
Figure 9. Stimulation of the CD8/ζ chimera results in activation of the tyrosine kinase pathway.

Phosphotyrosine immunoblot of whole cell lysates from cells either unstimulated (0), or stimulated with an anti-Ti β mAb (C305), or an anti-CD8 mAb (OKT8). Cells were stimulated in serum free medium for 2 minutes at 37° C prior to lysis. Lysates were subjected to SDS/PAGE, transferred to nitrocellulose, then blotted with an anti-phosphotyrosine mAb, 4G10. Each lane represents lysate from approximately 2.5 x 10^6 cells.

	Jurkat CD8			JCD8/ζ2			Jβ⁻CD8/ζ14		
	1	2	3	4	5	6	7	8	9
110 —									
84 —									
47 —									
33 —									
24 —									
16 —									
	0	C305	OKT8	0	C305	OKT8	0	C305	OKT8
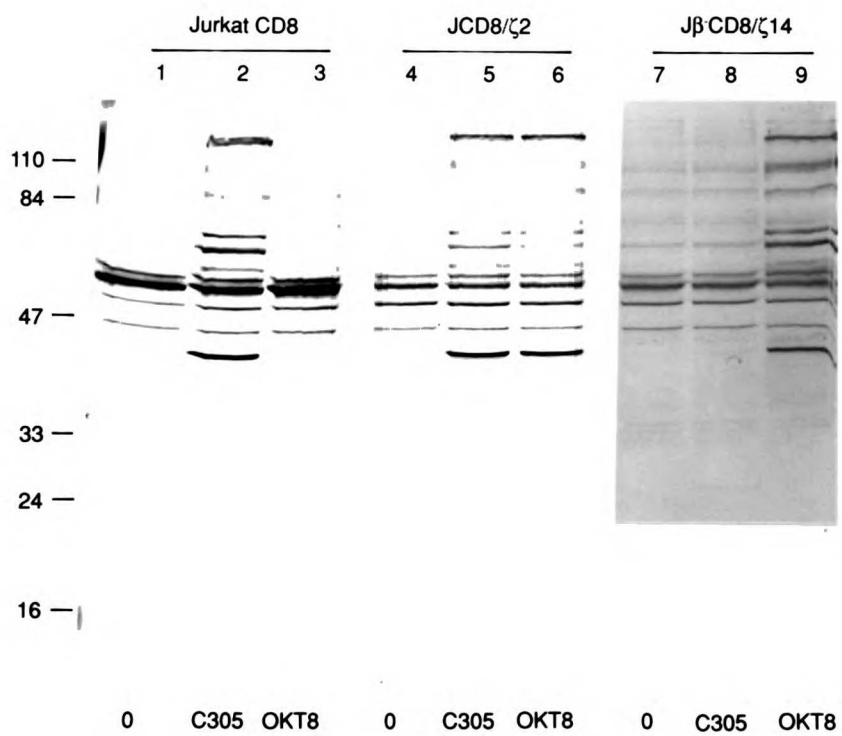
does not result in induction of tyrosine phosphoproteins (Figure 9, lane 3).
Thus, the CD8/ζ chimera, in the absence of Ti and CD3 γ, δ, and ε, is capable of
activating the tyrosine kinase pathway in a manner analogous to that of an
intact TCR.

Since JCD8/ζ 2 expressed two discernible forms of ζ on its surface, -
endogenous ζ and the CD8/ζ chimera -, each of which could be stimulated
independently, we addressed the specificity of receptor-induced ζ
phosphorylation. Immunoprecipitates of ζ derived from the three clones,
either unstimulated, or stimulated with C305 or OKT8, were analyzed by
western blotting with an anti-phosphotyrosine antibody (Fig. 10; upper panel).
A small fraction of the ζ immunoprecipitates were blotted with ζ antiserum
to control for differences in protein content between samples (lower panel).
Analysis of the lysate derived from TCR-stimulated Jurkat CD8 cells (Fig. 10
lane 2) reveals a typical pattern of ζ phosphorylation with the multiplicity of
bands from 16-21kD most likely representing the varying degree of
phosphorylation of the seven cytoplasmic tyrosine residues of ζ. In this
experiment, a small degree of constitutive ζ phosphorylation is detected in
Jurkat CD8; however, this is not augmented by stimulation of the wild type
CD8 protein (Figure 10, lanes 1 vs 3). Whereas phosphorylation of ζ is seen
with stimulation of the TCR in JCD8/ζ 2, though weaker than that seen in
C305-stimulated Jurkat CD8, no induced phosphorylation of the chimera is
apparent (lane 5). Conversely, stimulation of the CD8/ζ chimeric receptor on
both JCD8/ζ 2 and Jβ-CD8/ζ 14 results in a high degree of phosphorylation of
the chimera exclusively, seen as the induced broad band from 34 to 39kD.
This result indicates that the receptor-activated kinase responsible for

phosphorylation of ζ recognizes its substrate only in a stimulated receptor complex.

Figure 10. Phosphorylation of ζ occurs exclusively in activated receptors.

100 x 10^6 cells were stimulated with the indicated antibodies for 2 minutes, then lysed and immunoprecipitated with a rabbit anti-ζ antiserum (#387). 95% of each immunoprecipitate was blotted with an anti-phosphotyrosine mAb, 4G10, (upper panel) while 5% was blotted with the precipitating anti-ζ antiserum, #387 (lower panel). The band migrating at approximately 24 kD in all OKT8 immunoprecipitates (lanes 3,6,9) is likely the light chain of the OKT8 mAb which reacts with the alkaline phosphatase-conjugated goat anti-mouse blotting reagent. The two prominent species seen in the lower gel represent the chimera, at 35kD, and endogenous ζ, just below 16kD.

	Jurkat CD8			JCD8/ζ2			Jβ⁻CD8/ζ14		
	1	2	3	4	5	6	7	8	9

33 –
24 –
16 –

0   C305   OKT8   0   C305   OKT8   0   C305   OKT8

33 –
24 –
16 –
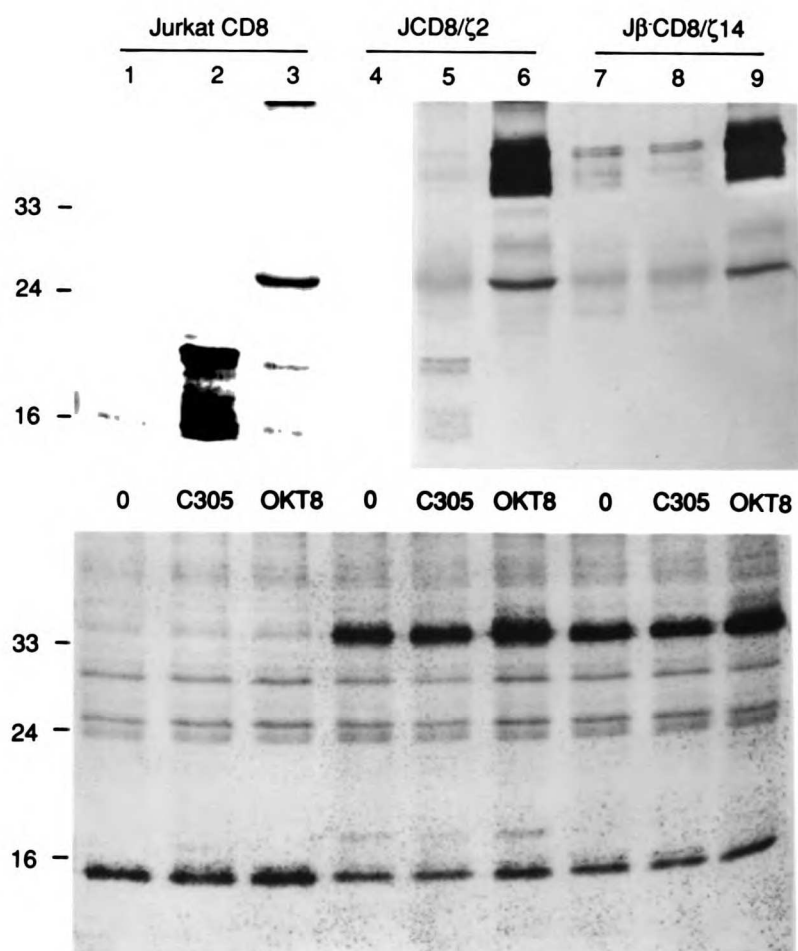
Stimulation of CD8/ζ results in late events of T cell activation

T cell activation results from the delivery of receptor-mediated signals to the nucleus where they act to induce expression of specific genes. One such gene encodes the activation antigen CD69, whose surface expression is induced within hours of T cell receptor stimulation and appears to be dependent on activation of protein kinase C [102]. Although the function of CD69 in T cell activation is not well understood, it provides us with a marker of distal signal transduction events. Flow cytometry reveals a very small degree of basal CD69 expression on unstimulated cells (Fig 11, row 1). Maximal levels are induced on all cells with phorbol myristate acetate, PMA, an activator of protein kinase C (row 2). Stimulation of the TCR results in induction of CD69 on Jurkat CD8 and JCD8/ζ 2 (Figure 11, panels c and g) but not on the TCR-negative clone, Jβ⁻CD8/ζ 14 (Figure 11, k). Moreover, stimulation of cells with OKT8 induces CD69 on both cells expressing the CD8/ζ chimera (Figure 11, h and l). Though a minimal degree of CD69 induction is apparent with stimulation of wild-type CD8 protein (Figure 11d), this level is no higher than that observed with stimulation of Jurkat CD8 with a class 1 MHC antibody w6/32 (data not shown).

Perhaps the most commonly used criterion to assess late activation events is the production of the lymphokine, interleukin-2 (IL-2) [103]. The IL-2 gene is tightly regulated, requiring the integration of a number of signals for its transcription [104], making it a valuable distal marker for assessing signalling through the CD8/ζ chimera. Stimulation of Jurkat CD8 and JCD8/ζ 2 cells with TCR antibodies in the presence of PMA, results in production of IL-2 (Table 2). Importantly, while treatment with OKT8 on Jurkat CD8

Figure 11. Stimulation of CD8/ζ induces expression of CD69.

Flow cytometry of cells stimulated overnight with the indicated stimuli, then stained with fluorescein-conjugated Leu 23 (anti-CD69; solid lines) or MOPC 195 (nonreactive control mAb; dotted lines). Stimulation was performed using fixed antibodies as described in Materials and Methods: IL-2 Bioassay. PMA was used at a final concentration of 50 ng/ml.

Jurkat CD8   JCD8/ζ 2   Jβ⁻CD8/ζ 14

Relative Cell Number

Fluorescence Intensity

Unstimulated

PMA

C305

OKT8
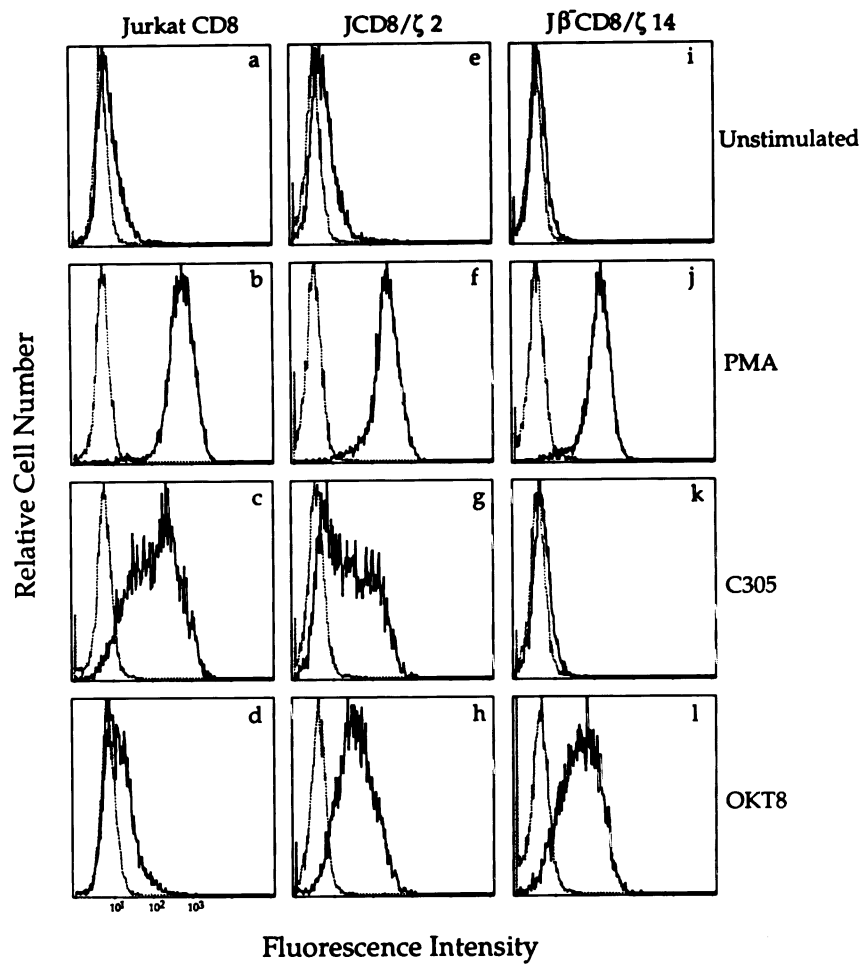
induces no IL-2, similar treatment of JCD8/ζ 2 results in levels of secreted IL-2

consistently higher than those produced in that cell with TCR stimulation.

Jβ⁻CD8/ζ 14 responded more weakly to all experimental stimuli in this assay,

but the data were qualitatively similar in that this cell reproducibly secreted

IL-2 in response to OKT8 but not to C305 (data not shown). These data

confirm that in addition to early signal transduction events, later activation

events occur upon stimulation of the CD8/ζ chimera, thus demonstrating its

ability to couple to the relevant signal transduction pathways in a physiologic

manner.

Treatment	IL-2 (Units/ml)			
	Jurkat CD8		JCD8/ζ2	
	Experiment #		Experiment #	
	#1	#2	#1	#2
Unstimulated	<0.1	<0.1	<0.1	<0.1
C305 + PMA	13.5	9.1	3.7	2.1
OKT8 + PMA	<0.1	<0.1	6.8	7.0
C305 + OKT8 + PMA	–	–	11.5	–
W6/32 + PMA	<0.1	<0.1	<0.1	<0.1
Ionomycin + PMA	30.4	4.2	24.2	24.6

Table 2. Induction of IL-2 production by the CD8/ζ chimera

JCD8/ζ 2 and Jurkat CD8 cells were stimulated with the indicated

mAb or ionomycin (1mM) in the presence of PMA (10ng/ml). Il-2

secretion was determined by the ability of culture supernatants of

stimulated cells to support the growth of the IL-2 dependent CTLL-

2.20 cells. Since PMA alone induces no IL-2 production in Jurkat,

yet has a small direct effect on the viability of the CTLL 2.20 cells,

values obtained with PMA alone were subtracted from each

response value, yielding the numbers shown above. Data from

two independent experiments are presented.

Discussion:

CD8/ζ is expressed independently of CD3 γ, δ, and ε

Progress in understanding the role of the various TCR subunits in signal transduction has been hindered by the complexity of the receptor structure and its stringent requirements for assembly and surface expression. While reconstitution experiments have proved invaluable in describing requirements for association of the receptor chains [95], and the existence and fate of partial complexes formed intracellularly [70, 105]), attempts at expressing partial complexes on the cell surface have essentially failed. In this study, we demonstrate successful surface expression of the TCR ζ chain independent of its companion chains by replacement of its transmembrane and nine amino acid extracellular regions with those of CD8.

We fail to detect evidence for any association between CD8/ζ and the additional TCR chains by two independent criteria: immunoprecipitation of CD3 or CD8/ζ, and down-modulation experiments. Although it is formally possible that the levels of CD3 associating with the chimera are below the levels of detection using either technique, this is highly unlikely. If associated CD3 were responsible for the chimera's signalling phenotype, antibodies against CD3 should elicit a response comparable to the chimera's in Jβ⁻CD8/ζ 14. Leu 4, an anti-CD3 ε antibody elicits no such response in that cell (data not shown). An alternative explanation for our data might invoke low level association of the chimera with partial complexes of CD3 or with CD3 existing in an anomalous conformation not recognized by the Leu 4 antibody. This also is unlikely, for the following reasons: First, thorough studies of intracellular partial receptor complexes have failed to detect ζ in a partial

complex containing any combination of the CD3 chains; instead, ζ has been observed either alone, in association with Ti, or as a subunit of the intact receptor [70, 106]. Second, in contrast, CD3 ϵ is identified alone, with CD3 δ, γ, or both intracellularly, or in the intact receptor, and importantly, is recognized by anti-CD3 ϵ antibodies in all contexts [70, 105]. Third, CD3 δ or γ are never observed beyond the endoplasmic reticulum alone or as a heterodimer independent of the CD3 ϵ chain, apparently because CD3 ϵ is necessary to stabilize these chains. If not complexed with CD3 ϵ chains, the δ and γ chains are recognized shortly after translation and are sent to a recently described ER-degradative pathway [107, 108]. This evidence argues strongly against the chimera's association with either partial complexes of CD3, or CD3 no longer recognized by Leu 4. Moreover, it supports the contention that the transmembrane domain of ζ contains sequences which dictate its association with the Ti and CD3 chains.

Our results are consistent with the accumulating evidence implicating the transmembrane domains of the TCR subunits in proper assembly of the receptor complex. Site-directed mutagenesis of the charged residues within the transmembrane domain of either Ti α or β have resulted in complete loss of receptor assembly and expression [96, 97, 109]. Moreover, the region of the Ti α chain required for its association with CD3 δ has recently been defined to a region of 8 amino acids within its transmembrane domain [95]. Finally, construction of chimeric Ti α and β chains demonstrates that, by retaining regions containing the transmembrane domains of these subunits, both a structural and functional association is maintained between the chimeric proteins and their CD3 counterparts [99].

ζ as a signal transducing molecule

By effectively reducing the complexity of the TCR from six distinct proteins (α,β,γ,δ,ε and ζ) to one, we demonstrate a function for the cytoplasmic tail of ζ in coupling the TCR to the appropriate signal transduction machinery in the T cell. Stimulation of the chimera results in activation of both the inositol phospholipid and tyrosine kinase second messenger pathways. In addition, distal activation events such as the induction of CD69 expression and production of IL-2 confirm the physiologic nature of the chimera-induced signals. Our findings that the signals transduced by the chimera are qualitatively indistinguishable from those generated by the TCR, strongly suggest that ζ is the subunit within the intact receptor which ultimately couples ligand binding to intracellular signalling events. Because the tyrosine kinase fyn has been coprecipitated with the CD3 complex, ζ may provide a direct link from Ti/CD3 to this putative receptor-activated kinase. Alternatively, ζ may link indirectly to a kinase, coupling instead to an as of yet undefined transmembrane or cytoplasmic protein. The CD8/ζ chimera should provide a means of studying ζ-associated proteins without the complication of the associated TCR chains and may help elucidate the nature of the interaction between the TCR and the inositol phospholipid and tyrosine kinase signal transduction pathways. Moreover, by constructing chimeras comprised of other cell surface molecules linked to ζ, it should be possible to assess any contribution the inherent structure of CD8 may have towards the activation of the cytoplasmic domain of ζ, and potentially to create T cells with specificity for a large number of other ligands independent of MHC restriction.

Our results bring to question the role of CD3 γ, δ, and ε, in the T cell receptor complex. Recent evidence from the previously described ζ-deficient murine hybridoma (MA 5.8) has implicated the CD3 chains as signal transducers, for while transfection of this cell with truncations of ζ essentially failed to reconstitiute antigen responsiveness, mAbs against Ti or CD3 elicited diminished but measurable responses [66]. One interpretation of these results would suggest a signalling role for CD3, with ζ required to communicate the binding of antigen by Ti to the signal transducing CD3 chains. Our data suggest an alternative interpretation. Whereas the truncations and mutations were sufficient to disrupt the communication between antigen-binding Ti and ζ, they were not sufficient to disrupt ζ's ability to transduce signals when stimulated with antibodies. Antibody stimulation may bypass the defect in ζ which prohibits its interpretation of the ligand binding event. This hypothesis is a reasonable one, for the truncations in ζ were relatively minor, with the largest truncation leaving 57 amino acids in the cytoplasmic domain [66]. One potential problem with the notion of ζ as the exclusive signalling component of the TCR arises in interpreting this variant's unreconstituted phenotype. Though this ζ-deficient cell expresses only 5% normal levels of TCR on its surface and is unresponsive to antigen, it does respond minimally to anti-TCR antibodies [86]. The crux of this apparent discrepancy with our model resides in the state of the T cell receptors expressed on the surface of the cell. Though ζ is not detectable in this cell even by PCR techniques [87], a ζ-like molecule may be expressed which is capable of rescuing expression of the small degree of receptors observed. This is not unreasonable, as the γ subunit of the Fcε receptor has recently been

identified in a murine T cell line incorporated as a subunit of the TCR complex [78]. In addition, since two homologues of ζ have been discovered to date, it is conceivable that a previously undefined ζ homolog may explain the remaining low level of TCR expression and the antibody response in MA 5.8. Alternatively, the CD3 chains could also couple, though less efficiently, to the appropriate signal transduction pathways in the absence of ζ.

If CD3 γ, δ, and ϵ do not couple to intracellular signalling, they may instead, serve a regulatory role in T cell receptor function. Alternatively, they may be required to transfer information from Ti or other ligand binding molecules to ζ, where it can be transduced in the form of a signal to the cytoplasm of the cell. Evidence from preliminary experiments examining signalling by CD2, a molecule requiring co-expression of the TCR for its signalling competence in T cells [110], is consistent with the latter role for CD3 in T cell signal transduction. It is hypothesized that CD2 signalling function converges quite proximally with that of the TCR and that CD2 may couple to TCR components directly. Because CD2 and ζ are expressed in NK cells independently of CD3 γ, δ, and ϵ [76] but stimulation of CD2 can activate NK cells [111], we postulated that CD2 may couple directly to ζ and thus, to the signal transduction machinery. In preliminary experiments to address this question, CD2 responses to activating antibodies were examined by calcium fluorimetry in the chimera-expressing cells. In contrast to the TCR-expressing cell, the CD3-negative, chimera-expressing cell was completely unresponsive to combinations of CD2 activating antibodies. Despite the presence of a competent ζ cytoplasmic domain, CD2 retains its requirement for CD3 suggesting a possible role for the CD3 or analogous chains in NK cells in

directing information from ligand binding molecules, e.g. Ti and CD2, to the signal coupling protein, ζ. In addition, a similar function for CD3 $\gamma\delta\epsilon$ may exist for other accessory molecules such as CD4, CD8, Thy-1 and Ly-6 which have been suggested to interact with the TCR [112, 113] .

Our finding that phosphorylation of ζ occurs exclusively in mAb-stimulated TCR and chimeric complexes, suggests that ζ, and thus the TCR, may undergo a conformational change upon activation. The fact that stimulation of CD8/ζ does not result in phosphorylation of endogenous ζ, and stimulation of the TCR fails to phosphorylate the chimera, suggests that the relevant kinase can distinguish between activated and unactivated substrate. Currently, two models exist to explain how extracellular ligand binding is interpreted by the TCR [114]. One hypothesis invokes receptor aggregation upon ligand binding, resulting in the initiation of intracellular signals. The other proposes a ligand-induced change in receptor conformation as a prerequisite for its functional activity. Both models are based on the demonstration that binding of antibodies against the TCR result in its activation, with a correlation existing between degree of crosslinking and intensity of the activation response. While our result does little to distinguish between the two models (antibodies were used in all experiments), evidence from a signal transduction mutant derived from Jurkat supports a ligand-induced conformational change in the TCR. In this cell, anti-Ti antibodies which effectively bind and thus cause aggregation of receptors, fail to elicit a response, whereas some but not all mAbs reactive with CD3 are capable of activating the receptor [115]. Our data with CD8/ζ are consistent with a ligand-induced conformational change in ζ. However, an

alternative interpretation exists for the data. The kinase responsible for ζ phosphorylation may be restricted to or sequestered by the receptor complex, and therefore rendered incapable of accessing the ζ outside its particular receptor complex.

The structural complexity of the TCR has previously prevented a simple analysis of structure-function relationships. This work has permitted the study of a single domain of a single chain, ζ, isolated from the complexities of the seven chain receptor and demonstrates its ability to couple to intracellular signal transduction events characteristic of those observed with TCR stimulation. Although a precise understanding of the integrated function of the seven chains of the holo-receptor is not possible through such an approach, it will now be possible to perform a more detailed analysis of the mechanism by which this domain interacts with intracellular signal transducing elements. Furthermore, a similar strategy employing replacement of the transmembrane regions, may provide a means of studying the role of CD3 γ, δ, and ε independent of ζ and Ti. Perhaps by analyzing the function of the individual components, a better understanding of interactions among the chains of this complex oligomeric receptor will emerge.

Materials and Methods:

CD8/ζ chimera construction

The polymerase chain reaction, PCR [116] was used to amplify the extracellular and transmembrane portion of CD8α (residues 1-187) from pSV7d-CD8α and the cytoplasmic portion of the human ζ chain (residues 31-142) from pGEM3zζ. DNA sequences are from [117]; CD8), and [74]; ζ). Plasmids pSV7d-CD8α and pGEM3zζ were kindly provided by Drs. Dan Littman and Julia Turner (Univ. of Ca., S.F.) and Drs. R.D. Klausner and A.M. Weissman (N.I.H.), respectively. Primers encoding the 3' sequences of the CD8 fragment and the 5' sequences of the ζ fragment were designed to overlap such that annealing of the two PCR products yielded a hybrid template. From this template the chimera was amplified using external primers containing Xba I and Bam HI cloning sites. The CD8/ζ chimera was subcloned into pTfneo [100] and sequenced via the Sanger dideoxynucleotide technique [118].

Antibodies

C305 and Leu 4 mAbs recognize the Jurkat Ti β chain and an extracellular determinant of CD3 ε, respectively. OKT8, acquired from the A.T.T.C., recognizes an extracellular epitope of CD8. The anti-ζ rabbit antiserum, #387, raised against a peptide comprising amino acids 132-144 of the murine ζ sequence [75], was kindly provided by Drs. R.D. Klausner, A.M. Weissman, and L.E. Samelson. The anti-phosphotyrosine mAb, 4G10, was a generous gift of Drs. D. Morrison, B. Druker, and T. Roberts. W6/32 recognizes an invariant determinant expressed on human HLA class 1 antigens. Leu23,

reactive with CD69, was obtained from Becton-Dickinson Monoclonal Center (Milpitas, Ca.). MOPC 195, an IgG2a (Litton Bionetics, Kensington, MD) was used as a control mAb in FACS analysis. Ascitic fluids of mAb were used at a final dilution of 1:1000 (a saturating concentration) in all experiments unless otherwise stated.

Cell lines and Transfections

The human leukemic T cell line Jurkat and its derivative J.RT3-T3.5 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) glutamine, penicillin and streptomycin (Irvine Scientific). Chimera-transfected clones were passaged in the above medium with the addition of Geneticin (GIBCO, Grand Island, NY) at 2 mg/ml. Electroporation of pTfneo-CD8/ζ into Jurkat or J.RT3-T3.5 was performed in a Bio-rad Gene Pulser using a voltage of 250V and a capacitance of 960μF with 20 μg of plasmid per 10^7 cells. After transfection, cells were grown for two days in RPMI before plating out in Geneticin-containing medium. Clones were obtained by limiting dilutions and screened for TCR and CD8/ζ expression by Flow Cytometry (see below). The Jurkat CD8 clone, transfected with the wild-type CD8 protein, was kindly provided by Drs. Julia Turner and Dan Littman.

Flow Cytometry

Approximately 1 x 10^6 cells/condition were stained with saturating concentrations of antibody, then incubated with fluorescein-conjugated goat anti-mouse Ab prior to analysis in a FACScan (Beckton Dickinson) as previously described [83]. Cells analyzed for CD69 expression were stained

directly with fluorescein-conjugated Leu 23 (anti-CD69 mAb) or MOPC 195 (control mAb).

$[Ca^{+2}]_i$ Measurement by Fluorimetry

Calcium sensitive fluorescence was monitored as previously described [115]. Cells were stimulated with soluble mAb C305 and OKT8 at saturating concentrations (1:1000 dilution of ascities). Maximal fluorescence was determined after lysis of the cells with Triton X-100; minimum fluorescence was obtained after chelation of Ca^{+2} with EGTA. $[Ca^{+2}]_i$ was determined using the equation $[Ca^{+2}]_i = K_d \times (F_{observed} - F_{min})/ F_{max} - F_{observed})$, with K_d = 250 nM as described [119].

Inositol Phosphate Measurement

Cells were loaded with $[^3H]$myo-inositol (Amersham) at 40 μCi/ml for 3 hr. in phosphate buffered saline, then cultured overnight in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were stimulated for 15 min. with the indicated antibodies at 1:1000 dilution of ascities in the presence of 10 mM LiCl to inhibit dephosphorylation of IP_1. The extraction and quantitation of soluble inositol phosphates were as described [44].

Surface Iodinations

Cells were labeled with ^{125}I using the lactoperoxidase/glucose oxidase (Sigma) procedure as described [83].

Immunoprecipitations

Cells were lysed at 2×10^7 cells/200 ml in 1% NP40 (Nonidet P40), 150 mM

NaCl, and 10 mM Tris pH 7.8 in the presence of protease inhibitors, 1mM

PMSF, aprotinin, and leupeptin. Lysis buffer for lysates to be analyzed for

phosphotyrosine content was supplemented with phosphatase inhibitors, as

described [48]. Iodinated lysates were supplemented with 10 mM

iodoacetimide to prevent post-lysis disulfide bond formation. Digitonin lysis

was performed in 1% Digitonin, 150 mM NaCl, 10 mM Tris pH 7.8, 0.12%

Triton X-100. After 30 min. at 4°C, lysates were centrifuged for 10 min. at

14,000 rpm., then precleared with fixed Staphylococcus aureus (Staph A;

Calbiochem-Behring). Alternatively, lysates of cells stimulated with antibody

prior to lysis were precleared with sepharose beads. The precleared lysates

were incubated with Protein A Sepharose CL-4B beads which had been

prearmed with the immuno-precipitating antibody. Washed

immunoprecipitates were resuspended in SDS sample buffer +/- 5% β-

mercaptoethanol and boiled prior to electrophoresis on 11% polyacrylamide

gels.

Stimulation of cells for assessment of phosphotyrosine content.

Cells were stimulated in serum free medium at 2×10^7 cells/200 µl with

antibodies at 1:250 dilution of ascities. After 2 min. at 37°C, the medium was

aspirated, and the cells lysed in 100 µl of 1% NP40 lysis buffer. Lysates were

precleared, then ultracentrifuged and samples resolved by SDS PAGE.

Immunoblots

Gels were equilibrated in transfer buffer (20 mM Tris-base, 150 mM Glycine, 20% methanol) for 30 min and transferred to nitrocellulose membranes in a Bio-Rad Western blotting apparatus run at 25 volts overnight. Membranes were blocked in TBST (10 mM Tris HCl [pH 8], 150 mM NaCl, 0.05% Tween 20) plus 1.5% ovalbumin, then incubated with either mAb 4G10 or rabbit anti-ζ antiserum (#387). The immunoblots were washed and incubated with a 1:7000 dilution of alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit antibody. After 1-2 hours, the blots were washed and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates as per manufacture's instructions (Promega).

IL-2 Bioassay

For stimulation, cells were coated with the indicated antibodies at saturating concentrations (1:1000 dil. of ascities) for 30 min. at 4°C. After removal of unbound antibody, cells were spun onto 24-well tissue culture plates which had been precoated with rabbit anti-mouse Ig (Zymed Labs) and blocked with medium plus 10% FBS. Phorbol myristate acetate, PMA (Sigma) and ionomycin (Calbiochem) were added at final concentrations of 10 ng/ml and 1 μM, respectively. Cell-free supernatants were harvested after 20 hr. of culture and assessed for IL-2 content utilizing the IL-2 dependent CTLL-2.20 T cell line in the MTT colorimetric assay as described [120].

CHAPTER III. FUNCTIONAL CHARACTERIZATION OF A SIGNAL TRANSDUCING MOTIF PRESENT IN THE T CELL ANTIGEN RECEPTOR ζ CHAIN.

Preface

A better understanding of how the TCR initiates the signal transduction cascade is rapidly emerging. Utilizing chimeric receptors composed of the cytoplasmic domain of ζ linked to heterologous extracellular domains, we and others have previously demonstrated a role for ζ in TCR-mediated signal transduction [121-124]. Stimulation of these chimeric molecules with monoclonal antibodies (mAbs) recapitulates both proximal and distal events normally associated with stimulation of the intact TCR. Furthermore, fusion proteins containing the cytoplasmic sequences of η and FcεRIγ are capable of mediating increases in $[Ca^{+2}]_i$ and targeted cytotoxicity [122]. Similarly, the cytoplasmic domain of CD3ε in the context of the extracellular and transmembrane domains of the IL-2 receptor α-chain is sufficient for the induction of tyrosine phosphoproteins and production of IL-2 [125]. Confirming the signalling capacity of CD3 is a study demonstrating that the TCR can function independently of the ζ cytoplamic domain [124]. Together, these results demonstrate a role for the ζ family of proteins and at least one of the CD3 chains in coupling the TCR to intracellular signal transduction machinery.

Since ζ is capable of transducing signals which are indistinguishable from those generated by the TCR, a more detailed analysis of the ζ sequence was initiated with the notion that identifying its functional elements may

provide valuable mechanistic information. The results obtained are detailed in this chapter.

Summary

A conserved sequence motif has been identified in a number of signalling subunits associated with hematopoietic cell antigen receptors. Here, we characterize signalling by a 17 amino acid motif that is triplicated in the T cell antigen receptor (TCR) ζ chain. Analysis of ζ truncations and constructs containing the isolated motif demonstrates that this motif is sufficient for the induction of both proximal and distal events associated with T cell activation. Stimulation of truncations which contain either one, two, or three copies of the motif results in induction of an identical pattern of tyrosine phosphoproteins. Moreover, triplication of the N-terminal ζ motif results in enhanced signalling suggesting a redundant role in signal amplification for the three motifs in ζ. Finally, we demonstrate the association of a recently identified protein tyrosine kinase (PTK) ZAP-70 with this motif, and provide evidence for its involvement in ζ function.

Truncations of CD8/ζ reveal a minimal functional unit that associates with ZAP-70.

To define the functional region(s) within the ζ cytoplasmic domain, constructs encoding C-terminal truncations of the CD8/ζ chimera [121] (see arrows in Fig 12 A) were transfected into the Jurkat T cell leukemic line. The designated clones were selected based on high levels of surface expression of the CD8 extracellular epitope as assessed by flow cytometry (Fig 12B). Characterization of the chimeras by surface iodination confirms that the expressed proteins are of the expected sizes (Fig 12C).

To determine which of the CD8/ζ truncations were capable of mediating intracellular signalling events, we examined the ability of the constructs to elicit a rise in intracellular calcium $[Ca^{+2}]_i$ (Fig 13A-D). Clone JζT34, which expresses a chimera containing 34 cytoplasmic residues, was completely unresponsive to saturating concentrations of anti-CD8 mAb, OKT8, or to further crosslinking with secondary Ab, rabbit anti-mouse IgG (RαmIgG) (Fig. 13A). However, the signal transduction pathway in this clone was intact as evidenced by the immediate and potent effect of anti-TCR mAb, C305. In contrast, stimulation of JζT46 with OKT8 alone resulted in a delayed but substantial increase in $[Ca^{+2}]_i$, which was further augmented by cross-linking with RαmIgG. More rapid responses characteristic of those made by the intact CD8/ζ were seen with stimulation of truncations T56 and 83, with only a minimal augmenting effect of subsequent crosslinking Ab. Like clone JζT34, clones expressing T12 and T24 were completely unresponsive to the combination of OKT8 and RαmIgG (data not shown) despite higher levels of surface expression of these molecules (Fig.12, B and C). Thus, these results

Figure 12. COOH terminal truncations of CD8/ζ: amino acid sequence, levels of surface expression, and structural characterization.

(A) The amino acid sequence of the ζ cytoplasmic domain is depicted with arrows denoting the C-terminus of each CD8/ζ truncation. The number assigned to each truncation refers to the number of amino acids remaining in the ζ cytoplasmic domain. All constructs share both the extracellular and transmembrane domains of CD8. The conserved tyrosine and leucine-based motif is shown underlined.

(B) Surface Expression of the CD8/ζ truncations as assessed by flow cytometry. Jurkat transfectants expressing the designated CD8/ζ truncations were stained with saturating concentrations of mAb OKT8 (anti-CD8), followed by labeling with fluorescein-conjugated goat anti-mouse IgG (solid line). Non-specific fluorescence was assessed utilizing a control mAb, MOPC 195 followed by similar labeling with goat anti-mouse IgG (dotted line.)

(C) Surface radio-iodinated cells (2 x 10^7) were lysed in 1% NP40 and immunoprecipitated with OKT8. Immunoprecipitates were resolved by SDS PAGE followed by autoradiography. Mobilities of markers are denoted at the left of all gels.

69

A.

Amino Acid Sequence of Human ζ Cytoplasmic Domain:

```
          T12              T22              T34
           ↓                ↓                ↓
RVKFSRSAEPPAYQQGQNQLYNELNLGRREEYDVLDKR
        T46              T56
         ↓                ↓
RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG
        T83
         ↓
MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR
```

B.

JζT12   JζT22   JζT34

JζT46   JζT56   JζT83

Relative cell number

10¹ 10² 10³ 10⁴   10¹ 10² 10³ 10⁴   10¹ 10² 10³ 10⁴

Fluorescence intensity

C.

CD8/ζ Truncations

T12 T22 T34 T46 T56 T83

kD

32.5 —

27.5 —

18.5 —

1   2   3   4   5   6
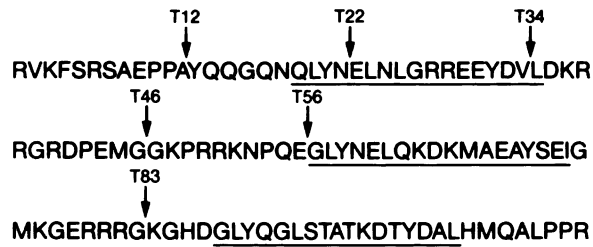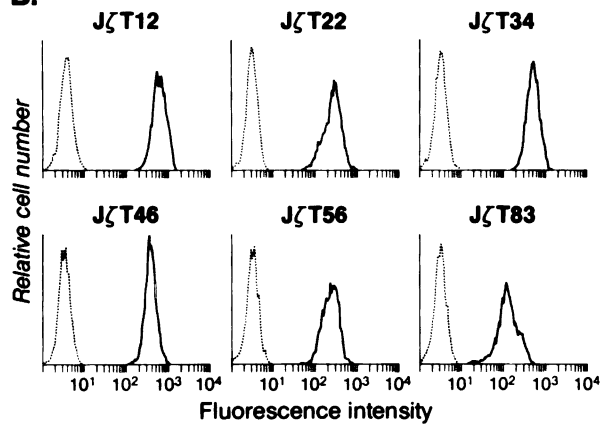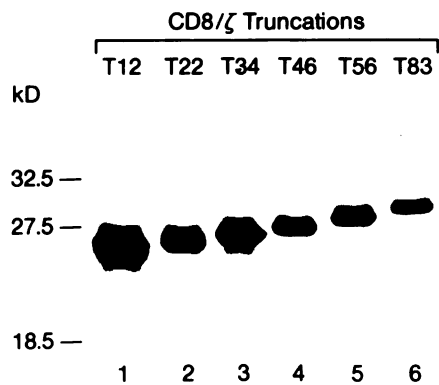
Figure 13. Analysis of calcium mobilization by Jurkat clones expressing C-terminal truncations of CD8/ζ.

(A-D) The designated clones were loaded with Indo-1 and stimulated with an anti-CD8 mAb OKT8 (1 μg/ml), followed by crosslinking with RαmIgG (2 μg/ml). JζT34 (A), unresponsive to OKT8 and to RαmIgG, was stimulated with the anti-TCR mAb, C305 (1 μg/ml). Stimuli were added at the indicated times (arrows) and changes in $[Ca^{+2}]_i$ were monitored by fluorimetry as described in Materials and Methods.

A. JζT34
B. JζT46
C. JζT56
D. JζT83

[Ca²⁺]ᵢ (nM)

Time (min)
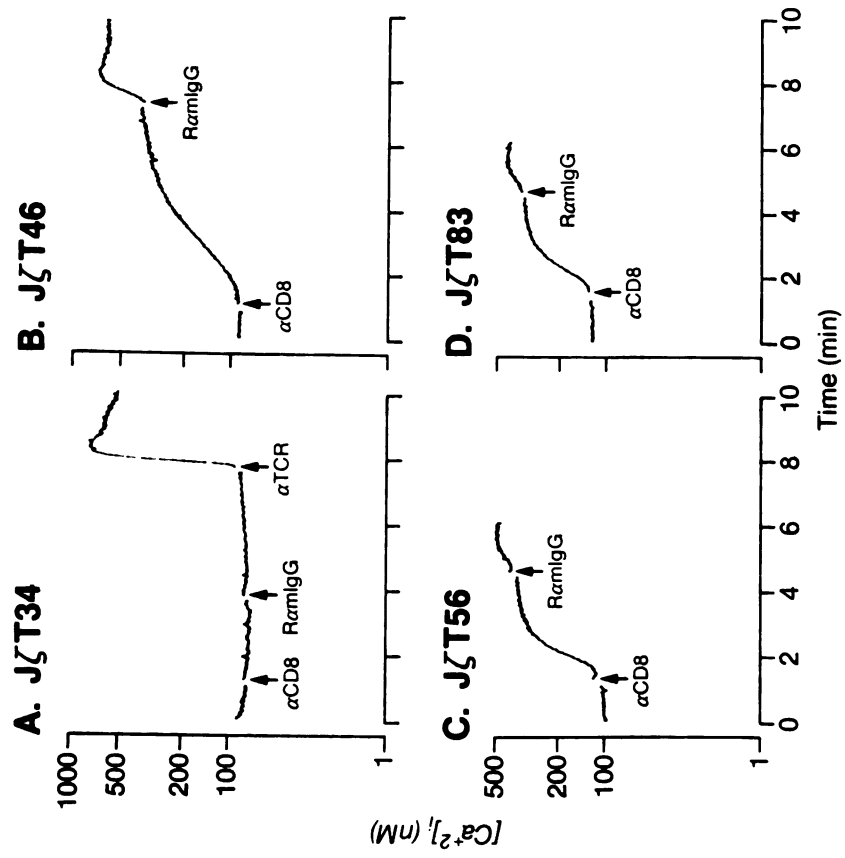
define a functional region within the first 46 cytoplasmic amino acids of ζ and indicate the requirement of sequences between cytoplasmic residues 34 and 46 for calcium mobilization.

Since TCR-mediated changes in $[Ca^{+2}]_i$ are dependent on increased PTK activity [16], we examined the status of tyrosine phosphoproteins in lysates prior and subsequent to stimulation of the truncated CD8/ζ chimeras. An increased number of tyrosine phosphoproteins is observed upon stimulation of either CD8/ζ or the intact TCR as visualized by western blotting with an anti-phosphotyrosine mAb (Figure 14, lane 2). Though a slight augmentation in one or two substrates can be seen in stimulated lysates from JζT12 and JζT22, the first marked induction in tyrosine phosphoproteins is seen with stimulation of truncation T46 (lane 10). Stimulation of T56, which contains 10 additional cytoplasmic amino acids, results in more efficient induction of tyrosine phosphoproteins revealing a pattern of substrates which is virtually indistinguishable from that observed with CD8/ζ (Figure 14, lane 2). Similar results were obtained with stimulation of ζT83. These data are consistent with the fluorimetry data presented above, and demonstrate that the first 46 cytoplasmic amino acids of ζ appear to be sufficient for activation of the appropriate PTKs.

We have recently described a 70 kD tyrosine phosphoprotein, ZAP-70, which associates stably with ζ upon stimulation of the antigen receptor [8]. Recent isolation and sequencing of a cDNA encoding ZAP-70 reveals that it is a PTK, exhibiting the highest degree of amino acid identity to syk, a 72kD PTK isolated from splenic cells [9, 126]. To define the site within ζ required for association with ZAP-70, Jurkat clones expressing the CD8/ζ truncations were

Figure 14. Induction of tyrosine phosphoproteins in Jurkat clones expressing C-terminal truncations of CD8/ζ.

Phosphotyrosine immunoblot of whole cell lysates from cells either unstimulated (-) or stimulated (+) with anti-CD8 mAb, OKT8. Cells were stimulated in phosphate buffered saline (PBS) at 37°C with OKT8 (2μg/ml) for 2 minutes followed by crosslinking with RαmIgG (2 μg/ml) for an additional 2 minutes. Cell lysates were resolved by electrophoresis and analyzed by western blotting with an anti-phosphotyrosine mAb, 4G10. Each lane represents 2×10^6 cell equivalents.

examined. Lysates from unstimulated or stimulated cells were immuno-
precipitated with the indicated mAb, resolved by SDS PAGE, and analyzed by
western blotting with an anti-phosphotyrosine mAb. Figure 15A demonstrates
no detectable ZAP-70 associated with any of the truncations in the basal state.
However, OKT8 stimulation induces the phosphorylation and association of
ZAP-70 with truncations T46, 56, and 83, while no induced association is
detected with T12, 22, and 34 (Fig. 15B). Interestingly, despite the fact that all
truncations, with the exception of T12, contain at least 2 cytoplasmic tyrosine
residues, tyrosine phosphorylation of ζ sequences is seen only in those
constructs which associate with ZAP-70 (28-33kD bands observed in figure 15B).
To demonstrate that all clones are equally capable of transducing the necessary
signals required for ZAP-70 association, cells were stimulated through the TCR
and ζ immunoprecipitates analyzed as described above. Figure 15C
demonstrates that both TCR-mediated ZAP-70 association and ζ
phosphorylation (19-21 kD phosphorylated proteins) occur to similar degrees in
all clones. These results provide striking correlative evidence linking tyrosine
phosphorylation of ζ and association of ZAP-70 with the ability of ζ to activate
both the PTK and inositol phospholipid pathways.

Signal Transduction through a 17 amino acid motif

Examination of the 46 cytoplasmic amino acids sufficient for ζ function
revealed a motif composed of two pairs of tyrosine and leucine residues
spaced in the following fashion: YxxLxxxxxxxYxxL. This motif, which is
present in three copies within ζ (see underlined sequences in Fig 12A) and
singly represented in the ζ homologue, FcεRIγ likely represents a signal

Figure 15. Association of the CD8/ζ truncations with ZAP-70 tyrosine kinase.
(A and B) 10^8 cells expressing the indicated truncations were stimulated as
described in Figure 14, lysed in buffer containing 1% NP40, and
immunoprecipitated with anti-CD8 mAb, OKT8. Immunoprecipitates were
analyzed for tyrosine phosphoproteins by western blotting with anti-
phosphotyrosine mAb 4G10 (The arrow depicts the tyrosine phosphorylated
form of ZAP-70.).

(C) Cells were stimulated with anti-TCR mAb C305 for 2 min. in PBS at 37°C,
lysed, and ζ immunoprecipitates analyzed as described above (A,B).

A.

kD	T12	T22	T34	T46	T56	T83	Stimulation	IP Ab

αCD8

B. αCD8 αCD8

C. αTCR αζ

1 2 3 4 5 6
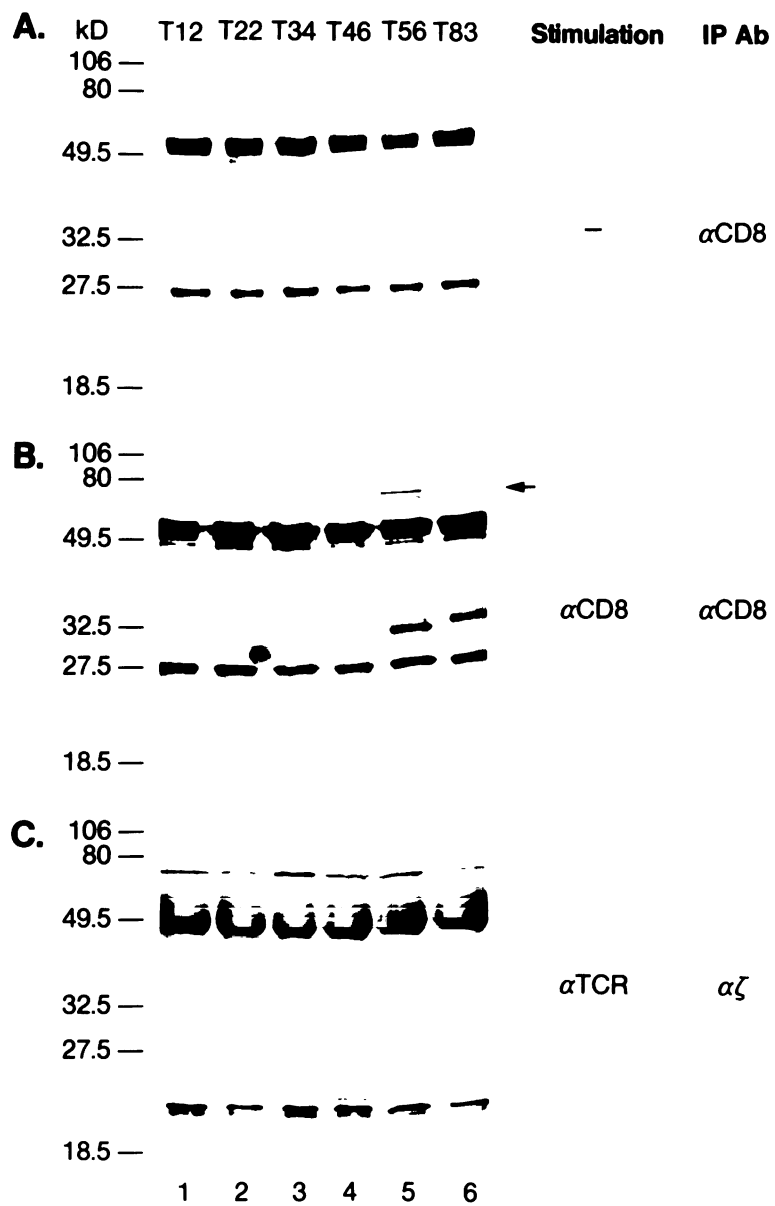
transducing module serving to couple ζ to intracellular PTKs (see discussion).
To study the function of this motif, fusion proteins whose cytoplasmic
sequences are depicted in Figure 16A were constructed and transfected into
the Jurkat line. Briefly, a truncated CD8 molecule, designated CD8$_T$, was
created, replacing cytoplasmic residues 5-6 with a Bgl II site into which
isolated sequences encoding the motifs plus two adjacent N-terminal residues
could be introduced. While constructs encoding ζ motifs 1, 2 and 3 were
made, surface expression of ζ3 was too low for analysis of its function. To
gain insight into why the tyrosine and leucine-based motif is triplicated in ζ if
one copy is apparently sufficient for function, a fusion protein containing a
triplication of the most membrane proximal motif was constructed. To
accomplish this and allow for separation between motifs in the triplicated
construct, a linker encoding 5 amino acids composed of arginine and glycine
residues was added at the C-terminus of motif 1 (designated ζ1L). An
additional restriction site attached to the linker enabled the introduction of
three copies of motif 1 into CD8$_T$, yielding ζ1L(3X). Clonal surface expression
and structural characterization of the chimeras were assessed by flow
cytometry (Fig 16B) and surface iodination (Fig 16C).

Calcium fluorimetry was performed to assess the signalling function of
the chimeras containing isolated motifs. (Figure 17). While in the previous
fluorimetry experiments, additional crosslinking of the primary mAb OKT8
had only a modest effect on calcium mobilization by the CD8/ζ truncations
(Figure 13), crosslinking was absolutely required for activation by this new
group of chimeras. Thus, sequences surrounding this 17 amino acid motif or
the spacing between it and the plasma membrane are important for optimal

Figure 16. CD8/ζ chimeras containing isolated motifs: amino acid sequence, levels of surface expression, and structural characterization.

(A) The cytoplasmic sequences of CD8/ζ chimeras containing isolated motifs. A Bgl II site was added to the sequences encoding the first four cytoplasmic residues of CD8 resulting in an additional arginine and serine at the C-terminus of CD8$_T$. Annealed oligos encoding the ζ sequences shown were introduced via this Bgl II site as described in Materials and Methods.

(B) Surface Expression of constructs depicted in (A) as assessed by flow cytometry. Staining was performed as described in Figure 12B legend. JCD8/ζ expresses the intact CD8/ζ chimera.

C) Structural characterization of constructs by surface iodination (see legend Figure 12C)

A.

Construct	Cytoplasmic Sequence
CD8$_T$	RNRRRS
ζ1	RNRRRS-QLYNELNLGRREEYDVL
ζ2	RNRRRS-GLYNELQKDKMAEAYSEI
ζ1L	RNRRRS-QLYNELNLGRREEYDVLRRGGR
ζ1L(3×)	RNRRRS-(QLYNELNLGRREEYDVLRRGGRGI) × 3

B.
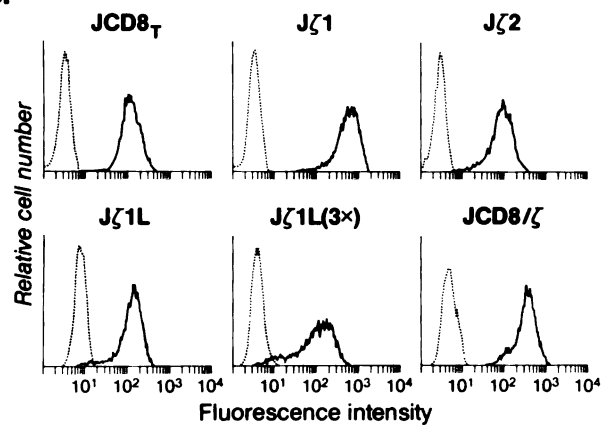
C.
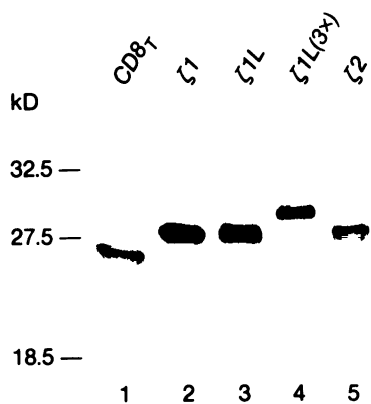
function. Therefore, unless otherwise stated, biotinylated OKT8 (bOKT8) and avidin as a crosslinking reagent were used for stimulations, as these reagents provided a more immediate and synchronous crosslinking than did OKT8 and RαmIgG (data not shown). As expected, no significant increase in $[Ca^{+2}]_i$ is seen with stimulation of the truncated CD8 molecule in JCD8$_T$, though a strong response is elicited with the addition of anti-TCR Abs. This demonstrates the requirement of ζ sequences for functional coupling of the chimeras to PLC. In contrast, crosslinking of chimeras $\zeta 1$ or $\zeta 2$ with OKT8 and avidin results in mobilization of intracellular $[Ca^{+2}]_i$ (17B and C). Interestingly, addition of the 5 amino acid linker onto motif 1, $\zeta 1L$, enhances its capacity to signal, (compare tracings 17D and 17B), perhaps by stabilizing the C-terminal end of the motif. However, this enhancement of function appears to be transient as evidenced by the precipitous fall in $[Ca^{+2}]_i$ seen following the very rapid, initial rise. The transient nature of the $[Ca^{+2}]_i$ increase mediated by $\zeta 1L$ is no longer apparent in the response of $\zeta 1L(3x)$ (Fig 17F), whose sustained plateau phase is more characteristic of that observed with either CD8/ζ or TCR stimulation. This enhancement in signalling by $\zeta 1L(3x)$ relative to $\zeta 1L$ was observed in multiple clones and was still detectable when expression of $\zeta 1L$ exceeded that of the triplicated construct. These results suggest that increasing the number of motifs from 1 to 3 can improve the efficiency of signal transduction and may indicate why teleologically, this motif has been triplicated in the ζ chain. However, despite its improved function, signalling by $\zeta 1L(3x)$ does not compare to that of CD8/ζ as demonstrated by the ability of CD8/ζ to respond to bOKT8 alone, and more robustly to avidin crosslinking (Fig 17F). Note that in contrast to the ramping

Figure 17. Calcium mobilization by chimeras containing the isolated motifs.

(A-F) The indicated clones were loaded with the calcium-sensitive dye Indo-1

and analyzed for chimera-induced increases in $[Ca^{+2}]_i$ by fluorimetry.

Biotinylated OKT8 (bOKT8) was used at a final concentration of 0.5 μg/ml and

avidin at 1 μg/ml.
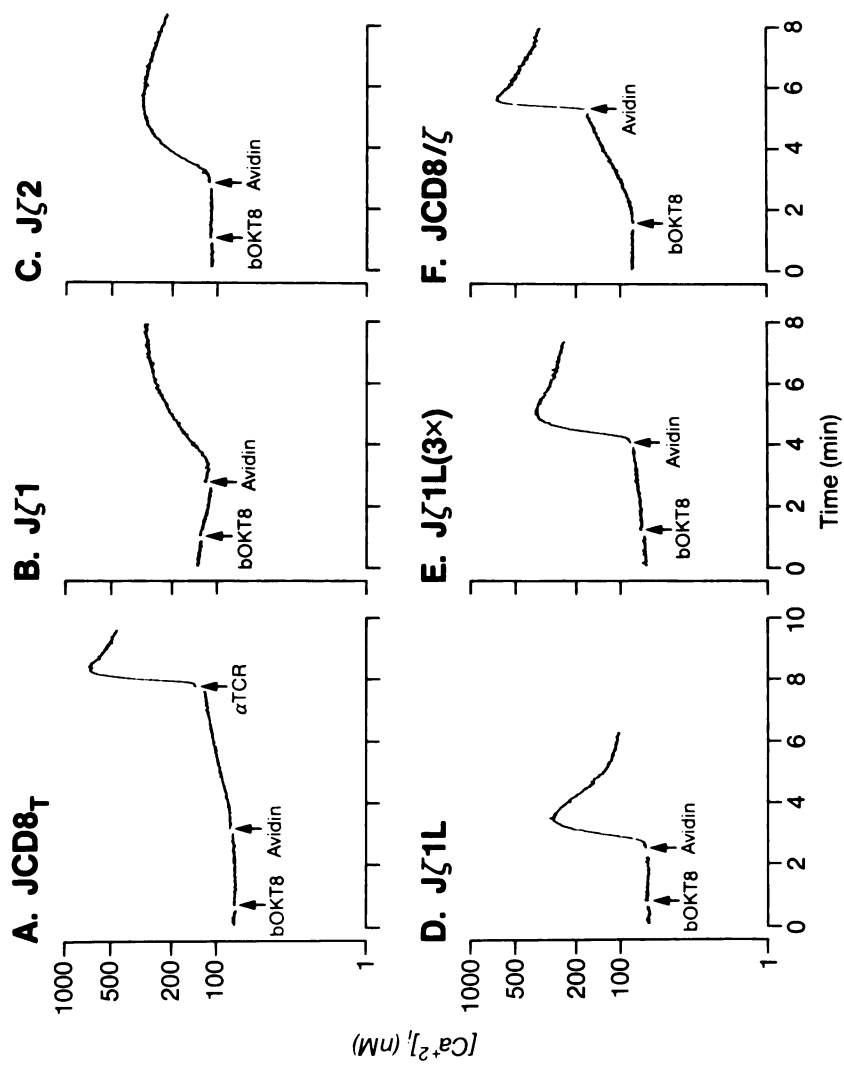
effect seen upon stimulation of CD8/ζ with bOKT8, purified OKT8 elicits a rapid, strong response from this chimera [121], suggesting that the degree of biotinylation may have compromised the CD8 binding epitope of the antibody.

To examine the ability of the 17 amino acid motif to induce PTK activity, tyrosine phosphoproteins from cell lysates were analyzed as previously described above (see Figure 14). Immunoblotting of cell lysates from JCD8$_T$ with an anti-phosphotyrosine mAb (Figure 18, lanes 1 and 2) reveals that although a higher than normal degree of phosphorylation is present in the basal state, no significant augmentation is seen with stimulation of the truncated CD8. However, stimulation of ζ1, ζ1L, and ζ1L(3x) results in progressively greater induction of tyrosine phosphoproteins, recapitulating the quantitative increase in signalling efficiency observed with these constructs in the fluorimetry experiments (lanes 3-8). The low degree of tyrosine phosphorylation induced by ζ1 is similar to that observed with stimulation of ζ2 (data not shown). Notably, the pattern of substrates seen with stimulation of ζ1L(3x) (lane 8) is nearly identical to that induced by the intact CD8/ζ, though the intensity of tyrosine phosphorylation induced by the latter is clearly greater. Thus, the sequences in the first motif are sufficient to associate with at least one PTK, which can induce the phosphorylation of substrates that appear to be similar to those phosphorylated by engagement of CD8/ζ or the TCR.

Our results with the CD8/ζ truncations demonstrated a correlation between the ability of the ζ truncations to associate with ZAP-70 and their ability to function. To determine whether ZAP-70 could associate with the 17

Figure 18. <u>Induction of tyrosine phosphoproteins by the isolated ζ motifs</u>

Cells were stimulated at 37°C in PBS with bOKT8 (2 μg/ml) for two min.

followed by crosslinking with avidin (4 μg/ml) for an additional 2 minutes.

Phosphotyrosine content in lysates was analyzed as described (Figure 14,

legend).
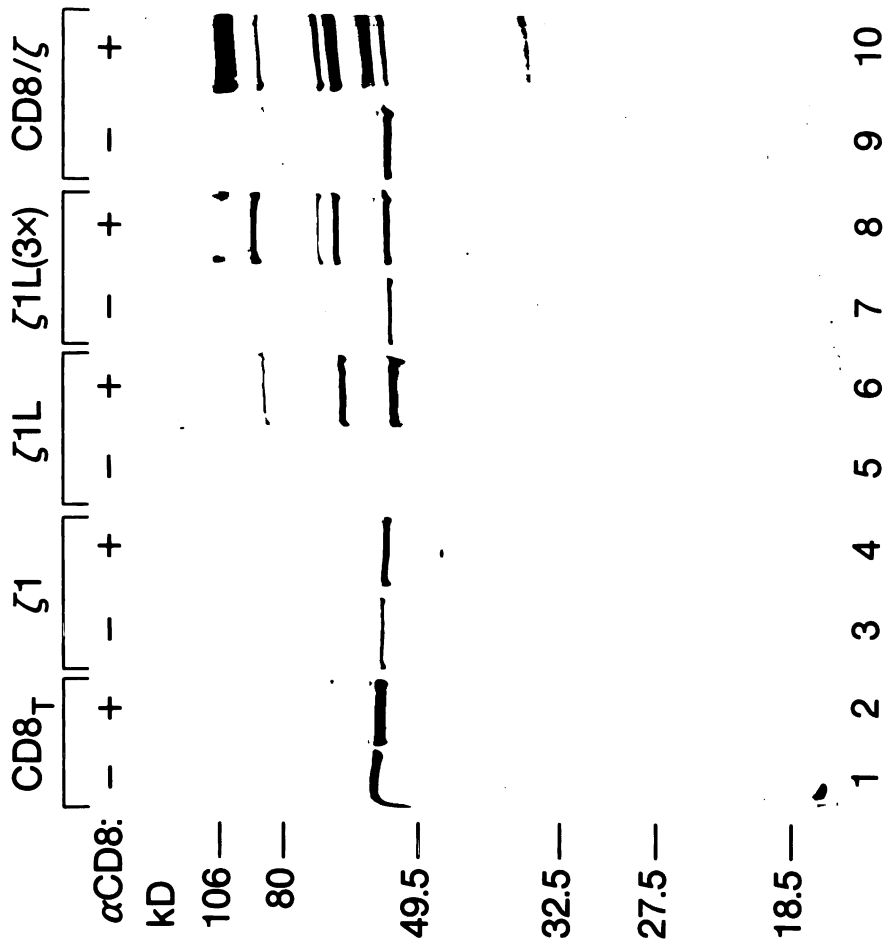
amino acid motif, lysates from unstimulated or OKT8-stimulated cells

expressing the isolated motif chimeras were subjected to anti-CD8

immunoprecipitations followed by immunoblotting with an anti-

phosphotyrosine mAb. As depicted in Figure 19A, no association of ZAP-70

with the truncated CD8 molecule is detected nor is it observed after

crosslinking of ζ1, or ζ1L. However, stimulation of ζ1L(3x) results in the

detectable induction of ZAP-70 phosphorylation and association (see arrow),

confirming the ability of this PTK to recognize and bind to the 17 amino acid

motif. Apparently, a chimera expressing only a single copy of the motif in

this context is impaired in its ability to associate stably with ZAP-70. Utilizing

a sensitive in-vitro kinase assay of CD8 immunoprecipitates, association of

ZAP-70 can be detected with ζ1L, though association with ζ1 and ζ2 has not

been convincing. However, direct immunoprecipitation of ZAP-70 with an

αZAP-70 antiserum reproducibly reveals its induced tyrosine

phosphorylation following stimulation of these latter two constructs (data not

shown). More efficient association of ZAP-70 is apparent with stimulation of

the intact CD8/ζ chimera (lane 10), demonstrating that while the sequences in

motif 1 are sufficient for activation, they alone do not confer optimal

function. Following immunoprecipitation with OKT8, the lysates were

subjected to immunoprecipitation with PLCγ1 mAbs to assess the state of PLC

tyrosine phosphorylation. The middle panel of Figure 19 demonstrates the

induced phosphorylation of PLC with stimulation of ζ1L, ζ1L(3x) and CD8/ζ.

A small fraction of each PLC immunoprecipitate was blotted with anti-PLC

Ab to normalize for differences in protein level between samples (lower

panel). These data indicate that the first 17 amino acid motif is sufficient to

Figure 19. ZAP-70 associates with sequences in the first ζ motif.

Cells (1.8×10^8) were stimulated as described (Figure 18, legend). OKT8 immunoprecipitates from lysates were resolved by electrophoresis, and analyzed for phosphotyrosine content by western blotting with anti-phosphotyrosine mAb, 4G10 (top panel). Immunoprecipitates of PLCγ1 (from 5×10^7 cells) were analyzed in a similar fashion (middle panel). The bottom panel represents a fraction of the PLCγ1 immunoprecipitate blotted with the immunoprecipitating mAb demonstrating comparable levels of PLCγ1 in each lane.

induce the tyrosine phosphorylation of PLCγ1 and ZAP-70 kinase, and, when triplicated, is capable of forming a stable association with ZAP-70.

The capacity of a T cell to produce the lymphokines required for proliferation and differentiation depends on the biochemical nature and duration of the TCR-mediated signals [104]. A common means of assessing the functional integrity of the TCR involves quantitating the expression and secretion of the lymphokine, interleukin 2 (IL-2). In place of the standard bioassay for IL-2, we have monitored the transcriptional activity of NF-AT (nuclear factor of activated T cells), perhaps the best characterized of the several nuclear factors which facilitate transcription of the IL-2 gene [127]. Similar to activation of the IL-2 gene, induction of NF-AT requires the integration of a number of TCR-generated signals and is blocked by the immunosuppressant Cyclosporin A [128-130]. We utilized a plasmid containing 4 tandem copies of the NF-AT DNA binding element placed upstream of the chloramphenicol acetyltransferase (CAT) reporter gene [131]. Figure 20 depicts results obtained from two experiments in which NF-AT-induced CAT activity is measured from cells harvested after 8 hours of stimulation with the indicated mAbs in the presence of an activator of protein kinase C, PMA. As expected, engagement of CD8$_T$ (hatched bar) resulted in no induction of NF-AT activity in either experiment. Similarly, crosslinking of Class I MHC molecules (black bars), whose expression is higher than that of the CD8/ζ chimeras, had little or no effect in any clone. In contrast, a three- to four-fold induction in NF-AT activity was seen with stimulation of either ζ1, ζ1L, or ζ2, indicating that the signals transduced by the isolated motifs are sufficient to manifest distal activation events.

Figure 20. Induction of NF-AT activity by isolated motifs.

A construct containing 4 tandem copies of the NF-AT binding site linked to the chloramphenicol acetyltransferase (CAT) gene was transfected into the designated clones. After 40 hours, cells were stimulated and assayed for CAT activity as described in Materials and Methods. Results are expressed as a fold induction, comparing values obtained from cells stimulated with OKT8 (hatched bars) or an anti-class 1 MHC mAb, W6/32 (black bars) in the presence of PMA, to those obtained with PMA treatment alone. Values were normalized against the average fold induction observed in response to ionomycin and PMA (20.76 ± 2.4 in experiment 1 and 21.1 ± 1.2 in Experiment 2).
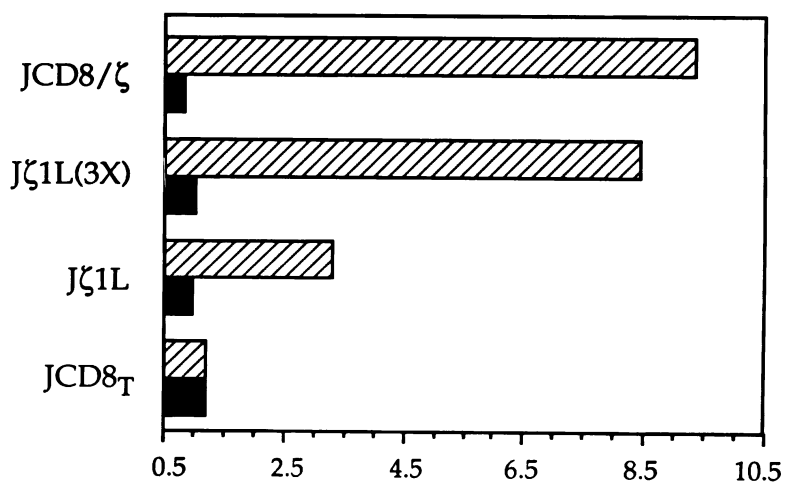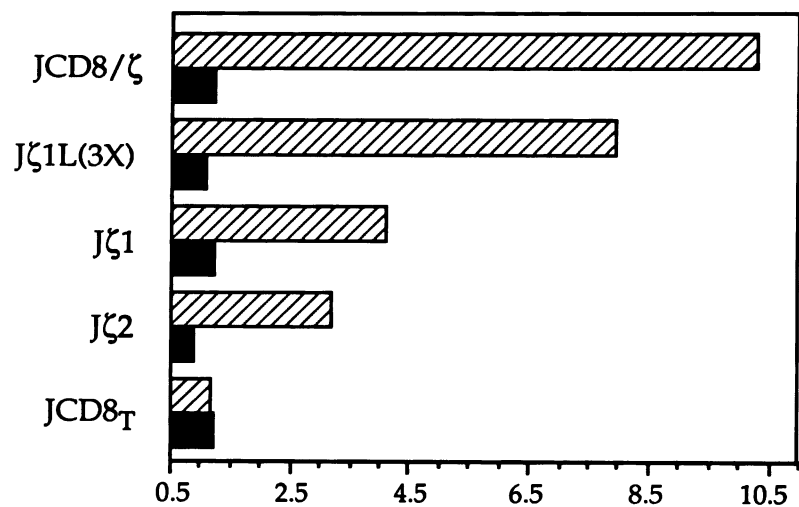
Fold Induction

Furthermore, ζ1 and ζ2 are capable of inducing surface expression of the late activation antigen CD69 (data not shown). Consistent with the proximal signalling data, triplication of motif 1 increased the signalling efficiency of ζ1L(3x), resulting in a greater than 8-fold induction in NF-AT activity with its stimulation. This degree of induction was surpassed only by the intact CD8/ζ which provided an average of 10 fold increase in NF-AT activity. These data demonstrate that the signals transduced by the first or second 17 amino acid motif are capable of effecting both early and late activation events and that multimerization of this motif in ζ can serve a function in signal amplification.

Discussion

Here we characterize signalling by the first and second of three signal transducing modules present in the TCR ζ chain. When isolated from its normal context, the 17 amino acid motif described herein, is capable of transducing signals sufficient for the induction of both early (Ca^{+2} mobilization and induction of tyrosine phosphoproteins) and late (induction of CD69 expression and NF-AT activity) events associated with T cell activation. Previous studies utilizing deletions and truncations of chimeric ζ fusion proteins suggested the existence of at least two functional domains in the ζ chain [123, 124] and led to the identification of a conserved sequence which is triplicated in ζ [124]. A 26-28 amino acid sequence, which encompasses the 17 residue motif, had been previously described based on its homology to sequences contained within the cytoplasmic domains of a number of antigen receptor-associated molecules [132]. These include ζ, FcεRIγ, each of the CD3 chains, and the membrane immunoglobulin-associated MB1 and B29 gene products (see Figure 21). Work from two groups has provided direct evidence for the role of these motifs in signal transduction, with the CD3ε motif capable of inducing tyrosine phosphoproteins and IL-2 production [125] and the ζ motifs sufficient to effect calcium mobilization and redirected cytolysis [133]. We confirm and extend the findings of the latter study demonstrating that signals transduced by a reduced motif of 17 amino acids are sufficient for induction of both CD69 and the transcriptionally active NF-AT complex. Our studies also suggest that one role for the redundancy of these motifs within the ζ chain is signal amplification. Finally, we provide a mechanism by which this motif may

Figure 21. Sequence alignment of activation motifs contained in subunits of hematopoietic cell antigen receptors. Conserved residues are highlighted in boxes.

ζ1	G	Q	N	Q	L	Y	N	E	L	N	L	G	R	R	E	E	–	Y	D	V	L	D
ζ2	P	Q	E	G	L	Y	N	E	L	Q	K	D	K	M	A	E	A	Y	S	E	I	G
ζ3	G	H	D	G	L	Y	Q	G	L	S	T	A	T	K	D	T	–	Y	D	A	L	H
CD3γ	P	N	D	Q	L	Y	Q	P	L	R	D	R	E	D	D	Q	–	Y	S	H	L	Q
CD3δ	R	N	D	Q	V	Y	Q	P	L	R	D	R	D	D	A	Q	–	Y	S	H	L	G
CD3ε	V	P	N	P	D	Y	E	P	H	R	K	G	Q	R	D	L	–	Y	S	G	L	N
IgE FcRγ	K	S	D	A	V	Y	T	G	L	N	T	R	N	Q	E	T	–	Y	E	T	L	G
IgE FcRβ	P	D	D	R	L	Y	E	E	L	–	H	V	Y	D	P	I	–	Y	S	A	L	E
MB-1	E	D	E	N	L	Y	E	G	L	N	L	D	D	C	S	M	–	Y	E	D	H	S
B29	E	E	D	H	T	Y	E	G	L	N	I	D	Q	Q	A	T	–	Y	E	D	H	V

function, namely through its association with ZAP-70 PTK.

Identification of the functional motif in ζ provided the basis for interpretation of the functional data acquired with the CD8/ζ truncations. Not surprisingly, truncations T46, T56, and T83 which contain at least 1 motif are competent signal transducers. However, the complete loss of function observed with removal of the motif's C-terminal leucine in T34, demonstrates for the first time the absolute requirement of this residue or its position for motif function. Mutational analysis within the cytoplasmic domain of CD3ε chain has identified the two YXXL pairs as the functionally important components of its motif, though the N-terminal of the pair was more vulnerable to alterations in sequence [125]. Our results suggest a functional requirement for the C terminal YXXL group in the ζ motif, further supporting the importance of this highly conserved feature of the motif. Comparison of signalling by T56 and ζ1 suggests that, although the isolated 17 amino acid module is sufficient for coupling, the sequences flanking the motif contribute to its functional integrity. This point is underscored by the requirement of secondary crosslinking of ζ1 and ζ1L(3x) for detectable signalling function.

The three functional motifs in the ζ chain may serve unique or redundant functions. Each motif could couple to distinct kinases and potentially utilize different subsets of cellular substrates for its signalling function. However, the pattern of tyrosine phosphoproteins induced with stimulation of T56, T83, and CD8/ζ, constructs which encode, one, two, or three motifs, respectively, appears to be identical. Furthermore, our results, together with those of others [133] demonstrate that each isolated motif is

functional by all independent criteria examined, suggesting that the motifs in ζ may function by similar mechanisms. Thus, each motif does not appear to serve a unique signalling function. Rather, the enhancement in signalling observed with triplication of motif 1 in ζ1L(3x) may reflect a synergistic or cooperative role for the motif triplication. Consistent with this hypothesis, clones expressing truncations and internal deletions of chimeric ζ proteins which retain only the first or third motif require 10 to 100 fold higher concentrations of antibody for detectable IL-2 production [123, 124]. This reduced efficiency of signalling by a single motif is also observed in comparisons of the dose response curves of CD3ε (which contains a single copy of the motif) and ζ in their ability to induce IL-2 secretion [125]. Finally, when transfected into a basophil leukemic cell line, ζ appears to induce degranulation more efficiently than its endogenous counterpart, the single motif-containing FcεRIγ chain [123]. Thus, triplication of the signalling module in ζ may have evolved as a means of amplifying the signals transduced, thereby increasing the sensitivity of the TCR to ligand stimulation.

Notably, only a slight augmentation in intensity of tyrosine phosphoproteins is observed with stimulation of ζ1L(3x) relative to ζ1L, despite the apparent enhanced avidity of ZAP-70 for ζ1L(3x) and the enhanced signalling by this chimera. This apparent lack of correlation between degree of phosphorylation and function is also observed with the phosphorylation of PLCγ1 upon stimulation of ζ1L, ζ1L(3x), and CD8/ζ (in Figure 19). Despite the progressive enhancement in signalling by these chimeras as assessed by calcium fluorimetry and induction of NF-AT activity, equivalent degrees of

PLC phosphorylation are induced. Similarly, one sees no significant difference in the degree of tyrosine phosphorylation induced by stimulation of T56 and CD8/ζ (Fig. 14), although, as discussed above, the intact chimera is a more potent signal transducer. Thus, at a single time point, enhancement of some signalling events may not necessarily reflect an increase in the numbers of a particular substrate phosphorylated, but rather the kinetics or duration of its phosphorylation. Perhaps by regulating localization of kinases and substrates during receptor stimulation, the cell can effectively prolong signal transduction without the need for further recruitment of effector molecules.

The data obtained with the CD8/ζ truncations and constructs containing isolated motifs provide strong correlative evidence for the involvement of ZAP-70 kinase in ζ function. An intact motif appears to be required for both association of ZAP-70 with ζ and ζ function (Figure 15). Furthermore, stimulation of all chimeras capable of transducing signals resulted in induced tyrosine phosphorylation of ZAP-70. Finally, efficiency of signalling was linked to the stability of chimera-induced ZAP-70 association. While sequences in ζ1L are sufficient for ZAP-70 binding as assessed by an *in vitro* kinase assay, triplication of motif 1 increased the avidity of the interaction, with a concomitant enhancement seen in function. Because similar modifications of constructs encoding motif 2 were not made, we do not have direct evidence for its ability to bind ZAP-70. However, based on the effect triplication of motif 1 had on our ability to detect ZAP-70 association, it is not unreasonable to assume that the phosphorylation of ZAP-70 induced by stimulation of ζ2 involved a transient association. Further evidence

supporting the functional significance of ZAP-70 is the observation that all receptors described to date whose associated subunits contain the motif, have been found to associate with a 70-72kD tyrosine phosphoprotein upon stimulation [8, 134-136]. Furthermore, the membrane immunoglobulin-associated 72kD phosphoprotein is also a PTK and likely represents syk, a PTK homologous to ZAP-70 [126, 134]. Thus, syk may play a role analogous to that of ZAP-70 in other receptor systems. ZAP-70 appears to be an important component in the TCR signal transduction pathway, though cell lines or transgenic animals deficient in this kinase may ultimately be required to define its role.

While a simple model invoking a direct, ligand-induced recruitment of ZAP-70 to ζ could be proposed, recent work suggests that the association of ZAP-70 with ζ requires expression of at least one src family PTK [9]. Whether this requirement indicates a requisite phosphorylation event within the motif or in ZAP-70 or both, is still unclear. Genetic evidence demonstrates a critical role for the src family PTK, lck, in TCR-mediated signal transduction and thymic selection [33, 35]. Furthermore, a number of groups have described the association between ζ and fyn, another src family PTK [20, 137-139]. A recent study utilizing an *in vitro* phosphorylation assay suggests that a minimum of 41 cytoplasmic residues in ζ are required for this association although the stoichiometry of binding was admittedly low. These residues include 7 amino acids beyond the C-terminal leucine of the first motif [138]. However, our results, in conjunction with work of others [133] demonstrate that the motif is functional independent of its surrounding sequences, and hence, suggest that the fyn association may not be absolutely required for ζ

function. This conclusion is supported by genetic data demonstrating that antigen receptors in mature, peripheral T cells are functional independent of fyn expression [24, 140]. While fyn may not be required for antigen receptor-mediated signal transduction in most circumstances, it may play a role in augmenting receptor function. The improved signalling capacity observed with stimulation of truncation T56 relative to ζ1 may be explained by the addition of the residues C-terminal of the motif required for fyn binding. A more detailed characterization of the interactions between the src kinases, ZAP-70, and the motif will be required before a complete understanding of ζ function can emerge.

Materials and Methods:

Chimera constructions

The CD8/ζ truncations were constructed by the polymerase chain reaction (PCR) utilizing the CD8/ζ construct as a template. Unique 3' primers, which included a stop codon and Bam HI site, were used with a constant 5' primer corresponding to CD8 sequences immediately 5' of the unique Nae I site. The PCR products replaced the Nae I/Bam HI fragment from CD8/ζ and the complete construct was excised with Xba I/Bam HI and subcloned into ptfneo [121]. The CD8/ζ chimeras containing isolated motifs were engineered by annealling oligonucleotides which together encoded the motifs flanked by Bgl II and Bam HI sites. These annealed oligos were cloned into the Bgl II and Bam HI sites of the CD8T construct (described in text). All PCR products and motifs derived from oligonucleotides were sequenced using the Sanger dideoxy-nucleotide technique.

Antibodies

OKT8, which recognizes an extracellular epitope of CD8, was acquired from the ATCC. Rabbit anti-mouse IgG (RαmIgG) was purchased from ZYMED. 4G10, is an anti-phosphotyrosine mAb (UBI); 387 is a rabbit α-ζ antiserum raised against a peptide comprising amino acids 132-144 of the murine ζ sequence [75]. Monoclonal antibodies (mAbs) against PLCγ1 were provided by Dr S.G. Rhee. The mAb C305 binds to the Jurkat Ti β chain and W6/32 to an invariant determinant on human HLA class 1 antigens. MOPC 195 (Litton bionetics, Kensington, MD) was used as a control mAb in FACS analysis.

Biotinylation of OKT8

Purified OKT8 at 1 mg/ml was dialyzed against 0.1 mM NaHCO₃, 0.1 mM

NaCl pH 8.4, and incubated for 1 h at room temperature with 0.1 mg/ml NHS

Biotin (Pierce). Free biotin was removed by extensive dialysis against PBS.

Cell Lines and Transfections

Jurkat cells were maintained in RPMI 1640 media supplemented with 5%

fetal bovine serum, glutamine, penicillin, and streptomycin (Irvine

Scientific). Chimera transfectants were passaged as above with the addition of

Geneticin (GIBCO, Grand Island, NY) at 2 mg/ml. Transfections and isolation

of clones were as previously described [121].

Flow Cytometry and [Ca^{+2}]$_i$ Fluorimetry

For flow cytometry, 10^6 cells were stained with saturating

concentrations of antibody, then incubated with fluorescein-conjugated goat

anti-mouse Ab prior to analysis in a FACScan (Beckton Dickinson) as

previously described [83].

Calcium-sensitive fluorescence was monitored as previously described

[115]. The CD8/ζ truncations were stimulated with purified OKT8 (1 µg/ml)

and crosslinked with RαmIgG (2 µg/ml). Chimeras containing isolated

motifs were stimulated with biotinylated OKT8 (0.5 µg/ml) followed by

avidin (1 µg/ml).

Immunoprecipitations, Cell Stimulations, Surface Iodinations, and Western Blotting

Cells were lysed at 10^8/ml in 10mM Tris. pH7.8, 1% NP40, 150 mM NaCl, in the presence of protease and phosphatase inhibitors as described. Lysates were centrifuged for 10 min at 14K x G, precleared with Sepharose 4B beads,and ultracentrifuged for 20 min. at 100K x G. Precleared lysates were incubated for 2 h with protein A-Sepharose CL-4B beads that had been prearmed with the immunoprecipitating Ab. Immunoprecipitates were washed 5 times, and analyzed by SDS-PAGE under reducing conditions.

Clones expressing CD8/ζ truncations were stimulated at 10^8/ml in PBS with OKT8 at 2 µg/ml for 2 min at 37°C, followed by RamIgG at 2 µg/ml for an additional 2 min. Cells were lysed as described above. The second group of constructs was stimulated under similar conditions with biotinylated OKT8 at 2 µg/ml and avidin at 4 µg/ml.

Cell surface iodinations with ^{125}I were performed using the lactoperoxidase/glucose oxidase (SIGMA) procedure as described [83]. Western blotting was performed as previously described [121].

Assessment of NF-AT Activity

A construct containing 4 tandem NF-AT binding sites linked to the CAT gene was transfected by DEAE Dextran [131] into Jurkat cells expressing the CD8/ζ chimeras. Forty hours after transfection, 6 x 10^6 (experiment 1) or 2 x 10^6 (experiment 2) cells were stained with saturating concentrations of OKT8 or W6/32, washed, then spun onto plates precoated with Rabbit anti-mouse IgG,A and M (ZYMED). PMA was added at a final concentration of 50 ng/ml,

ionomycin at 1 µM. After 8 h, cells were harvested in 100mM Tris pH 7.8 and

analyzed for CAT activity as previously described [141]. Results are expressed

as the fold induction of treated cells over that of PMA treated cells.

CHAPTER IV.   STUDIES WITH SYNTHETIC PEPTIDES TO EXAMINE THE STRUCTURE AND FUNCTION OF THE ζ ACTIVATION MOTIF

Preface

Knowledge of a protein's structure can often provide insights into its function. For instance, crystallization of the class I MHC molecule revealed a pair of helices situated above a platform of β-strands which together form a groove into which antigenic peptides can bind [142, 143]. This structural information provided considerable insight into the function of MHC molecules, the nature of the antigens recognized by the TCR, and the molecular basis of MHC restriction. In addition, solution structures of the oncogenic, guanine nucleotide binding protein, ras, reveal an allosteric change upon binding of GTP, providing a mechanism by which GTP could regulate its activity [144, 145]. Finally, recent structural analyses of several SH2 (src homology 2) domains, have provided insight into the function of these domains in binding phosphotyrosine-containing proteins [146-148]. Given that clues to protein function can be gained from knowledge of its three-dimensional molecular composition, a study was initiated to obtain structural information on the activation motifs present in ζ. Peptides corresponding to each of the three ζ motifs and the motif found in CD3ε were synthesized and analyzed for secondary structure by circular dichroism and 2D-NMR. Preliminary results are presented and discussed below.

Summary

A conserved tyrosine and leucine based motif has been demonstrated to be both necessary and sufficient for TCR signalling function. This motif is triplicated in ζ and represented singly in each of the CD3 chains. Circular dichroism (CD) analyses of peptides encompassing the activation motifs (ζ1, ζ2, ζ3 and CD3ε) reveal a predominantly helical secondary structure which is induced or stabilized in the presence of trifluoroethanol (TFE). Under appropriate solvent conditions, each of the ζ motifs assumes a similar degree of helical structure; in contrast, a peptide encompassing the CD3ε motif exhibits only an intermediate degree of helicity. Although no discernable helical signal is detected by CD in the absence of solvent, preliminary 2D-NMR analysis of ζ3 in aqueous solution confirms the presence of helical structure within the motif, demonstrating that TFE stabilized a pre-existing helical conformation. Structural heterogeneity among the ζ motifs is suggested by CD analyses of truncated peptides derived from the ζ1 and ζ3 motifs. The spectra of ζ1 are most consistent with a paired helical structure, and those of ζ3 suggest a single C-terminal helix. Furthermore, phosphorylation of two conserved tyrosine residues within the ζ1 motif results in a disruption of its helical structure, suggesting a role for ζ phosphorylation in regulating motif conformation. Finally, ZAP-70 binds specifically to a doubly phosphorylated peptide encompassing the ζ1 motif, demonstrating that both tyrosine residues contribute to the interaction of the ζ1 motif with ZAP-70. Given the disruptive effect of this double phosphorylation on the motif's helical structure, a model for receptor activation is proposed.

Secondary structure analysis of the signal transducing motif:

Peptides encompassing ζ1, ζ2, and ζ3 assume helical structure

Having defined a 17 amino acid region in ζ that is capable of

transducing signals associated with TCR stimulation, studies were

undertaken to gain insight into the structural basis of its function. Synthetic

peptides encompassing the motif (Figure 22) were analyzed by circular

dichroism (CD) [149, 150]. Circular dichroism measures the differential

absorption of right and left circularly polarized light by optically active

substances. The asymmetrical nature of the amide chromophore in the

peptide backbone provides for distinct indicies of refraction for right and left

circularly polarized light, resulting in a net rotation of circularly polarized

light. The degree of rotation or net ellipticity is plotted as a function of wave

length, yielding spectra which reflect the summed contribution of the various

secondary structures assumed within the peptide. CD analysis of ζ1.1, ζ1.3,

and ζ1 in aqueous phosphate buffer pH 7.4 revealed a spectra characteristic of

random coil or unstructured protein (Figure 23A, upper thick line and data

not shown). However, with the addition of trifluoroethanol (TFE) or

acetonitrile, these peptides assume a helical structure. Figure 23A

demonstrates the effect of TFE on the mean residue ellipticity of ζ1.3, whose

sequence most resembles that used in the functional ζ1 chimera. With

increasing concentrations of TFE in water, one sees the progressive deepening

of the minimum at 208 nm and the appearance of a second minimum at 222

nm, features indicative of helical structure. Using the ellipticity at 222 nm as

a diagnostic of helix formation and means of estimating % helicity within a

peptide, the relationship between helical content and amphiphilic nature of

Figure 22. Amino acid sequence of synthetic peptides used in circular
dichroism studies.

Peptides were sythesized as described in Materials and Methods. In most

peptides, both N- and C-termini were capped by acetylation and amidation,

respectively.

Peptide	Amino Acid Sequence
ζ1.1	E Q L Y N E L N L G R R E E – Y D V L D K
ζ1.3	E Q L Y N E L N L G R R E E – Y D V L D
ζ1.4	L Y N E L N L G R R E E – Y D V L D K
ζ1	N Q L Y N E L N L G R R E E – Y D V L D K
ζ1TH1	L Y N E L N L G R R E E – Y D V L D K
ζ1TH2	N Q L Y N E L N L G R R E E – Y
ζ2	E G L Y N E L Q K D K M A E A – Y S E I G M
ζ3	D G L Y Q G L S T A T K D T – Y D A L H M
ζ3TH1	L Y Q G L S T A T K D T – Y D A L H M
ζ3TH2	D G L Y Q G L S T A T K D T – Y
CD3ε	N P D Y E P I R K G Q R D L – Y S G L N Q
fyn 35-51	T P Q H Y P S F G V T S A P N Y N

Figure 23.   Peptide ζ1.3 assumes a helical secondary structure.

A) Circular dichroism spectra of ζ1.3 measured at 4°C. The upper thick line represents the spectrum obtained in phosphate buffer, pH 7.4. The thin lines represent the effect of increasing concentrations of trifluoroethanol (% by volume in H_2O) on mean residue ellipticity, with the lower thick line indicating the maximum negative ellipticity achieved at 40% TFE. The thin lined spectra in order of decreasing ellipticity correspond to the following TFE %: 10, 12.5, 15, 17.5, 20, 25, and 50.

B) Effect on helical content of ζ1.3 with increasing concentrations of TFE. Helical content was estimated based on ellipticity at 222 nm as described in Materials and Methods.

A.

B.
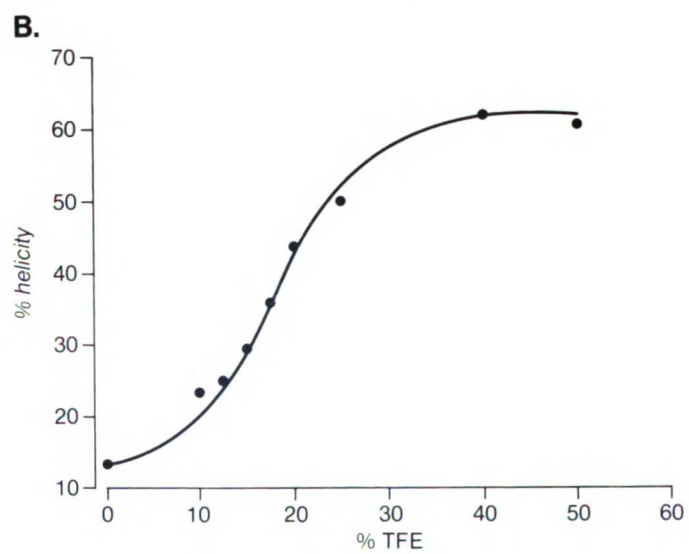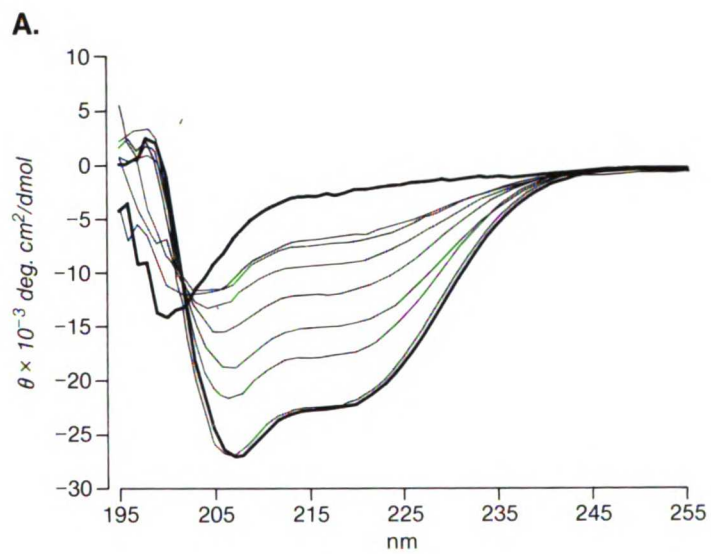
the solvent can be plotted. As seen in Figure 23B, peptide ζ1.3 achieves a maximum of 62% helicity at a concentration of 40% TFE. A cooperative structural transition is observed for ζ1.3 as a function of TFE concentration. The midpoint of the transition occurs at a concentration of 15-20% TFE. For some peptide hormones, these structural transitions have correlated with the biologically relevant conformation of the ligand [151].

To determine whether peptides encompassing each of the three ζ motifs assume a similar structure, CD analysis of ζ1, ζ2, and ζ3 was performed in 40% TFE (Figure 24). The CD spectrum of each of these peptides (spectra 3, 4, and 5) is characteristic of helical structure and reveals a relatively similar degree of helicity. In contrast, another peptide containing two similarly spaced tyrosines encompassing residues 35-51 of the *fyn* PTK (spectrum 1) appears unstructured under these same conditions. Interestingly, spectra of a peptide encoding sequences within the cytoplasmic domain of CD3ε which are homologous to the ζ motif reveals only an intermediate degree of helicity.

Structural heterogeneity suggested by truncations of ζ1 and ζ3 peptides

Our results demonstrate that peptides encompassing the three ζ motifs can assume a secondary structure which is predominantly helical. Calculations of helical content based on mean residue ellipticity at 222 nm [152] reveal that of the 20-21 residues in ζ1, ζ2 or ζ3, a total of 10-12 are helical in nature under these conditions (Table 3). Substitution of the asparagine in the first position of ζ1 with glutamic acid (ζ1.1) increased the helical propensity of the peptide suggesting that the N-terminus of the peptide may contribute to its helical structure. To define the location of the helical

Figure 24. Peptides encompassing each of the ζ motifs assume a helical structure.

CD spectra of fyn 35-51 (spectrum 1) CD3ε (2), ζ3,(3), ζ2(4) and ζ1(5) measured in 40% TFE at 4°C.
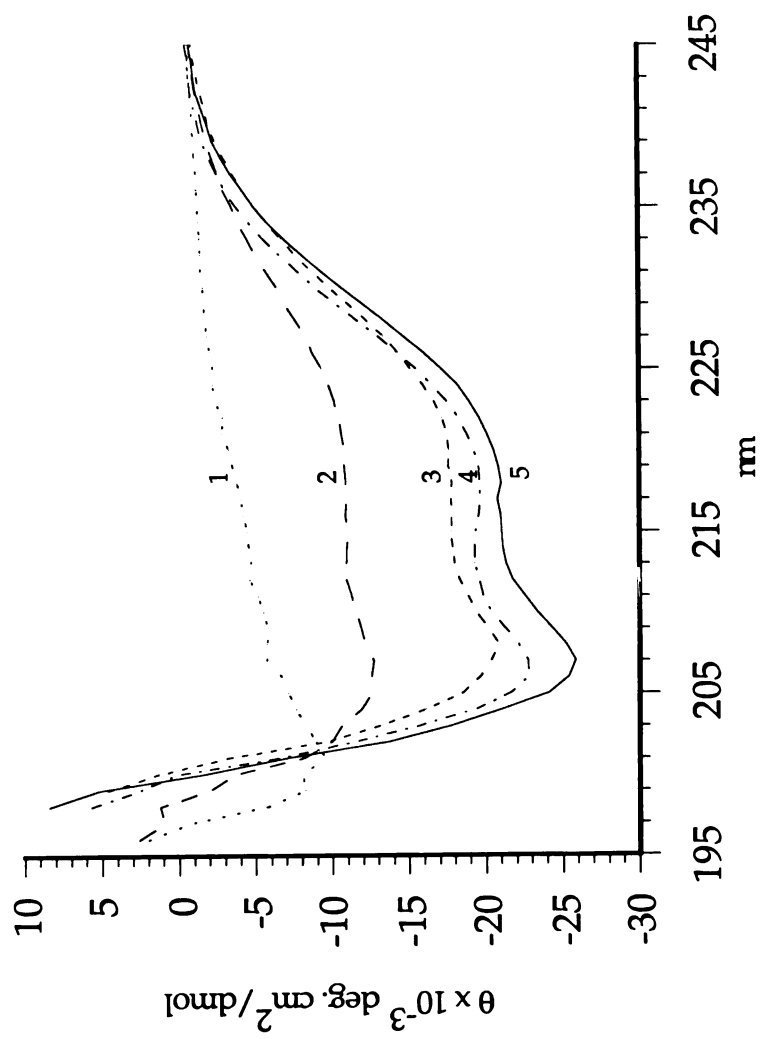
Table 3. CD analysis of truncated peptides derived from ζ1 is consistent with a paired helical structure

Peptide	Sequence	θ222(mre)	% helix	# a.a.	# helical residues
ζ1	NQLYNELNLGRREE-YDVLDK	19,600	58	20	12
ζ2	EGLYNELQKDKMAEAYSEIGM	18,200	54	21	11
ζ3	DGLYQGLSTATKDT-YDALHM	17,100	52	20	10
ζ1.1	EQLYNELNLGRREE-YDVLDK	21,900	64	20	13
ζ1.3	EQLYNELNLGRREE-YDVL	21,200	62	18	11
ζ1.4	LYNELNLGRREE-YDVL	19,000	57	16	9
ζ1TH1	NQLYNELNLGRREE	14,000	44	13	6
ζ1TH2	NLGRREE-YDVLDK	13,400	42	14	6
CD3ε	NPDYEPIRKGQRDL-YSGLNQ	10,400	34	20	7

residues within the motif, truncated peptide derivatives of motif 1 were analyzed for helical content. Truncation of 2 C-terminal residues from $\zeta 1.1$ ($\zeta 1.3$), resulted in a concomitant loss of 2 helical residues suggesting a role for the C terminus in helix formation. A contribution of the N-terminal end of the peptide was further suggested by truncation of 2 N-terminal amino acids from $\zeta 1.3$ ($\zeta 1.4$), revealing a loss of an additional 2 helical residues. Examination of $\zeta 1.4$, which retains 9 helical residues, suggested a model in which 2 helices, each encompassing the 4 amino acid conserved feature of the motif, (YXXL), are separated by a non-helical region. This model predicts that truncation of either putative helix should reduce the helicity of the peptide by approximately 50 percent; if however, the hypothesized spacer is contributing to the helicity, an asymmetrical effect of the truncations should be observed. The CD spectra of $\zeta 1$TH1 and $\zeta 1$TH2, peptides in which one of the YXXL pairs has been truncated, are consistent with a paired helical model, with a reduction in helical content of $\zeta 1$ from 12 to 6 residues being observed with loss of either helix. As suggested by its spectrum, the CD3ε motif peptide was less helical than its ζ counterparts, exhibiting only 6-7 helical residues.

Though data obtained in 40% TFE with peptides encompassing each of the ζ motifs are consistent with this model, spectra of truncations of $\zeta 1$ and $\zeta 3$ acquired in 20% TFE suggest that each YXXL group within a motif may not exhibit an equal propensity for helix formation (Figure 25). In 20% TFE, truncation of each putative helix in $\zeta 1$ ($\zeta 1$TH1 and $\zeta 1$TH2) results in a relatively equivalent loss in peptide helicity, with perhaps the suggestion of a greater contribution of the N-terminal region in $\zeta 1$ helicity (Figure 25A); this result is consistent with the paired helical model. However, an asymmetrical

effect is seen with similar truncations of $\zeta 3$, suggesting a requirement of the C-terminal residues for the integrity of significant helical structure; this is evidenced by $\zeta 3TH2$ exhibiting a spectra more reminiscent of random coil than helix (Figure 25B, dashed line). These data suggest there may be structural heterogeneity among the ζ motifs, with the spectra of $\zeta 1$ consistent with a paired helical structure, and those of $\zeta 3$, suggesting a single C-terminal helix.

To determine whether the helical structure observed by CD in TFE could be detected in the absence of organic solvent, two-dimensional NMR analysis was performed [153, 154]. A NOE spectrum was collected for a peptide encompassing $\zeta 3$ (an additional two residues were added to its C-terminus) at 5°C, pH 3.8 under aqueous conditions (Figure 26). $\zeta 3$ was selected as it was the most H_2O soluble of the three motif peptides, achieving a concentration of 8mM. Although no discernable helical signal was apparent by CD under these conditions, 15 distinct NH-NH cross peaks are noted in this NOE spectrum. Their intensity and position are consistent with the NH_i-NH_{i+1} cross peaks commonly observed in nascent and alpha-helices. This suggests that the ζ motif contains at least one helical region under physiologically relevant conditions and that TFE augmented a preexisting helical signal. A precise determination of the location of the helical residues in $\zeta 3$ will await the completion of the sequential assignments of the amide-amide cross peaks from the NOESY, COSY, and HOHAHA (Homonuclear Hartman Hahn) spectra. Similar analysis by 2D NMR of $\zeta 1$ and $\zeta 2$ is currently underway to determine their secondary structures unambiguously.

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Figure 25. Truncations of ζ1 and ζ3 in 20% TFE suggest structural heterogeneity among the ζ motifs.

A) CD spectra of ζ1TH1 (solid line) and ζ1TH2 (dashed line) in 20% TFE.

B) Spectra of ζ3TH1 (solid line) and ζ3TH2 (dashed line) under similar conditions.

A
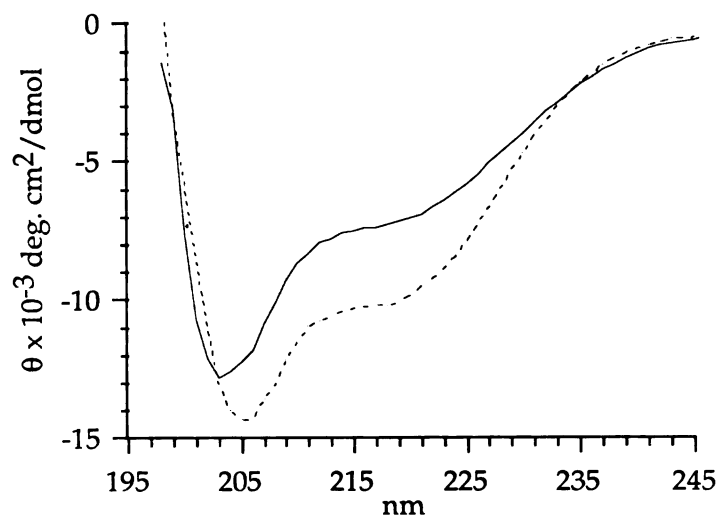
B
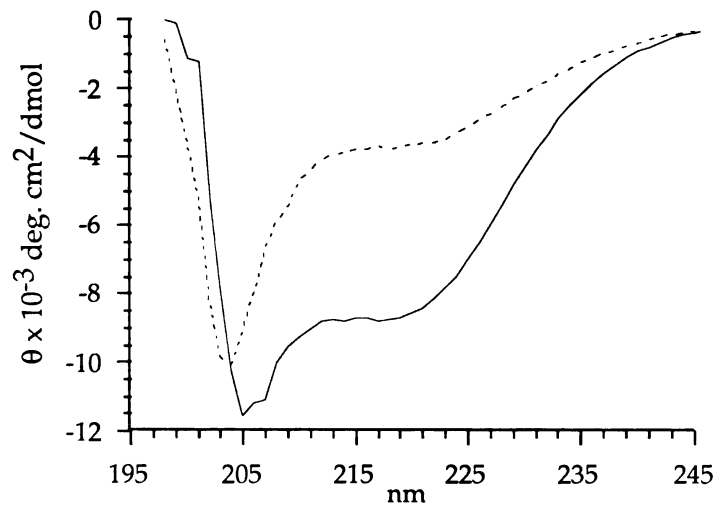
Figure 26. 2D NMR detects helical structure in ζ3 under aqueous conditions.
NOESY spectrum of ζ3 in H20 (10% ^{2}H$_2$0) at pH 3.8, 5 °C showing 15 cross
peaks between amide proton resonances (in yellow). The mixing time was 200
msec.
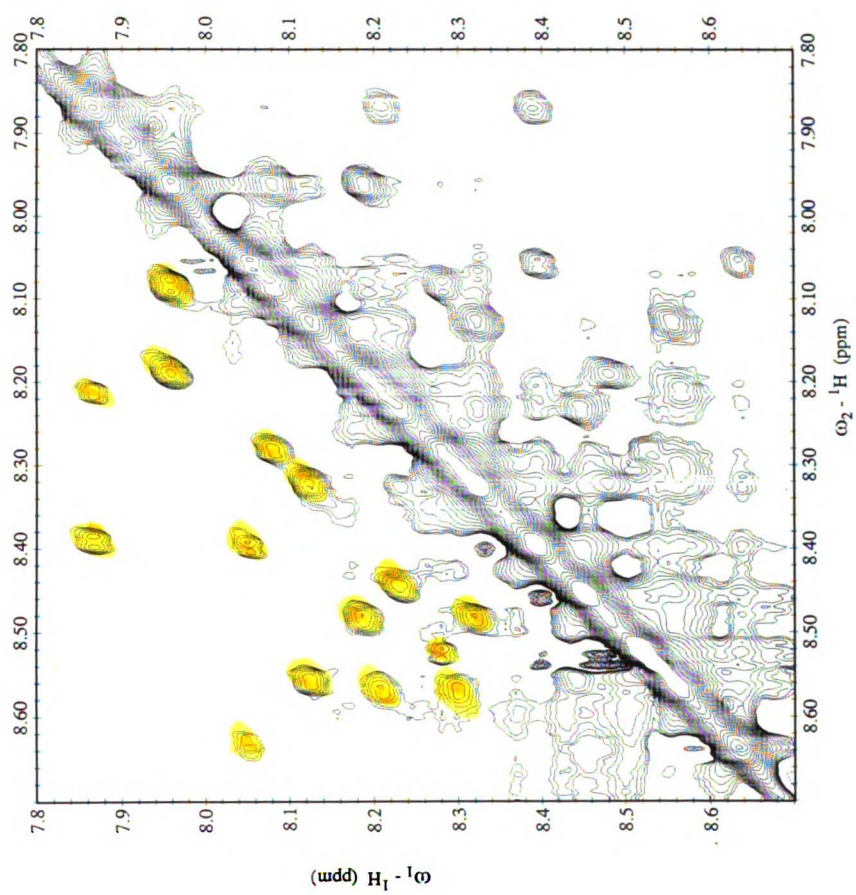
Phosphorylation of critical tyrosine residues in ζ1 disrupts its helical structure

Tyrosine phosphorylation of ζ occurs rapidly following TCR stimulation, though the role of this phosphorylation remains unclear [7, 8]. To determine whether the helical structure assumed by the motif was affected by tyrosine phosphorylation, ζ1 peptides were synthesized with phosphotyrosine and analyzed by CD (Figure 27). No effect on the helical nature of ζ1 is observed with phosphorylation of its N-terminal tyrosine residue (compare spectra 4 and 3). In contrast, phosphorylation of the C-terminal tyrosine results in a reduction of helicity as evidenced by the observed increase in mean residue ellipticity (#2). Finally, a more dramatic reduction in helicity is seen with phosphorylation of both tyrosine residues (#1), an effect possibly facilitated by a charge repulsion between the two negatively charged phosphate groups. Thus, depending on the residue(s) phosphorylated, phosphorylation appears compatible with three distinct conformations of the ζ motif, providing a basis for multiple roles of phosphorylation in ζ function.

Figure 27. The effect of tyrosine phosphorylation on the helical structure of
$\zeta 1$.

Phosphorylated $\zeta 1$ peptides were synthesized with the incorporation of
phosphotyrosine into either the N- or C-terminal tyrosine position or both.
CD spectra: (spectrum #3) $\zeta 1$; (#4) $\zeta 1$ in which the N-terminal tyrosine is
phosphorylated; (#2) $\zeta 1$ phosphorylated at the C-terminal tyrosine; and (#1)
$\zeta 1$ in which both tyrosine residues are phosphorylated. All spectra were
measured in 40% TFE at 4°C.
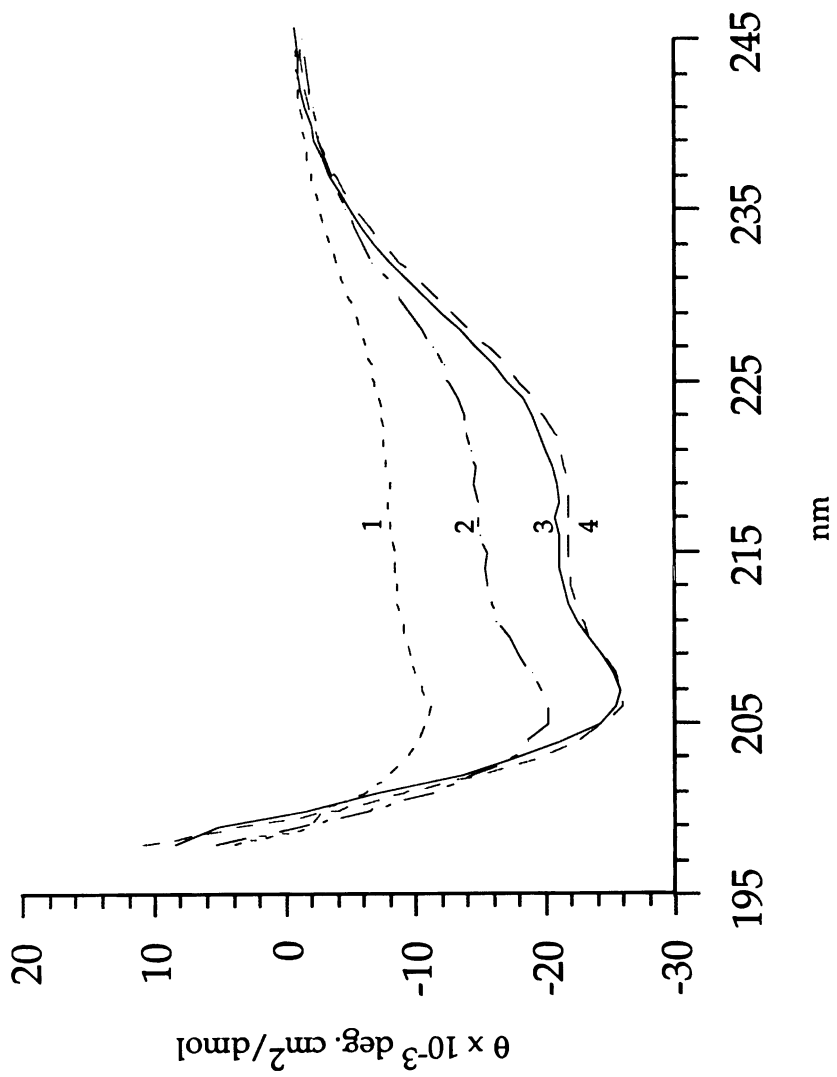
Preliminary functional analysis of ζ peptides:

Association of ZAP-70 with a tyrosine phosphorylated peptide encompassing the N-terminal ζ motif.

Having demonstrated that sequences within the most N-terminal ζ motif contain all information necessary for the association of ZAP-70 with ζ (chapter III), we set out to determine the requirements for its binding. A peptide encompassing ζ1 was synthesized with biotin incorporated at its N-terminal end to facilitate direct precipitation using avidin-coated, agarose beads. In attempts to improve the suboptimal association observed between ZAP-70 and chimeras expressing isolated motifs (see Chapter III), flanking residues were added to ζ1, yielding M1 (see Figure 28 for peptide sequences). This 28 amino acid peptide was synthesized in a non-phosphorylated form as well as in singly or doubly phosphorylated forms and tested for its ability to associate with ZAP-70 in Jurkat T cell lysates (Figure 29). No detectable binding of ZAP-70 by either M1, P1, P2, or an equal mixture of P1 and P2 is apparent by blotting with a ZAP-70-specific antiserum (Figure 29; upper panel, lanes 1-4). In contrast, ZAP-70 is able to bind quite efficiently to the doubly phosphorylated peptide, P1,2 (lane 5). Demonstrating the concentration dependence of this association are lanes 5-8, depicting the quantity of ZAP-70 precipitated by serial 4 fold dilutions of P1,2 peptide prior to its incubation with a fixed amount of lysate; the association is detected using 0.25 μgs, but not 0.06 μgs of peptide (lanes 6 and 7), quantities which correspond to concentrations of 120 and 30 nM, respectively. Stimulation of the TCR on Jurkat cells prior to lysis does not significantly effect the ability of ZAP-70 to specifically recognize P1,2 (lanes 9-12). The two lower panels

Figure 28. Amino acid sequence of biotinylated M1 peptides.

Peptide	Amino Acid Sequence
M1	biotin | QQGQNQLYNELNLGRREEYDVLDKRRGR
P1	PO₄ | biotin | QQGQNQLYNELNLGRREEYDVLDKRRGR
P2	biotin | QQGQNQLYNELNLGRREEYDVLDKRRGR
P1,2	biotin | QQGQNQLYNELNLGRREEYDVLDKRRGR

Figure 29. ZAP-70 associates preferentially with a doubly phosphorylated tyrosine phosphopeptide encompassing the N-terminal ζ motif.

500 μl of lysate from 50 x 10⁶ unstimulated or stimulated Jurkat T cells were incubated with 1 μg of the designated peptides (unless otherwise indicated), precipitated with excess avidin-agarose beads, resolved by SDS PAGE, and analyzed by western blotting. The nitrocellulose was cut horizontally and blotted with the indicated antibodies. Lane 13 represents lysate from 2 x 10⁶ Jurkat cells used as a control for the various blotting antibodies. Cells were stimulated with a Tiβ-specific mAb (C305) at 1 μg/ml in PBS for 2 min at 37°C prior to lysis.

TCR stimulation: — + —

Peptide:	M1	P1	P2	P1,2	P1,2	P1,2	P1,2	P1+P2	P2	P1	M1	P1,2	P1	P2	—
(μg):	1	1	1	.015	0.06	0.25	1	1	1	1	1	1	1	1	—

ZAP-70

PLCγ1

lck

1 2 3 4 5 6 7 8 9 10 11 12 13
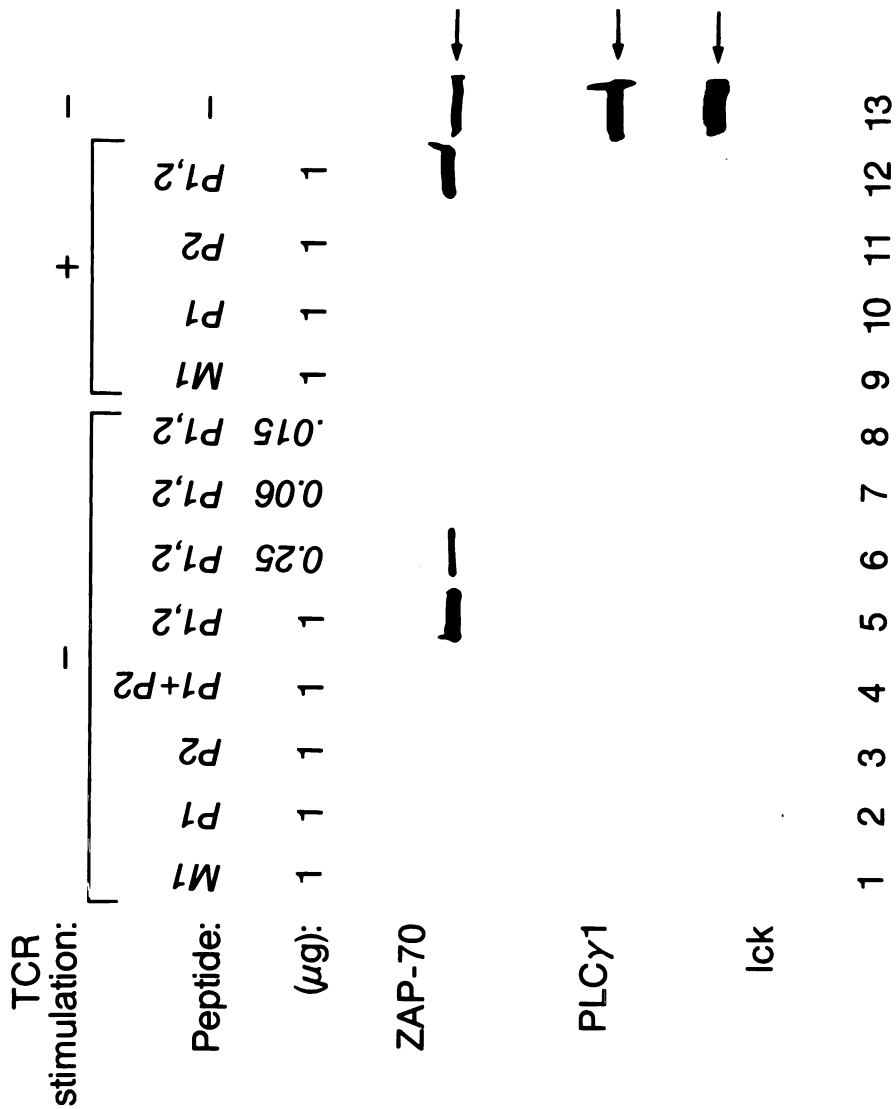
demonstrate the specificity of this interaction, as no detectable lck or PLCγ1 is precipitated by any of the peptides. This is noteworthy, since these proteins contain SH2 domains which bind tyrosine phosphorylated substrates with high affinity [155]. These data suggest tyrosine phosphorylation at both conserved tyrosine residues within a given motif is required for optimal association with ZAP-70.

To address further the specificity of the observed interaction and determine the complexity of the peptide precipitates, cell lysates from TCR-stimulated cells were precipitated with the indicated peptides, and blotted with an antiphosphotyrosine antibody, 4G10 (Figure 30). As many proteins implicated in signal transduction become phosphorylated on tyrosine residues following receptor stimulation, this mAb could identify proteins other than ZAP-70 which associate with the motif peptides. In this experiment, an increased amount of peptide was used (3 μgs, or approximately 1.5 μM) to detect associations of low stoichiometry. No associated tyrosine phosphoproteins are detected in precipitates of the unphosphorylated M1 (Figure 30, lane 1) However, a small amount of a 70 kD protein, likely to be ZAP-70, is precipitated by the singly phosphorylated peptides, P1 and P2. (lanes 2 and 3). This precipitation of ZAP-70 to a small degree by P1 and P2 is similarly detected with the ZAP-70 antiserum if 3 μgs of peptide are used (data not shown). A dramatic increase in the association of the 70 kD tyrosine phosphoprotein is observed in precipitates of the doubly phosphorylated P1,2 (lane 4), recapitulating the observation made in Figure 29. Importantly, although TCR stimulation had resulted in the induction of many tyrosine phosphoproteins, (compare lanes 5 and 6) no major species

Figure 30. ZAP-70 is the predominant tyrosine phosphoprotein associated
with the doubly phosphorylated, P1,2 peptide.

Lysates from 50 x 10^6 stimulated Jurkat cells, were precipitated with 3 μg of the
indicated peptides, resolved by SDS PAGE, and analyzed by western blotting
with an anti-phosphotyrosine mAb, 4G10. Precipitating peptides were as
follows: M1 (lane 1); P1 (lane 2); P2 (lane 3); and P1,2 (lane 4). Cells were
stimulated with a Tiβ-specific mAb (C305) at 1μg/ml in PBS for 2 min at 37°C
prior to lysis. Lanes 5 and 6 represent lysate from 2 x 10^6 cells, either
unstimulated or stimulated, respectively.
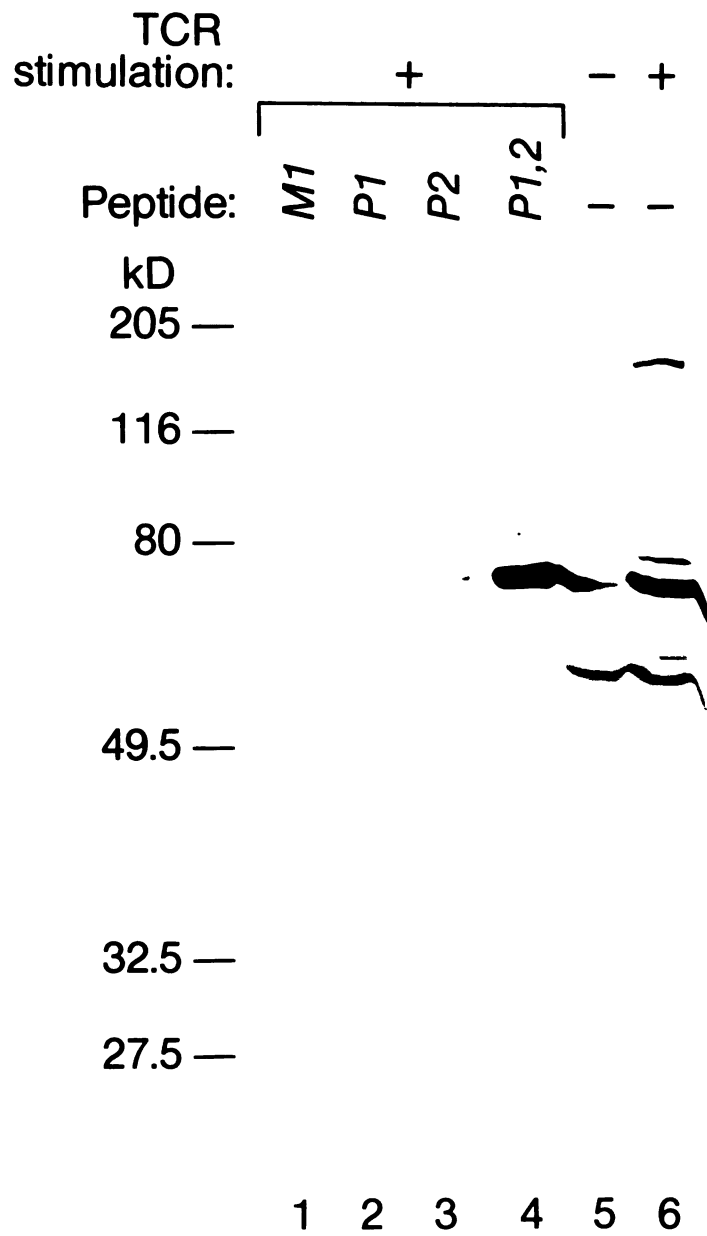
other than the 70 kD protein is detected. Admittedly, a single faint band is detected below ZAP-70 at approximately 65kD in lane 4; the relevance of this band is currently under investigation. Thus, although ZAP-70 is capable of binding the singly phosphorylated forms of the motif weakly, it binds the doubly phosphorylated form much more optimally. Furthermore, the association of ZAP-70 with P1,2 appears to be quite specific as SH2 containing proteins such as lck, PLCγ1, and PTP 1C are not detected in P1,2 precipitates (Figures 29, 30, and data not shown).

Discussion:

A helical structure within the ζ motifs

The structural analyses presented of peptides encompassing the functional motifs reveal at least one, and perhaps two regions of helical secondary structure. CD data acquired with truncations of ζ1 are most consistent with a structure composed of two helices, each encompassing one of the conserved YXXL pairs (Figure 31). The intervening residues, because of their nonconserved and highly charged nature, may serve as a turn positioning the helices in a functionally optimal fashion. Though data obtained in 40% TFE with peptides encompassing each of the ζ motifs are consistent with this model, spectra of truncations of ζ1 and ζ3 acquired in 20% TFE suggest that each YXXL group within a motif may not exhibit an equal propensity for helix formation. This result raises the possibility that the motifs in ζ may not have identical structures though they appear to function in similar fashions.

Consistent with the notion that each of the YXXL pairs may not possess helical structure is data acquired with the CD3ε peptide, which revealed reduced helical content relative to ζ1, 2, and 3 in 40% TFE (Table 3). The placement of 2 proline residues - an amino acid incompatible with helical structure - within the region corresponding to the proposed N-terminal helix in the ζ motif, likely explains its reduction in helicity. Unlike the motifs present in other antigen receptor-associated subunits, each of the CD3 motifs contains a conserved proline between the N-terminal tyrosine and leucine residues, YXPL (see CD3ε sequence in Figure 22). This feature, which is likely to prevent the formation or stability of an N-terminal helix, may provide the

Figure 31. Putative structure of the N-terminal motif in ζ.

A schematic representation of the N-terminal ζ motif depicting two helices, which encompass the conserved feature of the motif (YxxL), separated by a short turn of 7 residues. The conserved spacing between the tyrosine and the leucine side chains provides a hydrophobic interface on one side of each helix. Here, the hydrophobic residues are shown interacting between the helices. They may also provide the basis of intermolecular interactions. The oxygen atoms in the hydroxyl group of each tyrosine residue and the N-terminal asparagine are highlighted in red, the nitrogen in the asparagine, in blue.
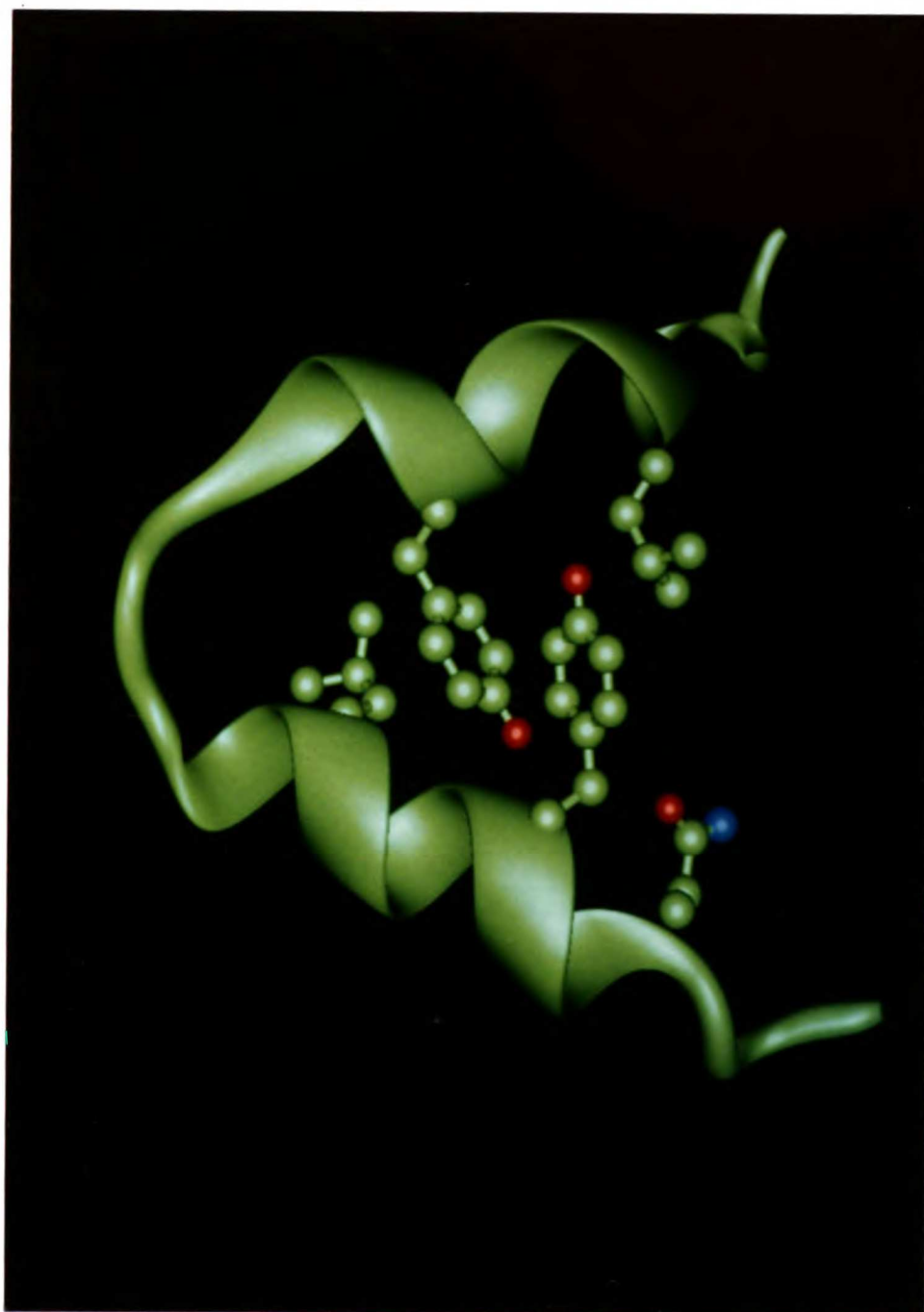
CD3 motifs with distinct signalling function or alternatively, effect the efficiency with which they couple to signalling components. Notably, a glycine residue is present in a similar position (YXGL) within motifs ζ3, MB1, B29 and FcεRIγ, and may also effect N-terminal helix formation. The fact that the N-terminus of ζ3 contains this, and an additional glycine residue, may explain the significant loss of structure observed in 20% TFE with truncation of this peptide at its C-terminal YXXL. Completion of the assignments from the ζ3 NMR spectra, in conjunction with similar analysis of other motifs, should determine whether one or two helices is present and whether structural heterogeneity exists among these motifs.

While helical structure within the motif was confirmed in aqueous solution by 2D-NMR analysis, it was not detected by CD analysis under similar conditions. This may suggest an instability of the helical conformation in aqueous conditions, with only a small percentage of the peptide assuming a helical structure at any given time. It is not uncommon for a peptide whose sequence encodes a helical segment as determined by NMR to appear unstructured in aqueous solution by CD analysis [156]. However, frequently the structure of such a peptide can be detected with the addition of TFE [157-159] TFE, an amphipathic solvent, may stabilize structure by allowing the exclusion of H_2O from residues or regions within the peptide which require a degree of hydrophobicity for formation or stability of secondary structure. The requirement for TFE may suggest a need for long range proton interactions normally provided by regions of the protein not present in the peptide; it may also reflect a requirement for extrinsic stabilizing forces provided by the surface of an associated protein. Illustrating

such a requirement for intermolecular interactions is data obtained with peptides encoding the leucine zipper region of the fos/jun family of transcription factors. CD analysis has demonstrated a requirement for dimerization of these peptides in formation of their helical structure [160]. The hydrophobic interface of the zipper region formed by the leucine heptad repeat is unstable unless associated with its hydrophobic counterpart. Moreover, the adjacent basic region of these proteins involved in contacting DNA, undergoes a DNA-dependent transition from random coil to α-helical structure [161]. While the structure of the ζ motif is apparently not stabilized by dimerization, regions surrounding the motif may act to stabilize its helical structure. Alternatively its structure may be stabilized *in vivo* by an association with another protein, possibly a tyrosine kinase. The spacing of the tyrosine and leucine residues in a helical context would provide a short, hydrophobic interface which may require the association of another protein to stabilize its helical conformation. We have previously demonstrated that, prior to receptor stimulation, a significant amount of tyrosine kinase activity is associated with ζ [8]. Thus, the motif may exist in the helical conformation in the basal state providing for its association with a tyrosine kinase. Alternatively, the helical structure may serve to mask the YxxL groups, preventing their phosphorylation by PTKs prior to TCR stimulation (Figure 31).

Relating the structure and function of the motif.

The observation that ZAP-70 associates preferentially with the motif in its doubly phosphorylated form suggests a requirement for two

phosphorylation events within a given motif for ζ function. A number of pieces of evidence support the involvement of ζ phosphorylation in motif function. First, ζ is one of the earliest substrates phosphorylated on tyrosine residues following TCR stimulation. Second, substitution of either tyrosine in the ζ motif with phenylalanine, renders it nonfunctional, consistent with an important role for phosphorylation of these residues [133]. Finally, a correlation is seen between tyrosine phosphorylation of the functional CD8/ζ truncations and their ability to associate with ZAP-70 [162]. A similar correlation has been noted between TCRζ phosphorylation and ZAP-70 association [9]. This requirement for phosphorylation is consistent with the previously hypothesized mechanism by which ZAP-70 associates with ζ., i.e. via an SH2/phosphotyrosine interaction [155,163]. ZAP-70 contains two closely linked SH2 (src homology-2) domains, structural domains which are known to mediate direct interactions with phosphotyrosine residues. Indeed, recent evidence defines a requirement for both functional SH2 domains in ZAP-70 for its stable association with ζ (Iwashima, M., et. al., manuscript in preparation). This finding, together with the specific binding of ZAP-70 to the P1,2 peptide, supports the binding of both SH2 domains to a single ζ motif which is phosphorylated on both tyrosine residues.

How does the function of the doubly phosphorylated motif relate to its structure according to the data acquired by CD? Our finding that two tyrosine phosphorylation events within the $\zeta 1$ peptide has a destabilizing effect on its helical structure suggests that phosphorylation may serve to facilitate or prolong a conformational opening within the motif, allowing for the recruitment of signalling molecules to the TCR. Given that the helical

structure of this peptide which optimally binds ZAP-70 is disrupted, we propose a model in which the unstructured state of the motif is its active conformation. Supporting this hypothesis is recent crystalographic data of an SH2 domain interacting with its cognate tyrosine phosphopeptide [146]. High resolution structures of the SH2 domains of src [146], lck [147], and abl [148], demonstrate that tyrosine phosphopeptides are bound in an unwound, or unstructured state, with the phosphate groups on the tyrosine and the side chain of the residue 3 amino acids C-terminal of the tyrosine inserting into two binding pockets present on the contact surface of the SH2 domain. As discussed above, association of ZAP-70 with ζ is likely to be mediated via two SH2/phosphotyrosine interactions. The spacing of the conserved residues within the ζ motifs, YXXL, is consistent with SH2 binding and likely explains the requirement of these particular residues for motif function. The unstructured nature of these residues in their SH2 binding state is also consistent with the disruption of secondary structure seen by CD with phosphorylation at both sites. Thus, phosphorylation within the motif may serve two roles in TCR-mediated activation: 1) to increase accessibility of the motif by facilitating or stabilizing a ligand-induced opening of its helical structure, and 2) to provide the means by which SH2-containing proteins, such as ZAP-70 are recruited to the TCR.

Materials and Methods:

Peptide Synthesis and Purification

Peptides were synthesized on an ABI Peptide Synthesizer, Model 430 (Applied Biosystems, Foster City, CA) using tert.-butyloxycarbonyl (tBOC) protected amino acids (Bachem, Torrance, CA) and benzhydroylamine resins (Applied Biosystems). For the incorporation of phosphotyrosine residues, the Boc-Tyr (OPO_3Bzl_2) derivative (Peninsula Laboratories, Belmont, CA) was employed [164]. Synthesis, deprotection, cleavage, purification, and characterization of the peptides were carried out as described [164, 165].

Circular Dichroism Analysis

Far ultraviolet circular dichroism (CD) measurements were recorded from 190 to 260 nm on a Jasco J500A CD spectropolarimeter. The peptide spectra, obtained in a 1 mm cell at 4°C, were averaged from 16 scans and ellipticity reported as $[\theta]$, mean residue ellipticity (deg. $cm^2/dmol$). Raw data expressed in milidegrees was converted to θ by the following equation: $\theta = \theta_{observed} \times mrw/10 \times d \times c''$, with $\theta_{observed}$ (in degrees); mrw (mean rotation weight) = Molecular weight of peptide/# residues; d = path length in cm and $c'' = gr/ml$ of peptide [152]. θ at 222 nm was used to derive estimates of helical content according to the method of Taylor and Kaiser [152]: $(\theta - 3000/-39,000) \times$ #amino acids in peptide = estimated total number of helical residues.

Peptide concentrations, ranging from 0.3-0.6 mg/ml were determined by measuring absorbance of tyrosine at 274 nm using an extinction coefficient, ε_{274} of 1420 cm^{-1} M^{-1} per tyrosine residue. [166]. Phosphate buffer of 1 mM

sodium citrate, 1 mM sodium borate, 1 mM sodium phosphate and 0.1 M

NaCl, pH 7.4., or trifluoroethanol (Sigma) in H_2O (% by volume) was used.

Precipitates of ZAP-70 with biotinylated peptides

Peptides were synthesized as described above with the exception that the N-

terminal residue of each was covalently linked to biotin. Lysates from 50×10^6

Jurkat cells (50×10^6 cells/500 μl) were incubated on a rotator at 4°C for 1.5-2

hours with the indicated biotinylated peptides. Avidin-coated agarose beads

(30 μl) (Vector Laboratories) were added to each sample which was rotated for

an additional hour before the bound complexes were washed 3 times with

0.5% NP40 lysis buffer. Samples were analyzed by western blotting with

either an anti-ZAP-70 rabbit antiserum [9], or the mAb 4G10 (UBI).

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CHAPTER V. CONCLUDING REMARKS

In this dissertation, a direct role for ζ in coupling to intracellular signalling pathways is demonstrated. Remarkably, the signals transduced by ζ appear to be identical to those generated by stimulation of the intact TCR. In addition, we have defined and characterized signalling by a short motif present in three copies within ζ, and have demonstrated the likely mechanism by which it functions, namely through its association with the PTK ZAP-70. Furthermore, preliminary structural analysis of peptides encompassing the motifs has revealed a predominantly helical secondary structure which can be disrupted by phosphorylation on critical tyrosine residues. This tyrosine phosphorylation ultimately provides the mechanism by which ZAP-70 is recruited to ζ. While considerable progress has been made by us and others in analyzing the function of ζ and the CD3 chains, a number of questions remain unanswered, and naturally, new issues have been raised. The following discussion will address some of these questions and discuss future directions for research toward a more complete understanding of signal transduction by the T cell antigen receptor.

The paradox of multiple functional components in the TCR

The finding that the TCR is composed of at least two (CD3ε and ζ) and possibly five (CD3 γ, δ, ε, ζ and η or FcεRIγ), distinct signalling molecules, raises a number of questions. If most growth factor receptors carry out both ligand binding and signalling functions utilizing a single transmembrane polypeptide [15], why then, has the TCR evolved with its unprecedented structural complexity, encoding for as many as ten potentially functional

activation motifs? Do these signalling modules function redundantly or do they serve to couple the receptor to distinct kinases or pathways? Evidence exists which suggests that at least a subset of motifs may possess distinct signalling characteristics. Comparison of the tyrosine phosphoproteins induced following stimulation of CD3ε and ζ chimeras reveals a variation in the intensities of phosphorylation of particular substrates [125]. Furthermore, no correlation is observed between the degree of induced tyrosine phosphorylation of each chimera and its ability to induce the secretion of IL-2. Similarly, phosphorylation of the η chain is not detected when expressed as a dimer in the TCR, although the receptor is capable of inducing proximal and distal activation events normally [167]. The authors argue that CD3ε, η, and ζ may be sufficiently unique to enable their coupling to distinct kinases or signalling pathways. The structural differences we detect in the CD3ε and ζ motifs may also reflect functional differences which have yet to be revealed. Perhaps the most compelling evidence suggesting a distinction between CD3ε and ζ in signalling are unpublished data which demonstrate that a truncated ζ chimera expressing only a single activation motif is functional in a rat basophilic leukemic cell, while a CD3ε chimera is not (Letourneur, F. and Klausner, R.D.). Of note is the failure of these cells to express detectable levels of ZAP-70. It is intriguing to speculate that CD3 function may require ZAP-70, while ζ may couple to both ZAP-70 and its homologue, syk, which is expressed in these cells [136]. Alternatively, the kinase responsible for phosphorylation of CD3ε may not be expressed in these cells, which would suggest the ability of src family kinases to distinguish between motifs. Studies comparing the abilities of the various motifs to associate with ZAP-70 and syk

may be useful in revealing distinct binding properties among motifs. They may also suggest whether the functional differences observed among the motifs represent qualitative or quantitative differences is signal transduction.

Independent of whether the various motifs function in unique or similar fashions, a teleological explanation for the apparent paradoxical redundancy within the TCR can be proposed. The TCR's structural complexity may have evolved as a means of amplifying its signal, enabling T cells to respond to a small number of receptors engaged in a low affinity interaction. This would be consistent with the belief that the affinity of the TCR for antigen/MHC is necessarily weak [168], for in order to protect the host against potentially autoreactive cells, T cells possessing high affinity receptors are deleted during thymic selection. Since the number of peripheral T cells which recognize a given antigen is likely to be extremely low, as is the number of MHC molecules expressing that antigen on a given cell, T cells would clearly benefit from an exquisitely sensitive triggering mechanism putatively provided by multiplication of motifs. Thus, by increasing the sensitivity of the TCR, multiple activation motifs may enable a rare surface interaction to result in sufficient secretion of lymphokines, proliferation, and effector function.

Consistent with such a role for multimerization of motifs in enhancement of signalling function is our result demonstrating enhanced signal transduction with triplication of a single ζ motif [162]. Reciprocally, T cells expressing truncations of ζ chimeras which retain only a single motif require 10-100 fold higher concentrations of antibody for detectable IL-2 production [123, 124]. However, inconsistent with this hypothesis is a study

in which a ζ deficient hybridoma is reconstituted with a severely truncated form of ζ possessing no activation motifs [124]. Stimulation of these TCR mutants with mAbs, antigen/MHC, or staphylococcal enterotoxin revealed surprising results. Independent of the stimuli used, TCR's devoid of functional ζ sequences were capable of inducing levels of IL-2 secretion which were comparable to those observed with stimulation of the wild type TCR. Interestingly, clones expressing the truncated form of ζ were defective in signalling through Thy-1, a murine T cell accessory molecule whose signalling properties require expression of the TCR. As proof that the signalling pathway utilized by ζ was intact in these cells, ζ was capable of signalling when expressed in a chimeric form. The fact that no impairment in TCR-mediated signalling was observed in the absence of ζ cytoplasmic sequences argued for a redundant, but not additive, role for ζ and CD3 in the TCR complex. The authors speculated that while CD3 may couple antigen binding to signal transduction, ζ may instead be utilized by accessory molecules such as Thy 1 and CD2. Notably, these results contradict those obtained in a similar study performed in an independently derived ζ mutant from the same parent hybridoma [169]. Reconstitution of this clone with truncated forms of ζ resulted in the expression of surface receptors completely unresponsive to antigen/MHC. Since neither finding has been reproduced in another T cell, one must exercise a degree of caution in interpreting these results. A similar experiment is currently underway in mice in which the ζ gene has been disrupted by homologous recombination. The forthcoming genetic results should reveal ζ's contribution to both thymic selection and peripheral immunity in the context of the intact receptor.

Events leading to the activated TCR complex

While the active TCR complex is likely to consist of ZAP-70 bound to at least one activation motif in either ζ or CD3, the sequence of events leading to the formation of this complex is still unclear. Evidence suggests that the interaction of ZAP-70 with the TCR may require at least one additional tyrosine kinase of the src family [33]. Stimulation of the TCR in a T cell deficient in functional lck expression fails to elicit tyrosine phosphorylation of the CD3 or ζ chains, and subsequent association of ZAP-70. Furthermore, a stable association between ZAP-70 and a CD8/ζ chimera expressed in a heterologous cell system depends on the expression of either lck or fyn [9]. In this system, a synergy between src kinases and ZAP-70 is suggested by the dramatic increase in tyrosine phosphorylation observed in cell lysates only if both kinases are expressed. Interestingly, expression of the CD8/ζ chimera is required for optimal synergy suggesting a degree of colocalizaton of lck/fyn and ZAP-70 with ζ. This notion is supported by several studies in which either lck or fyn is co-immunoprecipitated with ζ in T cells, NK cells, or in fibroblasts [20, 137, 138, 170]. Perhaps lck, brought into proximity with the TCR via its association with CD4/CD8 or via a weak association with ζ, may serve two functions in TCR-mediated signal transduction. First, it may phosphorylate tyrosine residues within the activation motifs resulting in the recruitment of ZAP-70 to ζ or CD3. Second, lck may also regulate the kinase activity of the TCR-associated ZAP-70, possibly via a phosphorylation of ZAP-70 itself.

Based on the cumulative data supporting ZAP-70's involvement in ζ function [162], the likely requirement of at least one src kinase for its

association [9], and the effect of phosphorylation on the motif's helical structure, we propose the following model for receptor activation: A given motif is likely to exist in its helical conformation prior to receptor stimulation, either providing the structural basis for its association with one or more tyrosine kinase or serving to mask the functional YXXL pairs from phosphorylation as depicted in Figure 31. Engagement of the TCR may result in the activation of ζ-associated lck or fyn, phosphorylation within the motif, and a subsequent disruption of its helical structure. Alternatively, ligand binding may directly result in a conformational opening within the motif allowing for its recognition and subsequent phosphorylation by lck or fyn. Two phosphorylation events within the motif would provide the means by which ZAP-70 is recruited via its two tandem SH2 domains. Once associated, ZAP-70's kinase activity may be increased, resulting in an induction in phosphorylation of cellular substrates. A schematic representation of the described model is depicted in Figure 32. As indicated, initiation of activation would be facilitated by lck during antigen presentation, with the colocalization of both TCR and the appropriate coreceptor CD4 or CD8.

Our finding that single phosphorylation events within the ζ1 peptide have distinct effects on its helical structure suggests the complex role phosphorylation may play in modifying motif structure, accessibility, and function. As ZAP-70 does not appear to associate tightly with a singly phosphorylated motif, regulation of phosphorylation of specific tyrosines within a motif may provide for multiple levels of regulation. Ligation of the TCR may not only effect the regulation of tyrosine kinases but also regulation of the tyrosine phosphatase/s responsible for dephosphorylation within the

motif. Further dissection of the molecular interactions between the
activation motifs, and the relevant tyrosine kinases and phosphatases will be
required before a complete understanding of TCR function can emerge.

Figure 32. Model of interactions between TCR, lck, and ZAP-70 during
recognition of antigen/MHC.

For simplicity, the individual activation motifs within the TCR subunits are
not depicted.

152
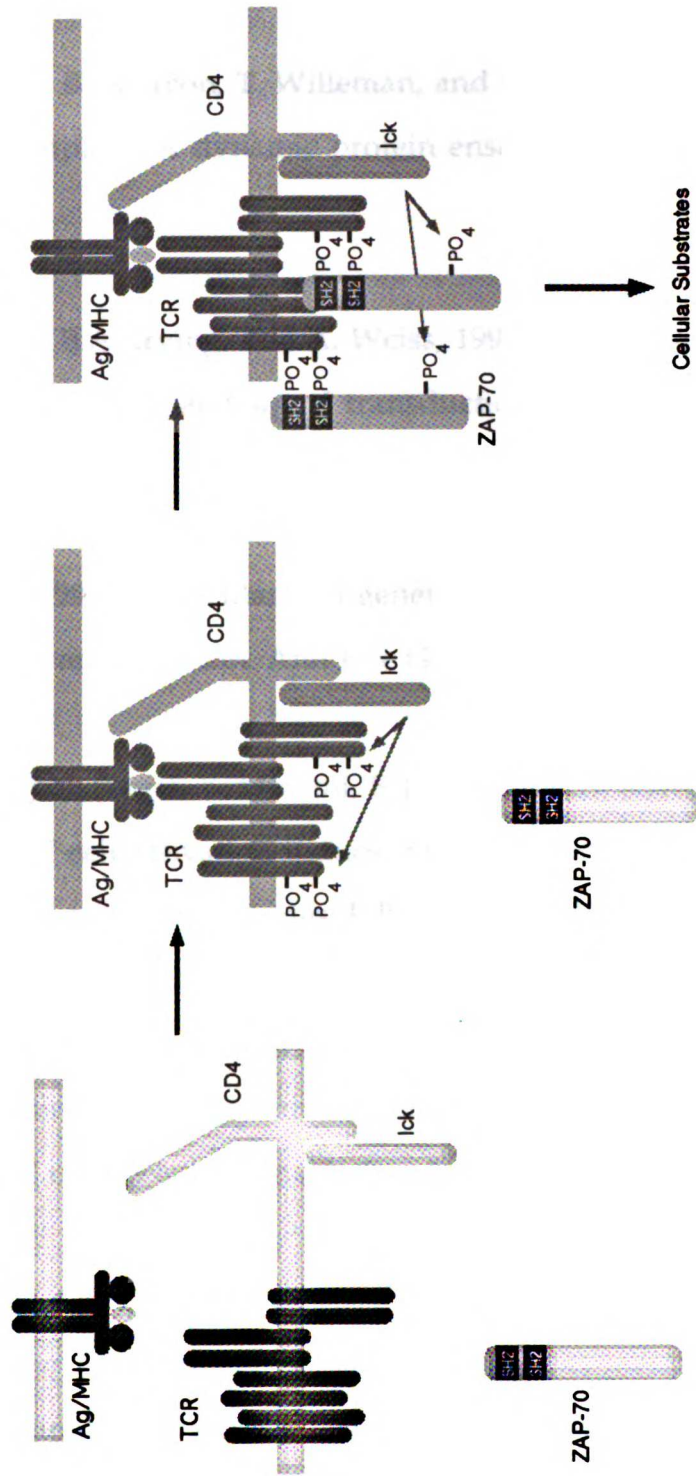
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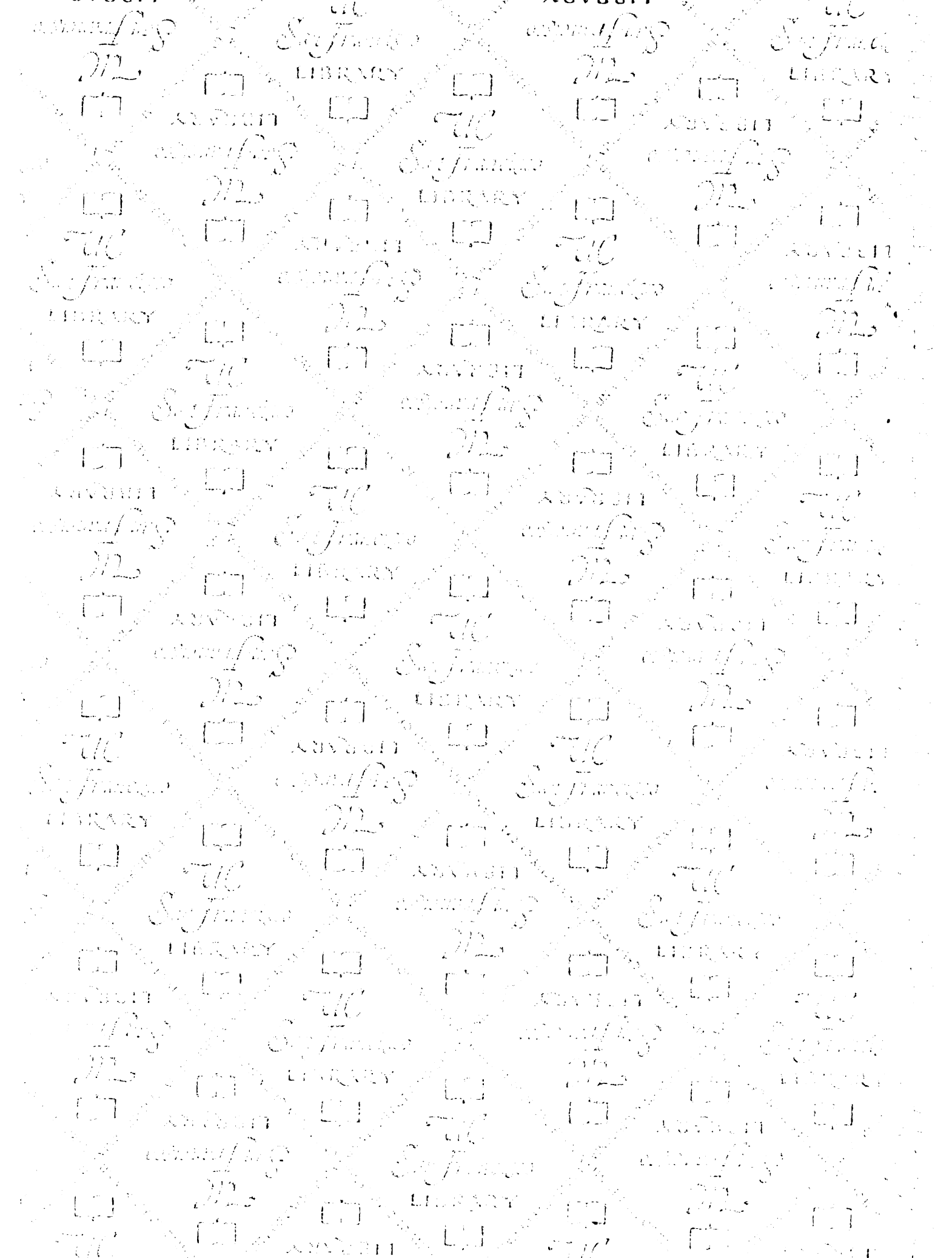
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