

Functional Characterization of a Signal Transducing Motif Present in the T Cell Antigen Receptor ζ Chain

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Summary

A conserved sequence motif has been identified in a number of signaling subunits associated with hematopoietic cell antigen receptors. Here, we characterize signaling by a 17 amino acid motif that is triplicated in the T cell antigen receptor 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 that contain either one, two, or three copies of the motif results in induction of an identical pattern of tyrosine phosphoproteins. Moreover, triplication of the NH₂-terminal ζ motif results in enhanced signaling, 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 ZAP-70 with this motif, and provide evidence for its involvement in ζ function.

T cells are activated during a complex cell-cell interaction in which recognition of an antigenic peptide results in the transduction of intracellular signals that ultimately dictate the immunologic fate of the cell. Central in initiating this process is the TCR, a multisubunit complex consisting of as many as seven distinct transmembrane proteins (1, 2). The clonally variable α and β subunits exist as a disulfide-linked heterodimer and together define the antigenic specificity of the receptor. These two antigen-binding subunits are expressed on the surface of the T cell in an obligate, noncovalent association with invariant chains, CD3 γ , δ , ϵ , and a dimer composed of members of the ζ family: ζ , η , or Fc ϵ RI γ (3-5). Although it has been difficult to assign a function to these invariant chains, recent evidence demonstrates that at least a subset of them serves to couple antigen recognition to intracellular signaling events.

Engagement of the TCR by antigen or by anti-receptor antibodies results in a cascade of biochemical changes within the cell, the earliest of which likely involves the activation of one or more protein tyrosine kinases (PTKs)¹ (6). Since none of the TCR chains possesses intrinsic tyrosine kinase activity, it is believed that the receptor associates with a cytoplasmic PTK. Evidence implicating the involvement of the src family PTKs, lck and fyn, in T cell activation has been

described (7-11), though the exact nature of their roles remains speculative. Recently, our laboratory has identified and characterized a novel 70-kD PTK, termed ZAP-70, that associates with the TCR ζ chain after receptor stimulation (12, 13). However, the precise role of ZAP-70 in TCR-mediated signal transduction is unclear.

After TCR stimulation, a number of cellular proteins become phosphorylated on tyrosine residues, among them being ζ (14), ZAP-70 (12), the protooncogene vav (15), and phospholipase C γ 1 (PLC γ 1) (16-18). Tyrosine phosphorylation of PLC γ 1 has been shown to augment its enzymatic activity, resulting in the production of inositol 1,4,5,-tris-phosphate and diacylglycerol (19). These two second messengers, in turn, are responsible for increasing intracellular Ca²⁺ concentration ([Ca²⁺]_i) and activating protein kinase C, respectively (20, 21). The function of tyrosine phosphorylation of ζ , ZAP-70, and vav is still unknown. The biochemical signals initiated by the TCR ultimately can converge upon the nucleus where they act to induce expression of lymphokines and influence cellular proliferation and differentiation.

Although the nature of the biochemical events that occur after TCR stimulation is becoming better defined, the mechanism by which the TCR subunits initiate the signal transduction cascade has, until recently, remained elusive. Utilizing chimeric receptors composed of the cytoplasmic domain of ζ linked to heterologous extracellular domains, we and others (22-25) have previously demonstrated a role for ζ in signal transduction. Stimulation of these chimeric molecules with mAbs recapitulates both proximal and distal events normally

¹ Abbreviations used in this paper: b, biotinylated; [Ca²⁺]_i, intracellular Ca²⁺; CAT, chloroamphenicol acetyltransferase; NF-AT, nuclear factor of activated T cells; PLC γ 1, phospholipase C γ 1; PTK, protein tyrosine kinase; R α mlgG, rabbit anti-mouse IgG.

associated with stimulation of the intact TCR. In addition, fusion proteins containing the cytoplasmic sequences of η and Fc ϵ RI γ are capable of mediating increases in $[Ca^{2+}]_i$ and targeted cytotoxicity (23). Similarly, the cytoplasmic domain of CD3 ϵ 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 (26). Confirming the signaling capacity of CD3 is a study demonstrating that the TCR can function independently of the ζ cytoplasmic domain (25). 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 that are indistinguishable from those generated by the TCR, we have pursued a more detailed analysis of the ζ sequence with the notion that identifying its functional elements may provide valuable mechanistic information. Here, we define and characterize signaling by the first and second of three 17 amino acid motifs present within ζ . Triplication of one of these motifs results in enhanced signal transduction suggesting a role in signal amplification for multiple copies of the motif within ζ . Finally, we demonstrate the association of the PTK ZAP-70 with this motif and provide evidence supporting its role in ζ function.

Materials and Methods

Chimera Constructions. The CD8/ ζ truncations were constructed by the PCR utilizing the CD8/ ζ construct as a template. Unique 3' primers, which included a stop codon and BamHI site, were used with a constant 5' primer corresponding to CD8 sequences immediately 5' of the unique NaeI site. The PCR products replaced the NaeI/BamHI fragment from CD8/ ζ and the complete construct was excised with XbaI/BamHI and subcloned into plasmid ptfneo (22). The CD8/ ζ chimeras containing isolated motifs were engineered by annealing oligonucleotides which together encoded the motifs flanked by BglII and BamHI sites. These annealed oligos were cloned into the BglII and BamHI sites of the CD8 τ construct. 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 American Type Culture Collection (Rockville, MD). Rabbit anti-mouse IgG (R α mIgG) was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). 4G10, is an antiphosphotyrosine mAb (Upstate Biotechnology, Inc., Lake Placid, NY); 387 is a rabbit anti- ζ antiserum raised against a peptide comprising amino acids 132–144 of the murine ζ sequence (27). mAbs against PLC γ 1 were provided by Dr. Sue Goo Rhee. The mAb C305 binds to the Jurkat Ti β chain and W6/32 to an invariant determinant on human HLA class I antigens. MOPC 195 (Litton Bionetics, Kensington, MD) was used as a control mAb in flow cytometric 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 N-Hydroxy succinimide biotin (Zymed Laboratories, Inc., South San Francisco, CA). 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, Santa

Ana, CA). Chimera transfectants were passaged as above with the addition of Geneticin (GIBCO BRL, Gaithersburg, MD) at 2 mg/ml. Transfections and isolation of clones were as previously described (22).

Flow Cytometry and $[Ca^{2+}]_i$ Fluorimetry. For flow cytometry, 10⁶ cells were stained with saturating concentrations of antibody, then incubated with fluorescein-conjugated goat anti-mouse Ab before analysis in a FACScan[®] (Becton Dickinson & Co.) as previously described (28).

Calcium-sensitive fluorescence was monitored as previously described (29). The CD8/ ζ truncations were stimulated with purified OKT8 (1 μ g/ml) and cross-linked with R α mIgG (2 μ g/ml). Chimeras containing isolated motifs were stimulated with biotinylated OKT8 (0.5 μ g/ml) followed by avidin (1 μ g/ml).

Immunoprecipitations, Cell Stimulations, Surface Iodininations, and Western Blotting. Cells were lysed at 10⁸/ml in 10 mM Tris pH 7.8, 1% NP-40, 150 mM NaCl, in the presence of protease and phosphatase inhibitors as described (22). Lysates were centrifuged for 10 min at 14,000 g, precleared with Sepharose 4B beads, and ultracentrifuged for 20 min at 100,000 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 five times, and analyzed by SDS-PAGE under reducing conditions.

Clones expressing CD8/ ζ truncations were stimulated at 10⁸/ml in PBS with OKT8 at 2 μ g/ml for 2 min at 37°C, followed by R α mIgG at 2 μ g/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 μ g/ml and avidin at 4 μ g/ml.

Cell surface iodinations with ¹²⁵I were performed using the lactoperoxidase/glucose oxidase (Sigma Immunochemicals, St. Louis, MO) procedure as described (28). Western blotting was performed as previously described (22).

Assessment of Nuclear Factor of Activated T Cells (NFAT) Activity. A construct containing four tandem NF-AT binding sites linked to the chloramphenicol acetyltransferase (CAT) gene was transfected by DEAE dextran (30) into Jurkat cells expressing the CD8/ ζ chimeras. 40 h after transfection, 6 \times 10⁶ (experiment 1) or 2 \times 10⁶ (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 Laboratories, Inc.). PMA was added at a final concentration of 50 ng/ml, ionomycin at 1 μ M. After 8 h, cells were harvested in 100 mM Tris pH 7.8 and analyzed for CAT activity as previously described (31). Results are expressed as the fold induction of treated cells over that of PMA treated cells.

Results

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 COOH-terminal truncations of the CD8/ ζ chimera (22) (see arrows in Fig. 1 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. 1 B). Characterization of the chimeras by surface iodination confirms that the expressed proteins are of the expected sizes (Fig. 1 C).

To determine which of the CD8/ ζ truncations were capable of mediating intracellular signaling events, we examined the

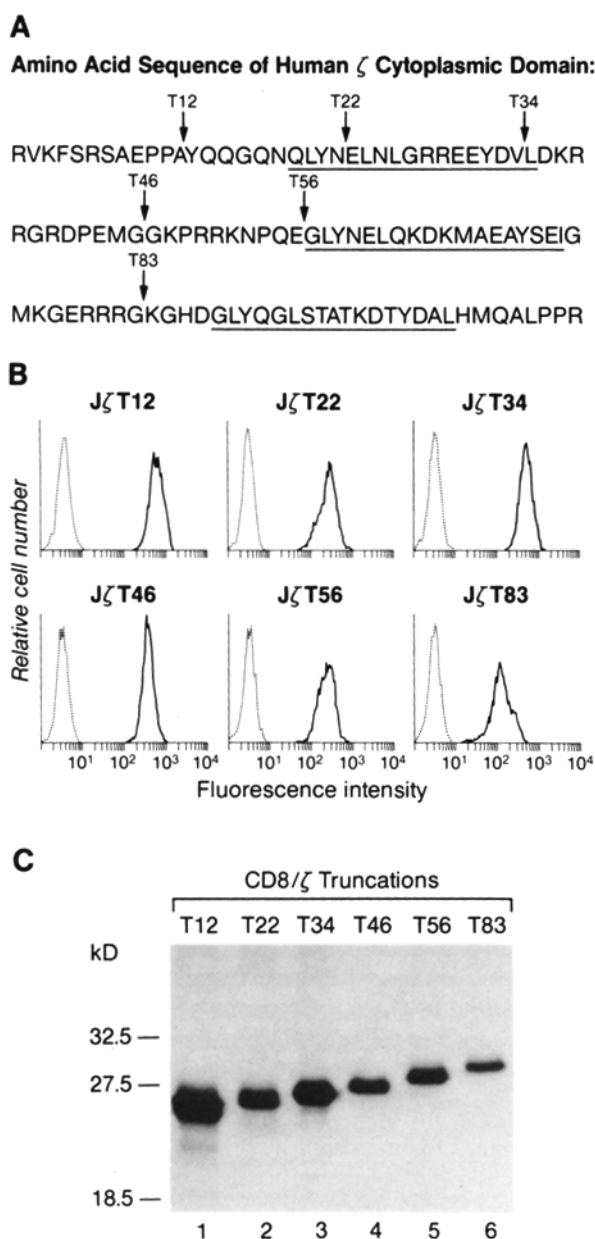


Figure 1. (A) The amino acid sequence of the ζ cytoplasmic domain is depicted with arrows denoting the COOH-terminus of each CD8/ ζ truncation. The number assigned to each truncation refers to the number of amino acids remaining in the ζ cytoplasmic domain. All constructs share both the extracellular and transmembrane domains of CD8. The conserved tyrosine and leucine-based motif (*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*). Nonspecific 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 (2×10^7) were lysed in 1% NP-40 and immunoprecipitated with OKT8. Immunoprecipitates were resolved by SDS-PAGE followed by autoradiography. (*Left*) Mobilities of markers are denoted.

ability of the constructs to elicit a rise in $[Ca^{2+}]_i$ (Fig. 2, A–D). Clone J ζ T34, which expresses a chimera containing 34 cytoplasmic residues, was completely unresponsive to saturating concentrations of anti-CD8 mAb, OKT8, or to further cross-linking with secondary Ab, R α mIgG (Fig. 2 A). 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 J ζ T46 with OKT8 alone resulted in a delayed but substantial increase in $[Ca^{2+}]_i$, which was further augmented by cross-linking with R α mIgG. 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 cross-linking Ab. Like clone J ζ T34, clones expressing T12 and T24 were completely unresponsive to the combination of OKT8 and R α mIgG (data not shown) despite higher levels of surface expression of these molecules (Fig. 1, B and C). Thus, these results 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 (32), we examined the status of tyrosine phosphoproteins in lysates before and after 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 (Fig. 2 E, lane 2). Though a slight augmentation in one or two substrates can be seen in stimulated lysates from J ζ T12 and J ζ T22, the first marked induction in tyrosine phosphoproteins is seen with stimulation of truncation T46 (Fig. 2 E, 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 (Fig. 2 E, lane 2). Similar results were obtained with stimulation of ζ T83. 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, that associates stably with ζ upon stimulation of the antigen receptor (12). 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 72-kD PTK isolated from splenic cells (13, 33). To define the site within ζ required for association with ZAP-70, Jurkat clones expressing the CD8/ ζ truncations were examined. Lysates from unstimulated or stimulated cells were immunoprecipitated with the indicated mAb, resolved by SDS PAGE, and analyzed by Western blotting with an antiphosphotyrosine mAb. Fig. 3 A 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, whereas no induced association is detected with T12, 22, or

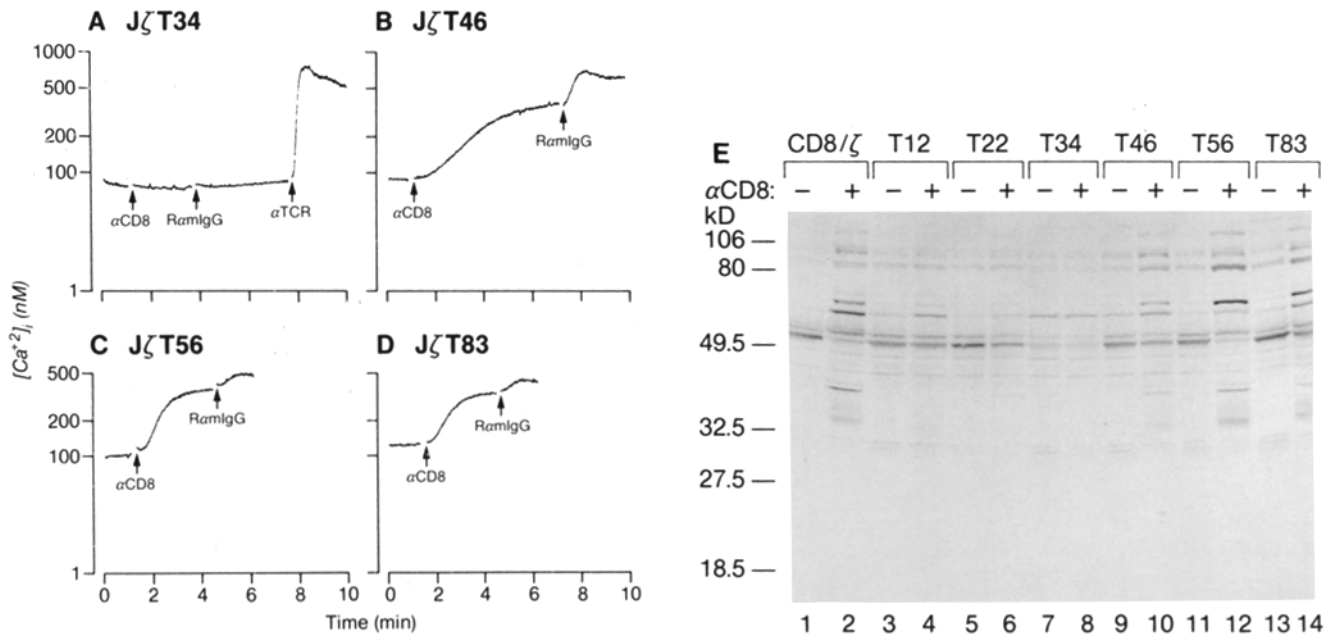


Figure 2. Analysis of proximal signaling by Jurkat clones expressing COOH-terminal truncations of CD8/ ζ (A–D). The designated clones were loaded with Indo-1 and stimulated with an anti-CD8 mAb OKT8 (1 μ g/ml), followed by cross-linking with R α mlgG (2 μ g/ml). J ζ T34 (A), unresponsive to OKT8 and to R α mlgG, was stimulated with the anti-TCR mAb, C305 (1 μ g/ml). Stimuli were added at the indicated times (arrows) and changes in [Ca²⁺]_i were monitored by fluorimetry as described in Materials and Methods. (E) Phosphotyrosine immunoblot of whole cell lysates from cells either unstimulated (–) or stimulated (+) with anti-CD8 mAb, OKT8. Cells were stimulated in PBS at 37°C with OKT8 (2 μ g/ml) for 2 min followed by cross-linking with R α mlgG (2 μ g/ml) for an additional 2 min. Cell lysates were resolved by electrophoresis and analyzed by Western blotting with an antiphosphotyrosine mAb, 4G10. Each lane represents 2 \times 10⁶ cell equivalents.

34 (Fig. 3 B). It is interesting that despite the fact that all truncations, with the exception of T12, contain at least two cytoplasmic tyrosine residues, tyrosine phosphorylation of ζ sequences is seen only in those constructs that associate with ZAP-70 (28–33-kD bands observed in Fig. 3 B). 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. Fig. 3 C 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: YxxLxxxxxx-xYxxL. This motif, which is present in three copies within ζ (see underlined sequences in Fig. 1 A) and singly represented in the ζ homologue, Fc ϵ RI γ , likely represents a signal 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 Fig. 4 A 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 BglII site

into which isolated sequences encoding the motifs plus two adjacent NH₂-terminal residues could be introduced. Whereas constructs encoding ζ motifs 1, 2, and 3 were made, surface expression of ζ 3 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 five amino acids composed of arginine and glycine residues was added at the COOH-terminus of motif 1 (designated ζ 1L). An additional restriction site attached to the linker enabled the introduction of three copies of motif 1 into CD8_T, yielding ζ 1L(3X). Clonal surface expression and structural characterization of the chimeras were assessed by flow cytometry (Fig. 4 B) and surface iodination (Fig. 4 C).

Calcium fluorimetry was performed to assess the signaling function of the chimeras containing isolated motifs (Fig. 5). Whereas in the previous fluorimetry experiments, additional cross-linking of the primary mAb OKT8 had only a modest effect on calcium mobilization by the CD8/ ζ truncations (Fig. 2), cross-linking 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 function. Therefore, unless otherwise stated, biotinylated OKT8 (bOKT8) and avidin as a cross-linking reagent were used for stimulations, as these

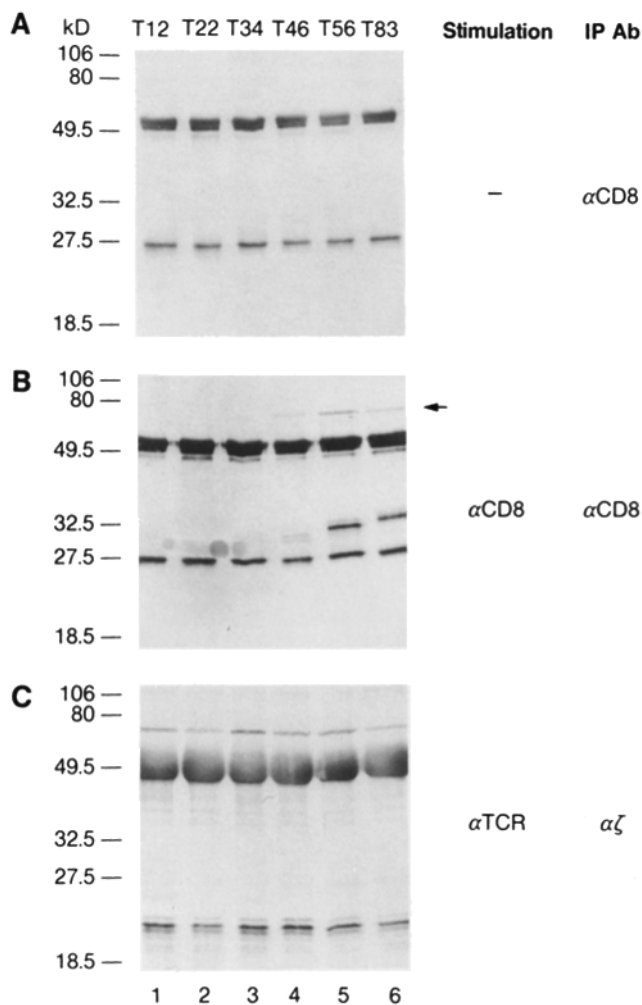


Figure 3. Association of the CD8/ ζ truncations with ZAP-70 tyrosine kinase. (A and B) 10^8 cells expressing the indicated truncations were stimulated as described in Fig. 2 E, lysed in buffer containing 1% NP-40, and immunoprecipitated with anti-CD8 mAb, OKT8. Immunoprecipitates were analyzed for tyrosine phosphoproteins by Western blotting with antiphosphotyrosine mAb 4G10. (Arrow) 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.

reagents provided a more immediate and synchronous cross-linking than did OKT8 and R α mIgG (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, cross-linking of chimeras ζ 1 or ζ 2 with OKT8 and avidin results in mobilization of $[Ca^{2+}]_i$ (Fig. 5, B and C). It is interesting that addition of the 5 amino acid linker onto motif 1, ζ 1L, enhances its capacity to signal, (compare tracings in Fig. 5, D and B), perhaps by stabilizing the COOH-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 after the very rapid, initial rise. The transient nature

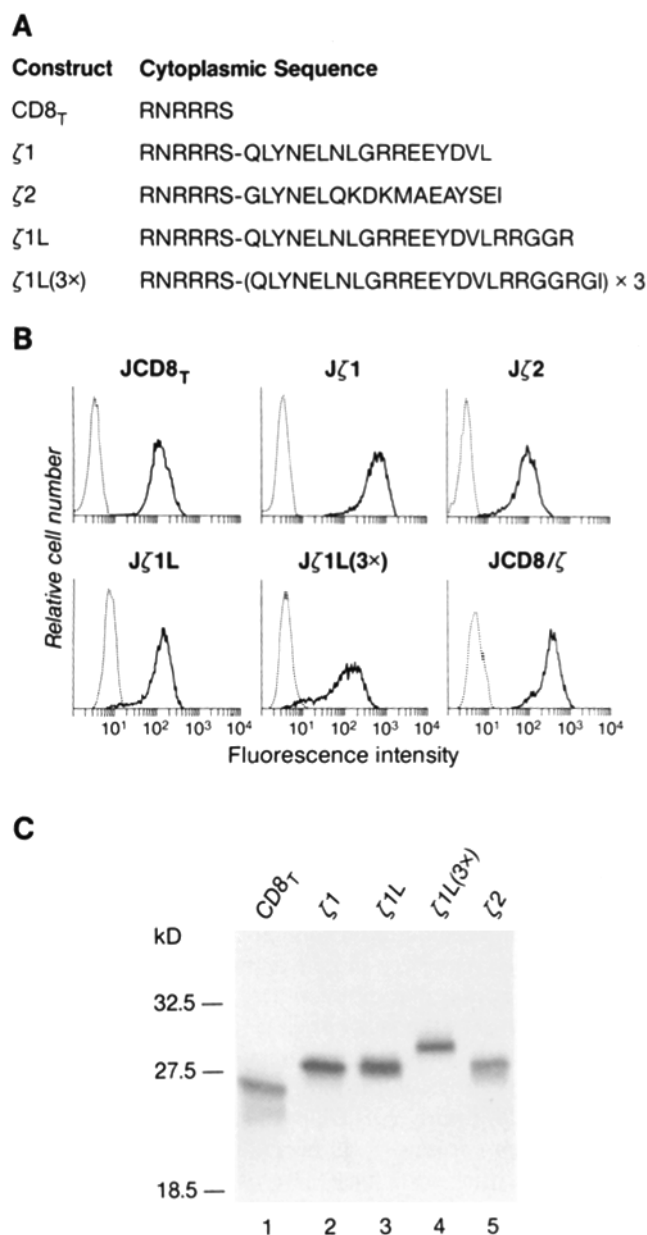


Figure 4. (A) The cytoplasmic sequences of CD8/ ζ chimeras containing isolated motifs. A BglII site was added to the sequences encoding the first four cytoplasmic residues of CD8 resulting in an additional arginine and serine at the COOH-terminus of CD8 $_T$. Annealed oligos encoding the ζ sequences shown were introduced via this BglII 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 the Fig. 1 B legend. JCD8/ ζ expresses the intact CD8/ ζ chimera. (C) Structural characterization of constructs by surface iodination (see legend to Fig. 1 C).

of the $[Ca^{2+}]_i$ increase mediated by ζ 1L is no longer apparent in the response of ζ 1L(3x) (Fig. 5 F), whose sustained plateau phase is more characteristic of that observed with either CD8/ ζ or TCR stimulation. This enhancement in signaling by ζ 1L(3x) relative to ζ 1L was observed in multiple clones and was still detectable when expression of ζ 1L ex-

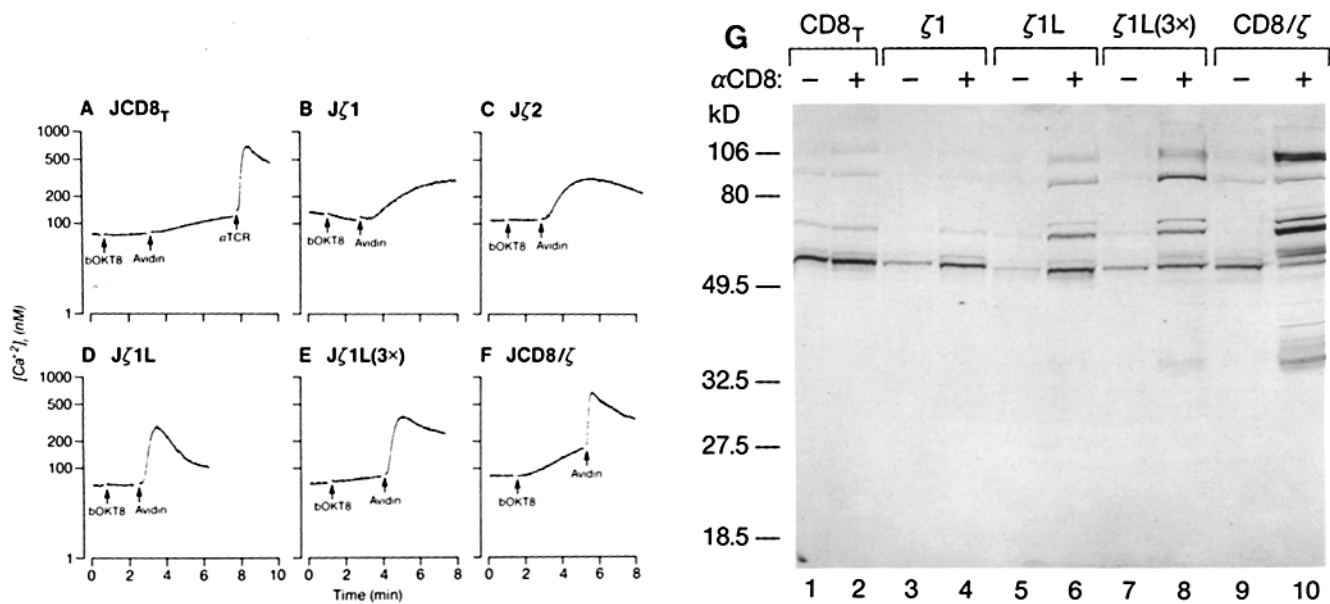


Figure 5. Calcium mobilization and induction of tyrosine phosphoproteins by the isolated motifs. (A-F) The indicated clones were loaded with the calcium-sensitive dye Indo-1 and analyzed for chimera-induced increases in $[Ca^{2+}]_i$ by fluorimetry. Biotinylated OKT8 (bOKT8) was used at a final concentration of 0.5 $\mu\text{g/ml}$ and avidin at 1 $\mu\text{g/ml}$. (G) Cells were stimulated at 37°C in PBS with bOKT8 (2 $\mu\text{g/ml}$) for two min followed by cross-linking with avidin (4 $\mu\text{g/ml}$) for an additional 2 min. Phosphotyrosine content in lysates was analyzed as described (Fig. 2 E, legend).

ceeded that of the triplicated construct. These results suggest that increasing the number of motifs from one to three 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, signaling by $\zeta 1L(3x)$ does not compare with that of CD8/ ζ , as demonstrated by the ability of CD8/ ζ to respond to bOKT8 alone, and more robustly to avidin cross-linking (Fig. 5 F). Note that in contrast to the ramping effect seen upon stimulation of CD8/ ζ with bOKT8, purified OKT8 elicits a rapid, strong response from this chimera (22), 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 (Fig. 5 G). Immunoblotting of cell lysates from JCD8 $_T$ with an antiphosphotyrosine mAb (Fig. 5 G, 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 $\zeta 1$, $\zeta 1L$, and $\zeta 1L(3x)$ results in progressively greater induction of tyrosine phosphoproteins, recapitulating the quantitative increase in signaling efficiency observed with these constructs in the fluorimetry experiments (Fig. 5, A-F). The low degree of tyrosine phosphorylation induced by $\zeta 1$ is similar to that observed with stimulation of $\zeta 2$ (data not shown). It is notable that the pattern of substrates seen with stimulation of $\zeta 1L(3x)$ (Fig. 5 G, 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 deter-

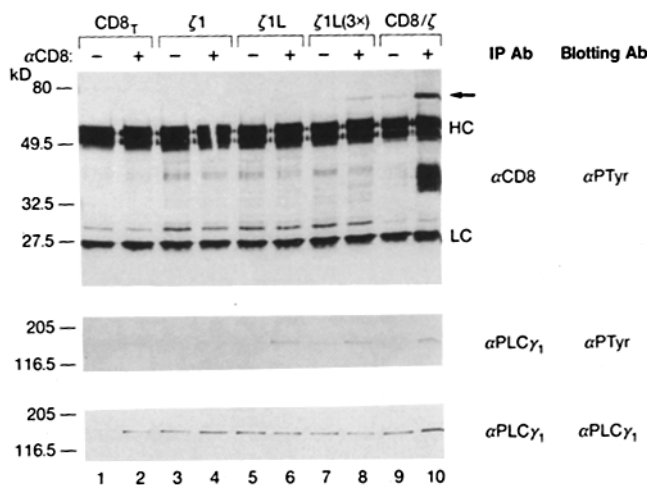


Figure 6. ZAP-70 associates with sequences in the first ζ motif. Cells (1.8×10^8) were stimulated as described (Fig. 5 G legend). OKT8 immunoprecipitates from lysates were resolved by electrophoresis, and analyzed for phosphotyrosine content by Western blotting with antiphosphotyrosine mAb, 4G10 (top). Immunoprecipitates of PLC $\gamma 1$ (from 5×10^7 cells) were analyzed in a similar fashion (middle). (Bottom) Fraction of the PLC $\gamma 1$ immunoprecipitate blotted with an immunoprecipitating mAb demonstrating comparable levels of PLC $\gamma 1$ in each lane.

mine whether ZAP-70 could associate with the 17 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 antiphosphotyrosine mAb. As depicted in Fig. 6, no association of ZAP-70 with the truncated CD8 molecule is detected nor is it observed after cross-linking of ζ 1, or ζ 1L. However, stimulation of ζ 1L(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. Using a sensitive in vitro kinase assay of CD8 immunoprecipitates, association of ZAP-70 can be detected with ζ 1L, though association with ζ 1 and ζ 2 has not been convincing. How-

ever, direct immunoprecipitation of ZAP-70 with an α ZAP-70 antiserum reproducibly reveals its induced tyrosine phosphorylation after 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 (Fig. 6, lane 10), demonstrating that although the sequences in motif 1 are sufficient for activation, they alone do not confer optimal function. After immunoprecipitation with OKT8, the lysates were subjected to immunoprecipitation with PLC γ 1 mAbs to assess the state of PLC tyrosine phosphorylation. The middle panel of Fig. 6 demonstrates the induced phosphorylation of PLC with stimulation of ζ 1L, ζ 1L(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 (Fig. 6, bottom). These data indicate that the first 17 amino acid motif is sufficient to induce the tyrosine phosphorylation of PLC γ 1 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 (34). A common means of assessing the functional integrity of the TCR involves quantitating the expression and secretion of the lymphokine, IL-2. In place of the standard bioassay for IL-2, we have monitored the transcriptional activity of NF-AT, perhaps the best characterized of the several nuclear factors that facilitate transcription of the IL-2 gene (35). 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 (36-38). We utilized a plasmid containing four tandem copies of the NF-AT DNA binding element placed upstream of the CAT reporter gene (30). Fig. 7 depicts results obtained from two experiments in which NF-AT-induced CAT activity is measured from cells harvested after 8 h of stimulation with the indicated mAbs in the presence of an activator of protein kinase C, PMA. As expected, engagement of CD8 τ (Fig. 7, hatched bars) resulted in no induction of NF-AT activity in either experiment. Similarly, crosslinking of class I MHC molecules (solid 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 fourfold induction in NF-AT activity was seen with stimulation of either ζ 1, ζ 1L, or ζ 2, indicating that the signals transduced by the isolated motifs are sufficient to manifest distal activation events. Furthermore, ζ 1, and ζ 2 are capable of inducing surface expression of the late activation antigen CD69 (data not shown). Consistent with the proximal signaling data, triplication of motif 1 increased the signaling efficiency of ζ 1L(3x), resulting in a greater than eightfold induction in NF-AT activity with its stimulation. This degree of induction was surpassed only by the intact CD8/ ζ which provided an average 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.

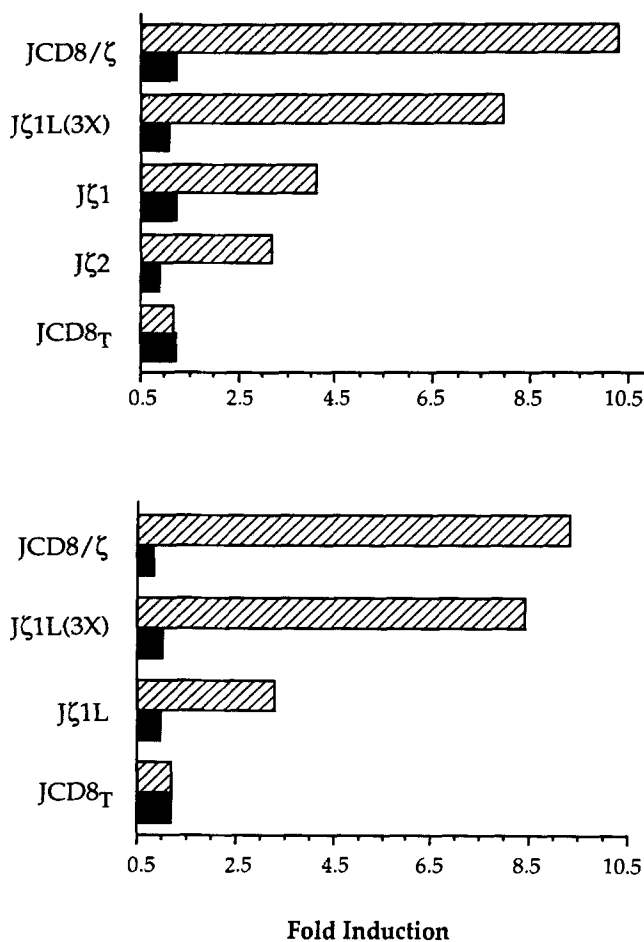


Figure 7. Induction of NF-AT activity by isolated motifs. A construct containing four tandem copies of the NF-AT binding site linked to the CAT gene was transfected into the designated clones. After 40 h, cells were stimulated and assayed for CAT activity as described in Materials and Methods. Results are expressed as a fold induction, comparing values obtained from cells stimulated with OKT8 (hatched bars) or an anti-class I MHC mAb, W6/32 (solid 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).

Discussion

Here we characterize signaling 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 (24, 25) and led to the identification of a conserved sequence which is triplicated in ζ (25). 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 (39). These include ζ , Fc ϵ R1 γ , each of the CD3 chains, and the membrane Ig-associated MB1 and B29 gene products. Work from two groups has provided direct evidence for the role of these motifs in signal transduction, with the CD3 ϵ motif capable of inducing tyrosine phosphoproteins and IL-2 production (26), and the ζ motifs sufficient to effect calcium mobilization and redirected cytotoxicity (40). 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 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 one motif, are competent signal transducers. However, the complete loss of function observed with removal of the motif's COOH-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 the CD3 ϵ chain has identified the two YXXL pairs as the functionally important components of its motif, though the NH₂-terminal of the pair was more vulnerable to alterations in sequence (26). Our results suggest a functional requirement for the COOH-terminal YXXL group in the ζ motif, further supporting the importance of this highly conserved feature of the motif. Comparison of signaling by T56 and ζ 1 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 cross-linking of ζ 1 and ζ 1L(3x) for detectable signaling 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 signaling 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 (40) 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 signaling function. Rather, the enhancement in signaling observed with triplication of motif 1 in ζ 1L(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 10–100-fold higher concentrations of antibody for detectable IL-2 production (24, 25). This reduced efficiency of signaling by a single motif is also observed in comparisons of the dose-response curves of CD3 ϵ (which contains a single copy of the motif) and ζ in their ability to induce IL-2 secretion (26). Finally, when transfected into a basophil leukemic cell line, ζ appears to induce degranulation more efficiently than its endogenous counterpart, the single motif-containing Fc ϵ R1 γ chain (24). Thus, triplication of the signaling module in ζ may have evolved as a means of amplifying the signals transduced, thereby increasing the sensitivity of the TCR to ligand stimulation.

It is notable that only a slight augmentation in intensity of tyrosine phosphoproteins is observed with stimulation of ζ 1L(3x) relative to ζ 1L, despite the apparent enhanced avidity of ZAP-70 for ζ 1L(3x) and the enhanced signaling by this chimera. This apparent lack of correlation between degree of phosphorylation and function is also observed with the phosphorylation of PLC γ 1 upon stimulation of ζ 1L, ζ 1L(3x), and CD8/ ζ (Fig. 7). Despite the progressive enhancement in signaling 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 , although, as discussed above, the intact chimera is a more potent signal transducer. Thus, at a single time point, enhancement of some signaling 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 (Fig. 3). Furthermore, stimulation of all chimeras capable of transducing signals resulted in induced tyrosine phosphorylation of ZAP-70. Finally, efficiency of signaling was linked to the stability of chimera-induced ZAP-70 association. Whereas sequences in ζ 1L 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 that 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 $\zeta 2$ 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 70–72-kD tyrosine phosphoprotein upon stimulation (12, 41–43). Furthermore, the membrane Ig-associated 72-kD phosphoprotein is also a PTK and likely represents syk, a PTK homologous to ZAP-70 (33, 41). 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.

Although a simple model invoking a direct, ligand-induced recruitment of ZAP-70 to ζ could be proposed, recent work (13) suggests that the association of ZAP-70 with ζ requires expression of at least one src family PTK. Whether this requirement indicates a requisite phosphorylation event within the motif or in ZAP-70 or both, is still unclear. Genetic evi-

dence demonstrates a critical role for the src family PTK, lck, in TCR-mediated signal transduction and thymic selection (8, 9). Furthermore, a number of groups have described the association between ζ and fyn, another src family PTK (10, 44–46). 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 seven amino acids beyond the COOH-terminal leucine of the first motif (45). However, our results, in conjunction with the work of others (40), 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 (47, 48). Whereas 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 signaling capacity observed with stimulation of truncation T56 relative to $\zeta 1$ may be explained by the addition of the residues COOH-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.

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