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Phosphorylated T cell receptor ζ-chain and ZAP70 tandem SH2 domains form a 1:3 complex *in vitro*

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The ζ polypeptide is part of the T cell antigen receptor (TCR). The ζ-chain contributes to efficient cell-surface expression of the TCR and accounts for part of its signal transduction capability. TCR recognition triggers a complex set of events that result in cellular activation. The protein tyrosine kinase (PTK) Lck phosphorylates the ζ-chain, which in turn associates with another PTK, ZAP70, and stimulates its phosphorylation activity. Here we report the expression of the intracellular part of the ζ-chain and its biochemical characterization. The recombinant protein does not dimerize by itself in solution. Circular-dichroic analysis reveals a random coil conformation. ζ, phosphorylated using recombinant Lck, associates with recombinant ZAP70 tandem-SH2 domains. All three T cell activation motifs in ζ bind ZAP70 tandem-SH2 domains *in vitro*, forming a 1:3 complex. This result extends the picture, derived from earlier studies, of a mechanism for signal amplification

Keywords: signal transduction; T-cell receptor ζ chain; protein-tyrosine kinase ZAP-70; ζ-chain phosphorylation; signal amplification.

The T cell antigen receptor (TCR) is a multisubunit structure that contains at least six different proteins (TCR α, β and ζ; CD3 γ, δ and ε). They are assembled in the endoplasmic reticulum and transported to the cell surface [1, 2]. Upon recognition of major histocompatibility complex plus foreign peptide by the α and β chain, the CD3 members and ζ initiate a signaling cascade to generate an immune response. Signaling through the TCR complex mediates activation of lymphocytes by induction of calcium ion mobilization, cell proliferation and release of lymphokines. One of the first events in the engagement of TCR is the phosphorylation of multiple tyrosine residues in CD3 and ζ [3].

The ζ-chain is an important signaling component of the TCR complex and capable of activating T cells independently of other subunits [4, 5]. ζ contains a short extracellular segment, a single transmembrane region and a cytoplasmic domain. It forms a disulfide-linked dimer in T cells. An amino acid sequence motif, first noted by Reth [6], is triplicated in the cytoplasmic domain of a single ζ-chain and is also present as a single copy in the subunits of CD3 and the B cell antigen receptor [4, 7–11]. It is responsible for the signal transduction capability of these chains. Each motif, called an immunoreceptor tyrosine activation motif (ITAM), consists of a pair of YXXL sequences separated by seven or eight amino acid residues. It has been shown that a single ITAM domain (17 amino acids) is sufficient to couple chimeric receptors to events associated with T cell activation [7, 10] and that the Tyr and Leu residues are required for the functional activity of the motif [7, 10, 12].

The Tyr residues in the ITAMs are phosphorylated upon TCR stimulation by protein-tyrosine kinases Lck or Fyn [13–16]. This phosphorylation leads to the recruitment of a second PTK, ZAP70, a member of the Syk subfamily [17, 18]. Its tandem-SH2 domains subsequently associate with phosphorylated ζ [7, 19]. The three-dimensional structure of the ZAP-70 tandem-SH2 domains in complex with a phosphopeptide derived from the first ITAM of ζ shows an extended conformation for the ζ peptide [20]. Both SH2 domains are required for efficient binding [19]. The N-terminal SH2 domain binds phosphorylated ζ with a 100-fold lower affinity, while no binding was observed for the C-terminal domain alone [21], suggesting a cooperativity in binding between these two SH2 domains. These binding studies are consistent with the finding that both tyrosines in one ITAM have to be phosphorylated for ZAP70 binding [14, 22].

For structural studies, we have expressed the intracellular part of ζ in *Escherichia coli*. The recombinant protein dimerizes in solution if a Cys residue is present at the amino terminus, but not otherwise. The CD spectrum reveals no apparent secondary structure and shows a spectrum typical for a random-coil polypeptide in the far-ultraviolet region. Recombinant ζ can be completely phosphorylated by a recombinant PTK, Lck. Recombinant ZAP70 tandem-SH2 domains bind the phosphorylated ζ-chain, expressed in *E. coli*, in a 3:1 molar ratio, fully occupying the Tyr(*P*) residues of its ITAM.

MATERIALS AND METHODS

Expression and purification of ζ. The ζ encoding sequence was amplified by PCR and fragments were subcloned into the pRSET vector (Invitrogen). The *E. coli*-expressed ζ-chain (ζ-*e*) contains the pRset His_6 tag sequence as an *Nde*I–*Nhe*I fragment

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Abbreviations. TCR, T cell antigen receptor; PTK, protein-tyrosine kinase; ITAM, immunoreceptor tyrosine activation motif; ζ-*e*, *E. coli*-expressed ζ-chain; ζ-*e*Cys, *E. coli*-expressed ζ-chain with N-terminal Cys; ζ-*eP*, *E. coli* expressed ζ-chain phosphorylated with the PTK Lck.

Enzyme. Protein-tyrosine kinase (EC 2.7.1.112).

at the N-terminus and encodes amino acids 54–163 [MKGS(H)$_6$GMAS-des(1–53)-ζ]. ζ-*e*Cys starts with MKKCG and amino acids 51–163 of ζ (without the His$_6$ tag) [MKKCG-des(1–50)-ζ]. Recombinant protein was expressed in *E. coli* strain BL21/pLysS.

E. coli cell pellets (ζ-*e*) were harvested by centrifugation and dissolved in 100 ml 50 mM Tris/HCl pH 8.0, 500 mM NaCl. After sonication, the lysate was cleared at 40000 rpm for 2 h. The soluble fraction was loaded on to a Ni-Sepharose column (Invitrogen) and purified according to the manufacturer's protocol. ζ-*e*Cys-expressing bacteria were lysed in 50 mM Mes pH 6.5, 400 mM NaCl. After centrifugation (40000 rpm, 2 h) the supernatant was diluted with 50 mM Mes pH 6.5 to a final NaCl concentration of 100 mM and loaded on to a S-Sepharose (Pharmacia) column (1.5×15 cm). Protein was eluted with a 0.1–1 M NaCl gradient. Fractions containing ζ-*e*Cys were identified by SDS/PAGE [23] and precipitated with 35% (mass/vol.) ammonium sulfate. The precipitated protein was redisolved in 20 mM Hepes pH 7.2, 150 mM NaCl. Both ζ-*e* and ζ-*e*Cys were purified by Superdex-75 gel filtration chromatography (Pharmacia). Eluted fractions containing ζ-protein were concentrated in a Centricon-10 (Amicon). The ζ-protein was stored at 4°C.

ZAP70-SH expression and purification. DNA encoding tandem-SH2 domains of PTK ZAP70 (residues 1–257) has been cloned into the pRSET vector (Invitrogen) and recombinant protein was expressed in *E. coli* strain BL21/pLysS. Bacterial pellets were resuspended in a lysis buffer containing 50 mM Tris pH 7.7, 150 mM NaCl, 5 mM dithiothreitol. The suspension was lysed by sonication and cleared by centrifugation. The SH2 protein contained in the supernatant was purified by affinity chromatography on a phosphotyrosine-Sepharose column. Bound SH2 domains were eluted with 20 mM free phosphotyrosine (20 mM Tris pH 7.7, 150 mM NaCl, 5 mM dithiothreitol); further purification was achieved by Superdex-75 gel filtration chromatography (Pharmacia).

PTK Lck expression and purification. Lck was expressed using SF9 cells and a Baculovirus vector. A fragment encoding residues 53 to 509 of human p56 Lck [des-(1–52)-Lck] was subcloned into pVL1392 transfer vector using standard PCR-based methods. Recombinant plasmids were transformed into DH5α *E. coli* cells and positive clones were selected by standard procedures. Recombinant baculovirus was obtained by co-transfecting linearized viral DNA (Pharmingen) and the transfer vector according to the manufacturers protocol (Pharmingen). High-titer viral stocks (10^9) were prepared from plaque-purified recombinant virus [24]. For protein production, SF9 cells were cultured in 2-l spinner flasks in TNM/FH media (Bioscience), supplemented with 10% bovine calf serum. Cells were infected with virus (multiplicity of infection 5) and harvested 64–72 h post-infection.

Infected SF9 cells were lysed by douncing and sonication in 25 mM Hepes pH 7.7, 25 mM NaCl, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 5 mM benzamidine. Lysates were cleared by centrifugation at 40000 rpm for 4 h. Supernatant was loaded on to a DEAE-Sepharose column (5×10 cm), washed with lysis buffer, and eluted with a 0.025–0.7 M NaCl gradient. Fractions containing Lck were identified by SDS/PAGE and Western blotting with anti-(human Lck) (UBI, Inc.), pooled, concentrated in a Centriprep-30 concentrator (Amicon), and exchanged into 25 mM Hepes pH 7.7, 25 mM NaCl, 5 mM dithiothreitol, 5 mM $MgCl_2$. Lck was further purified by ATP affinity chromatography [25]. After loading and washing, Lck was eluted from the ATP-Sepharose column (1×20 cm) with a 0.025–1 M NaCl gradient. Fractions containing Lck were pooled, concentrated and passed over a Superdex-200 gel filtration column (Pharmacia) in storage buffer (25 mM Hepes pH 7.7, 150 mM NaCl, 5 mM dithiothreitol). Protein was stored at −20°C.

Chemical crosslinking. Crosslinking of ζ-*e*, ζ-*e*Cys and of the complex ζ-*eP*/ZAP70-SH was performed in 50 mM Hepes pH 7.4, 150 mM NaCl. ζ-*e* and ζ-*e*Cys were crosslinked with 0.5, 1, 2.5 and 5 mM crosslinking reagent ethyleneglycol bis(-succinimidylsuccinate) (Pierce) and the complex ζ-*eP*/ZAP70-SH was crosslinked with 0.1, 0.5 and 5 mM. The reactions were incubated for 1 h on ice and were then quenched with 50 mM glycine for 20 min. Crosslinked products were analyzed under reducing conditions on SDS/PAGE.

Quantitative photometry. 10 μg ζ-*eP*, ZAP-SH and the complex of ζ-*eP*/ZAP-SH were separated on a 15% SDS/PAGE. Bands were detected by staining with Coomassie brilliant blue and the intensity was measured with a Fuji BAS 2000 scanner. The integrated density values obtained for ζ-*eP* and ZAP-SH bands were used to calculate the ratio of ζ-*eP* and ZAP-SH present in the complex.

Circular dichroism. Spectra were recorded using a 1-mm cell and an Aviv 62DS spectropolarimeter equipped with a thermoelectric temperature controller. Experiments were performed with buffers in the pH range 4–9 to which NaCl (0.1–1 M) was added. CD spectra were measured at 4°C and 20°C. The spectra shown in Fig. 2 were measured in 10 mM sodium phosphate pH 7.2, 100 mM NaCl at 20°C. The protein concentration was determined by the Bradford assay (BioRad) and the same protein concentration was used for all spectra.

Phosphorylation and ZAP70 SH2 domain binding. ζ-*e* was phosphorylated by recombinant PTK Lck at a molar ratio of 1:20. The reaction was performed in 20 mM Hepes pH 7.7, 150 mM NaCl, 5 mM $MgCl_2$, 10 mM ATP and 5 mM dithiothreitol at 37°C for 30 min. The pH was then adjusted to 6.0 with 100 mM Mes and the NaCl concentration was diluted to 50 mM. ζ-*eP* was bound to an S-Sepharose column (Pharmacia) and eluted with a 0.05–0.5 M NaCl gradient. Lck did not bind under these conditions. For binding studies ζ-*eP* and ZAP70-SH were incubated at room temperature for 30 min. The complex was purified by Superdex-200 gel filtration chromatography (Pharmacia); 0.5-ml fractions were collected and aliquots were analyzed on a 15% SDS/PAGE.

RESULTS

Expression of ζ. The intracellular part of ζ was over-expressed in *E. coli*. Two variants were generated, ζ-*e* (amino acids 54–163) and ζ-*e*Cys (amino acids 51–163). Fig. 1A shows purified ζ-*e* (lane 3) and ζ-*e*Cys (lane 4) on a Coomassie-stained SDS/PAGE. The latter has an amino-terminal Cys to promote dimerization. ζ-*e*Cys showed a molecular mass of approximately 30 kDa under non-reducing SDS/PAGE conditions, indicating formation of a dimer (Fig. 1A, lane 5). The disulfide bond was formed by air oxidation during the purification procedure without further modification. In addition, chemical crosslinking of both constructs showed that only ζ-*e*Cys can be efficiently crosslinked. With increasing concentrations of the crosslinking reagent in the reaction, only faint amounts of ζ dimer with an approximate molecular mass of 30 kDa can be detected under reducing SDS/PAGE conditions. In contrast, ζ-*e*Cys was almost completely crosslinked at high concentration of crosslinking reagent (Fig. 1B). Solution studies of ζ-*e* and ζ-*e*Cys by dynamic light scattering showed a monomodal distribution of molecules with an average molecular mass of 43±3 kDa for ζ-*e*Cys and of 25±3 kDa for ζ-*e*. The calculated molecular mass for ζ-*e* is 14 kDa and for the monomeric ζ-*e*Cys is 13.6 kDa.

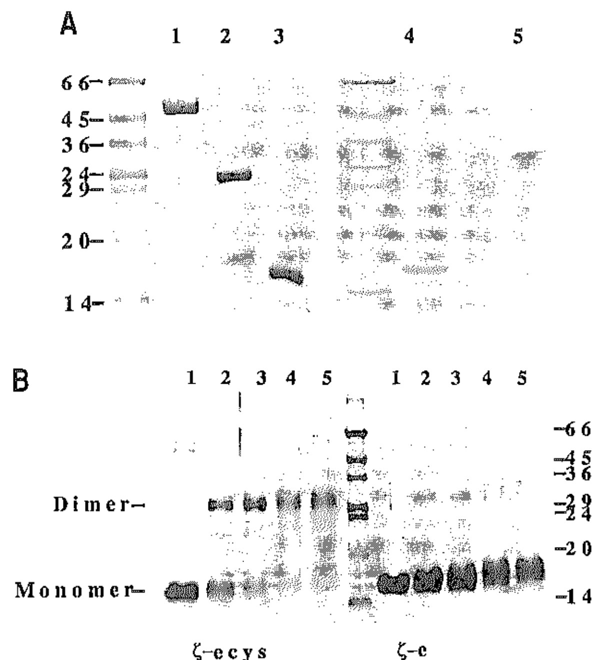

Fig. 1. SDS/PAGE analysis of expressed proteins. (A) Lane 1, purified recombinant PTK Lck; lane 2, ZAP70-SH; lane 3 ζ-*e*; lanes 4 and 5, ζ-*e*Cys. Lanes 1–4 were run under reducing conditions, lane 5 under non-reducing conditions. Bands were detected by staining with Coomassie brilliant blue. Numbers on the left are molecular masses (in kDa) of marker proteins. (B) Chemical crosslinking pattern of ζ-*e*Cys and ζ-*e*. Lane 1, no crosslinking reagent; lanes 2–5, 0.5 mM, 1 mM, 2.5 mM and 5 mM reagent, respectively. The protein was separated on a 15% SDS/PAGE under reducing conditions and protein bands were detected by staining with Coomassie brilliant blue. The sizes of molecular mass standards are shown in kDa.

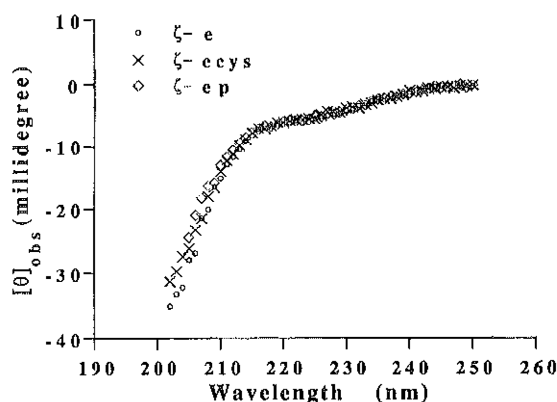

Fig. 2. CD analysis of ζ-*e*, ζ-*e*Cys and ζ-*eP*. CD spectra were measured over 250–200 nm. The observed change in ellipticity (10^3×day · cm · dmol^{-1}) is plotted as a function of wavelength (nm).

CD analysis of ζ. The far-ultraviolet CD spectra of ζ-*e* and ζ-*e*Cys showed the characteristics of an unfolded protein (Fig. 2). The spectra did not change under different buffer conditions, within a pH range of 4–9, nor did the protein concentration change the spectra obtained. In addition, no unfolding transition could be obtained by raising the temperature to 100°C. Moreover the high temperature did not change the protein's solubility. No changes in the CD spectrum were observed when ζ-*e* was completely phosphorylated (Fig. 2) (See below).

Phosphorylation of ζ. Despite its lack of detectable secondary or tertiary structure, the expressed intracellular ζ fragment could be efficiently phosphorylated *in vitro* by the recombinant tyrosine kinase Lck. Fig. 1A (lane 1) shows the purified Lck on a Coomassie-stained SDS/PAGE. Usually two bands appear, running close together. The doublet is probably due either to autophosphorylation or to insect-cell-specific phosphorylation. Phosphorylation shifted the ζ-*e*-specific band in reducing SDS/PAGE to a molecular mass of approximately 20 kDa (Fig. 3A). Completeness of phosphorylation was confirmed by mass spectroscopy. Incomplete phosphorylation, obtained by limiting the amount of active Lck in the reaction tube, yielded a ladder of bands by SDS/PAGE, corresponding to increasing numbers of Tyr(*P*) residues (Fig. 3B). Lck was able to phosphorylate both monomeric ζ-*e* and dimeric ζ-*e*Cys.

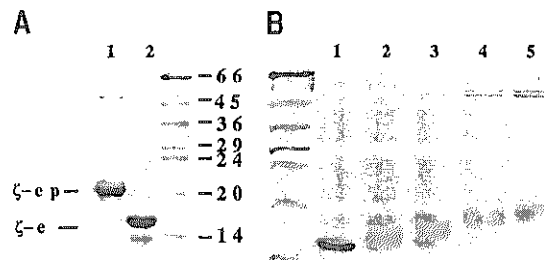

Fig. 3. Phosphorylation pattern of ζ-*eP*. (A) Lane 1, completely phosphorylated ζ-*eP*; lane 2, ζ-*e*. (B) Different isoforms of ζ-*e* upon limited phosphorylation. Lane 1, no Lck; lanes 2–5 increasing concentrations of Lck. Protein was separated on a 15% SDS/PAGE and bands were stained by Coomassie brilliant blue. The sizes of molecular mass standards are shown in kDa.

Complex formation of ζ and ZAP70-SH. To model the binding of ZAP70 to phosphorylated ζ chain, we prepared complexes of ζ-*eP* with recombinant tandem ZAP70 SH2 domains (ZAP70-SH). Fig. 1A (lane 2) shows a Coomassie-stained SDS/PAGE of the purified ZAP70 tandem-SH2 domains. The complex of ζ-*eP* with ZAP70-SH elutes as a peak at 13.2 ml from a Superdex-200 column (Fig. 4A). Comparing the position of the peak to the elution profiles of standard proteins indicates an apparent molecular mass of 120 kDa for the complex. That is close to the calculated molecular mass (110 kDa) expected for ζ-*eP* with three ZAP70-SH tandem domains bound, occupying all six of the Tyr(*P*) residues of the three ITAM domains.

Further evidence that ratio of ZAP70-SH to ζ-*eP* reaches 3:1 (i.e. full occupancy) was provided both by examining the intensities of bands of the constituents of the complex on SDS/PAGE and by observing a ladder of three bands of chemically cross-linked complex. SDS/PAGE of the peak fractions of the ζ-*eP*:ZAP70-SH complexes eluted from a gel filtration column(Fig. 4B), shows that the peak contained ζ-*eP* and ZAP70-SH polypeptides. The relative intensities of the bands from the constituents vary across the rather broad peak suggesting that the average stochiometry of the complex varies across the peak. Quantitative photometry of the bands compared with bands from known concentrations of the constituents suggest that the highest-molecular-mass complexes (lanes 2 and 3, Fig. 4B) contain ZAP70-SH/ζ-*eP* in 3:1 ratio while the lowest (lanes 9 and 10, Fig. 4B) has a 1:1 ratio.

Chemical crosslinking provided further evidence that all three ζ-*eP* binding sites could be occupied by ZAP70-SH by revealing that a ladder of three crosslinked species could be observed by SDS/PAGE (Fig. 4C). When fractions 2–6 (Fig. 4B) were pooled, concentrated, and incubated with increasing concentrations of the crosslinking reagent, three new major bands appeared on SDS/PAGE (labeled 1:1, 1:2 and 1:3 in Fig. 4C).

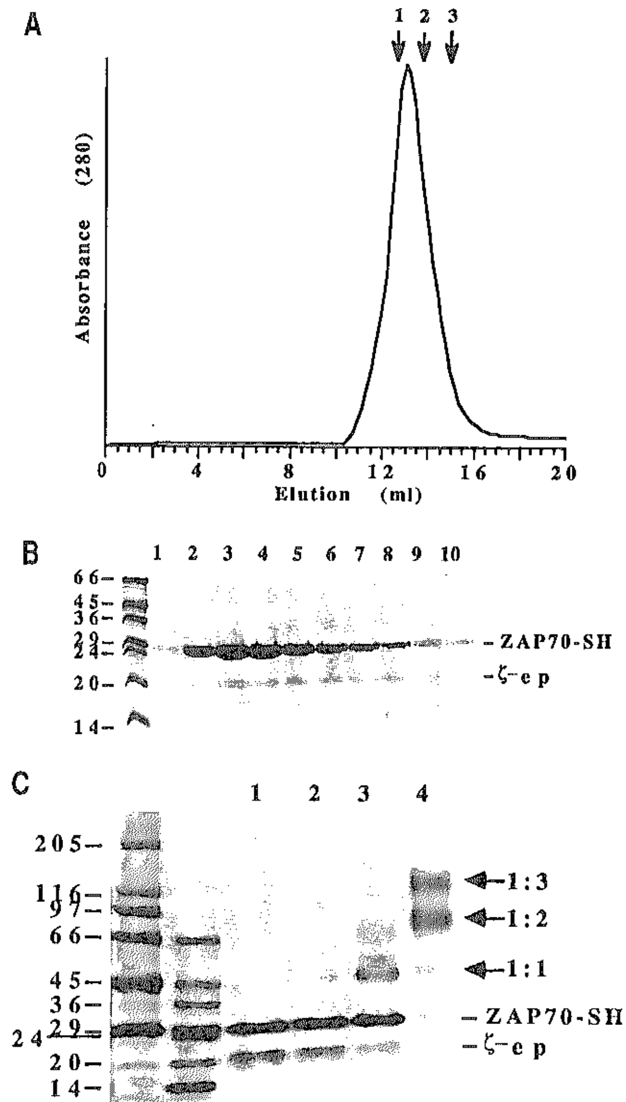

Fig. 4. Analysis of the ζ/ZAP70-SH complex. (A) Gel filtration elution pattern of the *ζ-eP*/ZAP70-SH complex. The numbers on top correspond to the elution position of molecular mass marker proteins: (1) aldolase, 155 kDa; (2) BSA, 66 kDa; (3) ovalbumin, 45 kDa. (B) SDS/PAGE (15%) analysis of eluted fractions. Fractions 1–10 correspond to the elution volume of 11.5–16 ml, each representing a 0.5-ml fraction. A sample of each fraction was separated on a 15% SDS/PAGE. Bands were detected by staining with Coomassie brilliant blue. The sizes of molecular mass standards are shown in kDa. (C) Chemical crosslinking of the *ζ-eP*/ZAP70-SH complex. Lane 1, no crosslinking reagent; lanes 2–4, 0.1 mM, 0.5 mM and 5 mM reagent, respectively. Crosslinked products were separated on a 6–15% gradient SDS/PAGE. Bands representing 1:1, 1:2 and 1:3 complexes are indicated with arrows. Crosslinking of unphosporylated *ζ-e* and ZAP-SH did not produce any higher-molecular-mass bands (data not shown). Bands were detected by staining with Coomassie brilliant blue. The sizes of molecular mass standards are shown in kDa.

Increasing concentrations of the crosslinking reagent (lanes 3 and 4, Fig. 4C) resulted in decreasing yield of the lower-molecular-mass bands and increasing yield of the higher-molecular-mass bands, as expected if partial crosslinking of the complex is occurring [26]. The presence of three new bands argues directly that the stochiometry of the complexes reaches 3:1, with bands representing either 1, 2 or 3 ZAP-SH crosslinked to phosphorylated ζ. The apparent molecular mass of the crosslinked bands, 50 kDa (Fig. 4C, lanes 2 and 3), 85 kDa (Fig. 4C, lane 4) and 120 kDa (Fig. 4C, lane 4), are also consistent with this interpretation. A similar result was obtained with phosphorylated *ζ-e*Cys. The ZAP-SH protein had to be maintained in 5 mM dithiothreitol to prevent disulfide-linked aggregation and precipitation. This dithiothreitol concentration reduced the disulfide bond in *ζ-e*Cys-*P* rendering it monomeric, thus resulting in the same elution profile of the complex with ZAP-SH (data not shown) that we obtained for monomeric *ζ-eP* in complex with ZAP-SH.

DISCUSSION

We have expressed the cytoplasmic segment of the T-cell receptor ζ-chain at high levels in *E. coli* and shown that, although ζ is a dimer in T cells, the cytoplasmic domain alone is not a dimer. The transmembrane region, not present in our construct, is likely to function as a dimerization module [27]. Our observations do not, however, exclude a very weak interaction within the intracellular part of ζ. Placing a Cys on the N-terminus of the cytoplasmic domain promotes dimerization by disulfide bond formation, as shown by chemical crosslinking and dynamic light scattering.

The results of CD measurements suggest an unfolded, non-globular, conformation for the cytoplasmic domain of ζ *in vitro*. They show that there are no significant amounts of α-helical or β-sheet structures. The absence of measurable thermodenaturation also indicates that the molecule lacks a stable ordered structure. Dimerization via an N-terminal Cys does not change the conformation as detected by CD. The higher apparent molecular masses for both ζ constructs, deduced from the diffusion coefficient in the dynamic light scattering experiments, may also be attributed to the non-globular shape of ζ in solution.

PTK ZAP70 associates only with phosphorylated ζ molecules of the T-cell receptor *in vivo* [17, 18]. Transfection studies in Cos cells show that expression of a Src family tyrosine kinase, such as Fyn or Lck, is required for ζ/ZAP70 association. Phosphorylation of ζ and/or ZAP70 thus appears to be a prerequisite for association. Our mass spectrometry data show that ζ can be completely phosphorylated *in vitro* by a recombinant PTK, Lck. The phosphorylation pattern as detected by SDS/PAGE suggests a random mechanism of Lck/ζ phosphorylation *in vitro* (Fig. 3). There seems to be no preferential order of phosphorylation. That is, we can detect no pauses in the ladder of species generated by recombinant Lck and no preferential intermediates. Once Lck starts to phosphorylate a ζ-chain *in vitro*, it continues until completion. It is unlikely that one of the six Tyr is more important for starting the phosphorylation sequence than the others, as mutagenesis of single Tyr shows phosphorylation *in vivo* [28]. Recent studies revealed differently phosphorylated forms of the ζ-chain *in vivo* dependent on the nature of the antigenic peptide [29, 30]. This suggests that the accessibility of single Tyr to protein-tyrosine kinases might differ in a given *in vivo* situation, possibly as a result of binding other proteins or factors not yet identified.

Our CD measurements also indicate that phosphorylation does not produce detectable conformational changes in the ζ-chain. This *in vitro* result suggests the possibility that enhanced binding to downstream effector molecules in the signaling pathway *in vivo* may be due solely to Tyr phosphorylation itself.

Phosphorylated Tyr residues in ζ bind to SH2 domains. A number of crystal structures [31–33] of SH2 domains in complex with phosphopeptides show that phosphopeptides are not part of a standard secondary structure when bound. The crystal structure of the ZAP-70 tandem-SH2 domains complexed to a phosphopeptide corresponding to the first ITAM of ζ shows a

mostly extended conformation for the ζ peptide, with nearly one α-helical turn [20]. Our *in vitro* data indicate that not only are the ITAMs themselves unstructured in the absence of bound SH2 domains, but that the inter-ITAM sequences are also not folded together into a compact core. Extrapolated to the *in vivo* state, this argues that the cytoplasmic domain of the unliganded ζ-chain may be a cytoplasmic string anchored in the membrane and that it may only adopt stable structures when bound to other proteins such as ZAP70 or the adaptor protein shc, implicated in coupling the TCR activation to the Ras signaling pathway [34].

Binding of multiple ZAP70 molecules to a single ζ-chain may be necessary to bring PTKs in close proximity to each other, in order to enhance kinase activity for full T-cell activation. Occupation of multiple SH2 binding sites by the same ligand could facilitate the auto- or trans-phosphorylation process and thus enhance the corresponding signals. It has been shown that ZAP-70 autophosphorylation is enhanced when it is bound to phosphorylated ζ, thus generating Tyr(*P*) with the capacity to bind SH2 domains of Fyn, Lck, GAP and Abl [35]. One possible model of a first step in T-cell activation might involve activation of Lck or Fyn following TCR recognition. This event could lead in turn to phosphorylation of ζ and CD3, and perhaps also to phosphorylation of ZAP-70 Tyr493 [36], either before or after binding to ζ. Our data provide evidence that multiple ZAP-70 molecules can bind to phosphorylated ζ. This association could in turn create the environment for effective auto- or trans-phosphorylation, enhanced by activation of ZAP-70 through Tyr(*P*)493 [36], and thereby generate binding sites for downstream molecules in this signal transduction pathway. How the ζ-chain or single ITAMs are used to create partial or complete activation signals is still unknown [7, 37–39].

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