Purification and Characterization of Human ZAP-70 Protein-tyrosine Kinase from a Baculovirus Expression System*

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Noah Isakov†‡, Ronald L. Wang‡, Julian D. Watts†, Ruedi Aebersold‡, and Lawrence E. Samelson‡

From the †Cell Biology and Metabolism Branch, NICHD, National Institutes of Health, Bethesda, Maryland, 20892, the ‡Department of Microbiology and Immunology, and the Cancer Research Center, Ben Gurion University of the Negev, Beer Sheva 84105, Israel, and the ¶Department of Molecular Biotechnology, University of Washington, Seattle, Washington 98195

The ZAP-70 protein tyrosine kinase is essential for T cell antigen receptor (TCR)-mediated signaling. The absence of ZAP-70 results in impaired differentiation of T cells and a lack of responsiveness to antigenic stimulation. In order to study the characteristics of ZAP-70 in vitro, we overexpressed an epitopically tagged human ZAP-70 in a recombinant baculovirus expression system and purified it by column chromatography. The kinase activity of purified, recombinant ZAP-70 required cation and purified it by column chromatography. The kinase activity of purified, recombinant ZAP-70 required cation and exhibited a strong preference for Mn2+ over Mg2+. The apparent K_m of ZAP-70 for ATP was ~3.0 μM. The activity of the recombinant ZAP-70, unlike that of the homologous protein tyrosine kinase, Syk, was not affected by binding of TCR-derived tyrosine phosphorylated immunoreceptor tyrosine-based activation motif peptides. Several proteins were tested as potential in vitro substrates of ZAP-70. Only α-tubulin and the cytoplasmic fragment of human erythrocyte band 3 (cfb3), which have a region of sequence identity at the phosphorylation site, proved to be good substrates, exhibiting K_m values of ~3.3 and ~2.5 μM, respectively ([ATP] = 50 μM). α- and β-casein were poor substrates for ZAP-70, and no activity toward enolase, myelin basic protein, calmodulin, histone proteins, or angiogenin could be detected. In contrast to the T cell protein tyrosine kinase, Lck, ZAP-70 did not phosphorylate the cytoplasmic portion of the TCRζ chain or short peptides corresponding to the CD3ε or the TCRζ immunoreceptor tyrosine-based activation motifs. Our studies suggest that ZAP-70 exhibits a high degree of substrate specificity.

Stimulation of the antigen-specific receptor on T cells (TCR)1 initiates a cascade of biochemical events leading to activation of the mature T cell. Among the earliest biochemical events that follows TCR triggering is the activation of protein-tyrosine kinases (PTKs) resulting in a transient tyrosine phosphorylation of multiple intracellular protein substrates (reviewed in Refs. 1 and 2) including the TCR/CD3 subunits (3–5). These multiple TCR subunits lack intrinsic PTK activity; instead, they interact with and activate cytoplasmic PTKs that couple the receptor to the signaling apparatus.

PTKs that have been implicated in TCR signaling include the Src family members, Fyn and Lck, and the TCRζ-associated-protein (ZAP-70) (reviewed in Refs. 1 and 2). Although Fyn is constitutively associated with nonpolymorphic components of TCR/CD3 subunits (6, 7), Lck is noncovalently associated with the cytoplasmic domain of the CD4 and CD8 co-receptor molecules (8, 9). Both Fyn and Lck can also directly associate with the inner leaflet of the plasma membrane via their N-terminal myristylation/palmitoylation signals (10), whereas ZAP-70, which lacks this sequence, is thought to reside in the cytosol in resting cells and then becomes rapidly recruited to the TCR upon its activation and phosphorylation (11, 12). Following interaction of the TCR with peptide-bound MHC molecules on the surface of antigen presenting cells, CD4 or CD8 interact with the MHC molecules, thus recruiting Lck into close proximity with the cytoplasmic tails of the TCR/CD3 subunits.

One function of the Lck and/or Fyn molecules that are activated directly or indirectly by TCR engagement is the tyrosine phosphorylation of specific regions within the TCR and CD3 subunits termed immunoreceptor tyrosine-based activation motifs (ITAMs). The ITAM, first noted by Reth (13), is found in three copies in the TCRζ chain and in one copy each in the CD3γ, δ, and ε chains (1, 13–17). The presence of ITAM cytoplasmic sequence in chimeric receptors has been shown to be sufficient to permit T cell activation following receptor cross-linking (14, 15, 22). ITAMs posses the general structure YXX(L/I)X(L/I)XX(L/I) and, when phosphorylated upon their tyrosine residues, serve as interaction sites for src-homology 2 (SH2) domains of various proteins. Association of the ZAP-70 kinase with tyrosine phosphorylated TCR subunits after T cell activation has been shown to be mediated by the tandem SH2 domains of this kinase binding to TCR ITAMs (3, 16, 18–21).

The role of ZAP-70 in the sequential PTK activation cascade has been studied in COS cells transiently transfected with TCR chimeras and PTKs (23). These experiments indicate that ZAP-70 association with the TCR/CD3 is dependent on signals that are provided by Lck and/or Fyn (23, 24), supporting the role of Lck and/or Fyn in early tyrosine phosphorylation of ITAMs within the TCR/CD3 subunits. ZAP-70 recruitment to the TCR via binding of the tandem SH2 domains of ZAP-70 to tyrosine phosphorylated ITAMs is a prerequisite for its activation and elicitation of subsequent biochemical events (25, 26), although the precise role of recruitment in activating ZAP-70 remains unclear. It is also yet unknown which of the proteins

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† To whom correspondence should be addressed. Tel.: 301-402-1400; Fax: 301-402-0078; E-mail: samelson@helix.nih.gov.

‡ The abbreviations used are: TCR, T cell antigen receptor; PTK, protein-tyrosine kinase; ITAM, immunoreceptor tyrosine-based activation motif; SH2, src-homology 2; mAb, monoclonal antibody; kb, kilobase pair(s); MES, 4-morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis (hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; Ab, antibody; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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that become tyrosine phosphorylated in response to TCR engag-ement are ZAP-70 substrates. Recent studies of a rare hu-man disease provide further support for the importance of ZAP-70 in T cell differentiation and activation. Mutations within the ZAP-70 gene that result in a lack of ZAP-70 protein give rise to a severe T cell immunodeficiency reflected by failure of the T cells to proliferate and become activated following antigen or mitogen stimulation (27–29). Thus, absence of ZAP-70 results in an inability of the TCR to couple to downstream signaling pathways. ZAP-70 appears to function in the very early phase of antigen-TCR-induced signal transduction, because agents that bypass the receptor, such as phorbol esters and ionomycin, induce normal proliferation of ZAP-70-deficient T cells. The fact that children who inherit ZAP-70 mutations lack CD8+ T cells also suggests an important role for ZAP-70 in differentiation/maturation and/or selection processes of T cell precursors within the thymus. Recently, the utilization of baculovirus expression vectors in insect cells proved to be an efficient method for high level expression of active enzymes. This system has been used to express a number of recombinant PTKs that are involved in T cell activation including Lck (30, 31) and Syk (32). We used this system for expression and purification of the human ZAP-70 PTK. Using the purified and highly active form of the recombinant ZAP-70, we carried out purification and characterization of this protein.

MATERIALS AND METHODS

Substrate Proteins and Antibodies—The 9E10 hybridoma producing anti-human Myc IgG, mAb (33) was obtained from the ATCC and ascites was prepared in Balb/c mice. Anti-phosphotyrosine mAb (4G10) was from Upstate Biotechnology Inc. Rabbit anti-ZAP-70 polyclonal antibody was a gift from Dr. H. R. Knoll (University of North Dakota). The peptide designated peptide-b contains amino acids 1–17 of a variant of the cytoplasmic portion of the TCR chain (TCRcyt, residues 52–164) has been described (31). Amino acid sequences of ITAM-containing synthetic peptides were derived from the human TCR or CD3e chains. Peptides that are either not phosphorylated or phosphorylated on both tyrosine residues within the ITAM motif were prepared by Michael Berne (Tufts University, Boston, MA) and were described previously (21). Additional nonphosphorylated ITAMcyt and ITAMe peptides were purchased from Peptide Technologies (Gaithersburg, MD). Poly(Glu:Tyr; 1:1), poly(Glu:Tyr; 4:1), angiotensin I, Val[5]angiotensin II, histone H1, dephosphorylated α-casein, β-casein, and myelin basic protein were from Sigma. Src kinase-specific substrate peptide (amino acids 6–20 of cdc2: KVEK1GE1GTGKVYK) was from Upstate Biotechnology Inc. The peptide containing the 30 C-terminal amino acid residues of α-tubulin (35) was a gift of H. R. Knoll (University of North Dakota). The cdb3 peptide designated peptide-a contains amino acids 2–16 of erythrocyte band 3 protein (6). Two additional lysine residues at the C terminus were added to permit the use of this peptide in the phospholipase A2 binding assay. The cdb3 peptide designated peptide-b contains amino acids 1–17 of a variant sequence of the erythrocyte band 3 protein (37) and was also a gift of H. R. Knoll.

Baculovirus Expression of Human ZAP-70—In order to express recombinant ZAP-70 in the baculovirus expression system, two fragments of ZAP-70 cDNA from two different plasmid constructions were ligated together in the baculovirus transfer vector pVL1393. This strategy generated a full-length insert of ZAP-70 with a C-terminal myc epitope tag, which lacked 5′ untranslated bases. The plasmid pGEX-3X-ZAP(SH2)2, has previously been described (18). A 504-base pair cDNA fragment encoding the N-terminal 162 amino acids of ZAP-70 was excised from pGEX-3X-ZAP(SH2)2, by digestion with BamHI and KpnI and purified by agarose gel electrophoresis and GeneClean II (Bio 101). The plasmid pBSK10.13myc contains a C-terminal epitopically tagged full-length cDNA of ZAP-70 in pl phi856c II SK (Stratagene) and was produced by using the polymerase chain reaction to add a 39-base pair oligonucleotide coding for a 13-amino acid human-c-myc epitope (MYC-SEELDLN) at the extreme 3′ end of the ZAP-70 coding sequence. A 1.4-kb cDNA fragment encoding the C-terminal 457 amino acids of ZAP-70 with a C-terminal myc epitopic tag was obtained by digestion of pBSK10.13myc with KpnI and SalI. The 1.4-kb fragment was subcloned into the superlinker plasmid pSL1180 (Pharmacia Biotech Inc.) and was used to transform the KpnI and SalI sites to facilitate the generation of a 3′ EcoRI site. The 1.4-kb fragment was subsequently recovered from pSL1180 by digestion with KpnI and EcoRI and purified by agarose gel electrophoresis and GeneClean II. The 504-base pair BamHI to KpnI N-ZAP-70 cDNA fragment and the 1.4-kb KpnI to EcoRI C-ZAP-70myc cDNA fragment were ligated together into BamHI/EcoRI digested pVL1393. DH5α E. coli transformed with the ligandation product, and transformed clones were screened for an 11.7-kb plasmid containing a 1.9-kb insert that could be excised by digestion with BamHI and EcoRI. One of the positive clones, designated pVL1393-ZAPmyc-6, was used to generate plasmid for baculovirus expression by Invitrogen (San Diego, CA). Baculovirus was used to infect Spodoptera frugiperda (SF9) or Trichoplusia ni (High Five) cells, and expression of the Myc-tagged ZAP-70 was tested by ZAP-70 immunoblotting of 9E10 immunoprecipitates from cell lysates.

For protein expression, High Five cells, which in preliminary screening exhibited higher expression levels of ZAP-70, were resuspended in viral stock at a density of 1–2 × 106 cells/ml and multiplicity of infection of 5 plaque-forming units/cell. Cells were grown for 48 h and harvested by nitrogen gasification, and cell ϕ300nm, and RNA were precipitated with ethanol in the presence of 0.5 M ammonium acetate and 100 μg/ml of polysine.

Purification of Recombinant ZAP-70 from High Five Cells—A cell pellet equivalent to 5 × 108 baculovirus infected High Five cells was resuspended in 4 ml of lysis buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1, % Triton X-100, 1 mM NaVO4, 5 mM EDTA, 10 μg/ml each leupeptin and aprotinin, and 25 mM p-nitrophenyl p'-guanidino benzoate) and incubated on ice for 30 min. Lysate was centrifuged at 4°C for 30 min at 10,000 g, and supernatant was immediately subjected to chromatography to minimize degradation and loss of activity. A Pharmacia fast protein liquid chromatography system was used in the purification procedures that were conducted at 4°C, and protein concentration was determined by Bradford assay (38).

Cell lysate was loaded on a Reactive Yellow-3 column (Sigma) pre-equilibrated with buffer A (150 mM NaCl, 25 mM Tris-HCl, pH 7.5). The column was washed with 100 ml of buffer B followed by protein elution with an 80-ml linear gradient from 0.15–1.5 × NaCl in 25 mM Tris-HCl, pH 7.5, at a flow rate of 0.5 ml/min and collection of 1-ml fractions into tubes containing 10 μl of a mixture of leupeptin (1 mg/ml), aprotinin (1 mg/ml), and 4-(2-aminoethyl)-benzenesulfonylfluoride (mM). The column was washed with the initial elution buffer containing the aprotinin peak of cdb3 was pooled and applied to a HiLoad 16/60 Superdex 200 column (Pharmacia) pre-equilibrated with buffer A, and proteins were eluted with buffer A at a flow rate of 1 ml/min. 1.5-ml fractions were collected into tubes containing 10 μl of a mixture of leupeptin (1 mg/ml), aprotinin (1 mg/ml), and 4-(2-aminoethyl)-benzenesulfonylfluoride (mM). Proteins in column fractions were visualized following electrophoresis on acrylamide gels by Coomassie Blue or silver staining, and their molecular weights were estimated by comparison with standard proteins of known weight.

In Vitro Kinase Assays for Tyrosine Kinase Activity—Routine assays of column fractions contained, in 20 μl of 25 mM Tris, pH 7.0, 1,50 mM NaCl, 50 μM ATP, 5 μCi of [γ-32P]ATP (at 4000 Ci/mmol−1, ICN), and varying amounts of enzyme. Reactions were carried out at 37°C for 6 min. The kinase reactions were terminated by the addition of 5 μl of 5 × SDS-sample buffer and heating for 5 min at 95°C.

The effect of pH on ZAP-70 activity was determined using 0.5 M stock buffers, MES-HCl (pH 5.0–6.5), Tris-HCl (pH 7.0–9.0), and CAPS-KOH (pH 9.5–11.0). Reaction mix in 20 μl contained: 50 mM of the appropriate buffer, 10 mM MnCl2, 50 μM ATP, 5 μCi of [γ-32P]ATP (at 4000 Ci/mmol−1, ICN), and varying amounts of enzyme. Reactions were carried out at 37°C for 6 min. The kinase reactions were terminated by the addition of 5 μl of 5 × SDS-sample buffer and heating for 5 min at 95°C.
For determination of the $K_m$ value for ATP, a serial dilution of ATP was made, and a kinase assay was carried out as before. $K_m$ and $V_{max}$ values for cfb3 as an exogenous substrate were determined by performing kinase assays on serial dilutions of the substrate proteins at a constant concentration of ATP (50 μM). Michaelis constants for ATP and for ZAP-70 substrates were determined using double-reciprocal plots.

Kinase dependence on cations and the effects of various potential inhibitors on ZAP-70 enzymatic activity were tested under similar assay conditions. In order to test ZAP-70 activity at low salt concentration, the purified ZAP-70 preparation was applied to a PD-10 Sephadex G-25M desalting column (Pharmacia) and eluted with 25 mM Tris, pH 7.5, plus protease inhibitors in the absence of salt. Phosphorylation of some of the short synthetic peptides was quantitated by the phosphocellulose paper binding method.

Electrophoresis and Immunoblotting—Samples of cell lysate or cell supernatants were resolved by electrophoresis on 10–12.5% acrylamide gels using Bio-Rad Mini-PROTEAN II Cell, and gels were either fixed and Coomassie-stained, followed by drying, or blotted onto nitrocellulose sheets (Schleicher & Schuell) at 15 V for 1 h in a Bio-Rad semi-dry transfer cell. Nitrocellulose sheets were incubated with anti-Myc (9E10) mAb or rabbit anti-ZAP-70 anti-serum, followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit Ig (Amersham Corp.), respectively. Immunoreactive proteins were visualized using ECL reagent (Amersham Corp.) and autoradiography. Radioactive gels or nitrocellulose sheets were developed by autoradiography, and the relative mass of protein bands were determined by comparison with prestained molecular weight markers (Amersham Corp.).

Gel analysis of phosphorylation of short peptides (2.3–12 kDa) was performed using Tris/Tricine SDS-PAGE followed by autoradiography. Tricine gels were prepared as described (39), using Tricine SDS-PAGE followed by autoradiography. Tricine gels were either fixed and Coomassie-stained, followed by drying, or blotted onto nitrocellulose sheets (Schleicher & Schuell) at 15 V for 1 h in a Bio-Rad semi-dry transfer cell. Nitrocellulose sheets were incubated with anti-Myc (9E10) mAb or rabbit anti-ZAP-70 anti-serum, followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit Ig (Amersham Corp.), respectively. Immunoreactive proteins were visualized using ECL reagent (Amersham Corp.) and autoradiography. Radioactive gels or nitrocellulose sheets were developed by autoradiography, and the relative mass of protein bands were determined by comparison with prestained molecular weight markers (Amersham Corp.).

Gel analysis of phosphorylation of short peptides (2.3–12 kDa) was performed using Tris/Tricine SDS-PAGE followed by autoradiography. Tricine gels were prepared as described (39), using gels of 16.5% total acrylamide monomer, with 6% bis-acrylamide for the separating gel and 4% total acrylamide monomer, with 3% bis-acrylamide for the stacking gel. The running buffers were 200 mM Tris, pH 8.8, for the anode and 100 mM Tris, 100 mM Tricine, and 0.1% SDS for the cathode. Prestained low molecular weight rainbow markers for the Tricine gels were from Amersham Corp.

Binding Assay—Preparation and purification of GST and GST-ZAP-70 fusion proteins was performed as described (18). Two micrograms of fusion proteins immobilized to glutathione-agarose beads (Sigma) at 2 μl of a 1:1 mixture of antimouse or anti-ZAP-70 antiserum immobilized to protein A-Sepharose beads (Pharmacia) and preincubated with purified recombinant ZAP-70 were incubated with 1 μl 32P-labeled (pTyr)2-ITAM$_a$ for 1 h at 37 °C on a rotator. The beads were then extensively washed, and bound radioactivity was determined by scintillation counting.

**RESULTS**

Purification of Recombinant Human ZAP-70—Recombinant C-terminal myc-tagged human ZAP-70 was overexpressed in High Five cells by infection with a ZAP-70-containing baculovirus, and purification was carried out as described under "Materials and Methods." The levels of expression of ZAP-70 in High Five cells varied from one experiment to another, and different proportions of ZAP-70 were found in large protein aggregates after each infection.

Initial preparations of High Five cell cytosol and particulate fractions showed the presence of substantial ZAP-70 autophosphorylation activity in both fractions (not shown); thus detergent-soluble cell extracts were prepared. Numerous initial purification procedures for ZAP-70 were tested, including affinity chromatography on various affinity dye resin columns, anti-Myc bound resin, or tyrosine phosphorylated TCR$_\gamma$ ITAM peptide-conjugated beads. Although most procedures resulted in a relatively low recovery of ZAP-70, Reactive Yellow-3 quantita
tively bound all of the ZAP-70 in the cell extract, and this ZAP-70 could be easily eluted with 1 M NaCl. This procedure was adopted in all further purification attempts. A sizing column such as the HiLoad 16/60 Superdex 200 was found to yield a good separation of the soluble ZAP-70 from contaminating proteins and aggregates giving rise to one peak of 71–72 kDa that reacted with anti-Myc Abs. In order to maximize the recovery of soluble ZAP-70, we attempted to solubilize ZAP-70-containing protein aggregates using various salt and pH conditions, sonication, or different detergents (including CHAPS, Nonidet P-40, Brij96, and TX-100). Under the different conditions tested we found small differences in recovery of soluble ZAP-70 and found that 0.15 M NaCl and 1% TX-100 gave the best yields. The purification protocol is described in Table I, which summarizes the yield and efficiency obtained at each purification step.

The proteins resulting from each purification step were analyzed by SDS-PAGE and visualized by Coomassie Blue staining (Fig. 1). Detection of ZAP-70 was performed by immunoblotting with an anti-myc tag mAb (9E10). Kinase activity for each chromatography step was determined by an in vitro kinase assay without (autophosphorylation) (Fig. 1) or with the exogenous ZAP-70 substrate, cfb3 (not shown). As a control, in some experiments the anti-Myc blotted nitrocellulose filters were stripped and rebotted with anti-ZAP-70 Abs and gave a similar pattern of stained bands.

The final purification step of ZAP-70 yielded only one major protein band of 71–72 kDa that reacted in immunoblot with anti-Myc or anti-ZAP-70 Abs and underwent autophosphorylation in an in vitro kinase assay. The retarded electrophoretic mobility of the recombinant ZAP-70 compared with native ZAP-70 is due to the additional 13 amino acids of the epitopic tag.

**Table I**

| Step       | Protein | Total activity | Specific activity | Purification | Yield |
|------------|---------|----------------|-------------------|--------------|-------|
| Extract    | 231     | 6,341          | 274               | 1            | 100   |
| Reactive Yellow-3 | 1.85   | 5,263          | 2,844             | 10.4         | 83    |
| Superdex 200 | 0.14    | 1,078          | 7,700             | 28.1         | 17    |

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α-chain was found to be a good ZAP-70 substrate and underwent phosphorylation with an apparent $K_m$ of $-3.3 \mu M$ and a $V_{max}$ of $-4.0 nmoI/min \cdot mg^{-1}$ (Fig. 3B).

To investigate substrate specificity of the recombinant ZAP-70 and compare it with that of recombinant Lck, we incubated the two enzymes with various substrate peptides (0.5–5 μg of protein/assay) in 20 μl of kinase buffer containing [γ-32P]ATP. We found that under conditions where ZAP-70 efficiently phosphorylated cfb3 and tubulin, it exhibited only a low level of phosphorylation of α- and β-casein (Fig. 4). In contrast, no phosphorylation by ZAP-70 of myelin basic protein, enolase, calmodulin, or histone proteins was observed (Fig. 4), even at high protein concentrations (such as 500 μg/ml) and longer exposure times of the autoradiograms (not shown). Lck was less efficient then ZAP-70 in phosphorylation of cfb3 and tubulin but in contrast to ZAP-70 showed a high level of phosphorylation of myelin basic protein and histone proteins. Perhaps the most striking and physiologically meaningful difference between Lck and ZAP-70 was observed when testing as a substrate a synthetic peptide corresponding to the cytoplasmic domain (residues 52–164) of the TCRζ chain (TCRζcyt) that includes the 6 tyrosine residues within the ITAM motifs. This peptide was a poor substrate for ZAP-70 despite being the most efficient substrate for Lck (Fig. 4 and Ref. 31). Under the assay condition used, ZAP-70 did not phosphorylate the src-specific substrate peptide, cdc2–20 angiotensin I, or [Val8]angiotensin II (not shown). A synthetic peptide consisting of the sequence poly(Glu:Tyr; 4:1) was a good substrate for ZAP-70, whereas poly(Glu:Tyr; 1:1) was a poor substrate (not shown).

TCC ITAM Motif-containing Peptides Are Substrates for Lck but Not for ZAP-70—To further explore the differences between the ZAP-70 and Lck kinase activities, we examined the ability of these kinases to phosphorylate either a synthetic polypeptide composed of the entire cytoplasmic domain of the TCRζ chain or peptides derived from the individual ζ-chain ITAMs. Only Lck phosphorylated the TCRζcyt and the ITAM-containing peptides (Fig. 4, far right lane of both panels and Fig. 5A). That the recombinant ZAP-70 was enzymatically active in this experiment is demonstrated by its ability to autophosphorylate and to phosphorylate cfb3.

To address the possibility that the inability of recombinant, insect cell-derived ZAP-70 to phosphorylate TCRζ chain was the result of inappropriate post-translational modification in the High Five cells, we also immunoprecipitated ZAP-70 from resting or TCR-activated Jurkat T cells. We tested the activity of T cell-derived ZAP-70 or Lck for comparison on TCRζcyt. We found that Jurkat cell activation by anti-TCR Abs did not significantly change the Lck autophosphorylation levels or TCRζcyt phosphorylation (Fig. 5B). In contrast, levels of autophosphorylation of ZAP-70 from activated Jurkat T cells were increased by 2–4-fold over the level seen in ZAP-70 isolated from nonstimulated cells (Fig. 5B, lower panel). Furthermore, Lck from resting or anti-TCR-stimulated Jurkat cell exhibited similar levels of phosphorylation of the CD3ζ and each of the TCRζ ITAM peptides, whereas none were phosphorylated by ZAP-70 from resting cells. The low levels of phosphorylation of ITAM peptides by ZAP-70 immunoprecipitates from activated Jurkat cells likely reflect the activity of kinases that co-immunoprecipitate with ZAP-70, such as Lck, which can associate with ZAP-70 in activated T cells (42). This conclusion is consistent with the observation that recombinant ZAP-70 was unable to phosphorylate ITAM peptides (Fig. 5A). The low levels of phosphorylation of ITAM peptides by ZAP-70 immunoprecipitates were not due to some general reduction in the catalytic activity toward exogenous substrates because the same immunoprecipitates exhibited high levels of phosphorylation of the cfb3 substrate (Fig. 5B, lower panel, last two lanes).

Shared Sequence of Substrates—Examination of the amino acid sequences of erythrocyte band 3 protein and α-tubulin revealed a short stretch of sequence (LXXDYEXΨ), where Ψ is a hydrophobic residue) that is shared by these two proteins. In
both proteins this sequence lies within a region known to be susceptible to tyrosine phosphorylation (36). Peptides based on these sequences were tested for their ability to serve as ZAP-70 substrates. The sequence of the peptides used are shown in Fig. 6A and have been aligned to highlight the shared residues. Cfb3 peptide a and b are based on variant sequences of the band 3 protein, which differ in having either aspartate or methionine at position 11. In a kinase reaction with 2 μg of the indicated peptides or 1 μg of cfb3 protein ZAP-70 phosphorylated the cfb3 protein, the α-tubulin peptide, and cfb3 peptide-a but not cfb3 peptide-b (Fig. 6B). Interestingly, although the cfb3 protein is a good substrate for Lck, cfb3 peptide-a and cfb3 peptide-b are not (not shown).

Engagement of the ZAP-70 Tandem SH2 Domains with Tyrosine Phosphorylated ITAM Peptides Does Not Modify ZAP-70 Catalytic Activity—The mechanism of activation of ZAP-70 is not completely resolved. Recent studies have shown that Syk binding to tyrosine phosphorylated FcγRI γ-chain ITAM peptide (43) or tyrosine phosphorylated peptides derived from the Igα and Igβ ITAMs (44) resulted in over 10-fold stimulation of the specific activity of Syk. Studies with immunoprecipitated ZAP-70 have shown that co-incubation with tyrosine phosphorylated ITAM motif-containing peptides does not affect ZAP-70 catalytic activity (45). To eliminate the possibility that the anti-ZAP-70 Abs or additional molecules in the immunoprecipitate used in previous studies may have interacted with ZAP-70 and/or the peptide and interfered with the activation process, we tested the activity of purified ZAP-70 before and after preincubation with nonphosphorylated or tyrosine phosphorylated ITAM peptides. We have previously shown that these ITAM peptides do bind to a GST fusion protein containing the tandem SH2 domain of ZAP-70 (18). The ability of a 32P-labeled (pTyr)2-ITAMz3 peptide to bind to the purified recombinant ZAP-70 was demonstrated by its ability to coprecipitate with recombinant ZAP-70 in anti-ZAP-70 or anti-myc immunoprecipitations (Fig. 7A). The other ITAMs would be expected to bind to ZAP-70 with equal or higher affinity (21). ZAP-70 autophosphorylation or phosphorylation of cfb3 were found not to be affected by the binding of tyrosine phosphorylated ITAM peptides (Fig. 7B).

To exclude the possibility that contaminating protein-tyrosine phosphatases may have influenced interpretation of the data, experiments were repeated with a nonhydrolysable synthetic ITAMz3 peptide analog in which phosphotyrosine residues were replaced by difluoro-phosphonomethyl phenylalanine. This peptide, termed F2(Pmp)2ITAMz3, was previously shown to bind ZAP-70 SH2 domains and function as a competitive inhibitor of the binding of ZAP-70 to the TCR (41). Preincubation of ZAP-70 with F2(Pmp)2ITAMz3 was found not to affect ZAP-70 autophosphorylation activity or ZAP-70 phosphorylation of the exogenous substrate, cfb3 (not shown).
DISCUSSION

Tyrosine phosphorylation has been shown to be a critical and obligatory mechanism in the TCR-triggered process leading to activation of T cells. At least three distinct PTKs (Lck, Fyn, and ZAP-70) are known to be involved in the initial steps that are regulated by protein tyrosine phosphorylation. ZAP-70 appears to be essential for both differentiation and maturation of pre-T cells and activation of mature T lymphocytes. Humans lacking ZAP-70 were found to have no CD8\(^+\) T cells in their thymus, and their mature peripheral blood T cells were nonresponsive to antigenic stimulation (27–29). Mice genetically engineered to be deficient in ZAP-70 are even more impaired. These mice lack both CD4\(^+\) and CD8\(^+\) positive T cells (46).

Despite the crucial role of this enzyme in T cell signaling, little is known about its downstream effector target proteins, and only very recent studies have begun to shed some light on its potential regulation during the activation process of T cells. ZAP-70 is known to undergo tyrosine phosphorylation following engagement of the TCR (11, 24), and mapping of the major in vivo phosphorylation sites have indicated that they include tyrosine residues 292, 492, and 493 (47). Further analysis of the importance of the individual tyrosine residues was performed using site-directed mutagenesis approaches and evaluation of the activity of the mutated ZAP-70 upon receptor engagement (48, 49). Additional studies suggested that tyrosine phosphorylation of ZAP-70 may function to serve as a docking site for SH2-containing molecules and that through this recruiting mechanism ZAP-70 controls the activation process (42).

In order to study the regulation of ZAP-70 in more detail, we devised a method for the purification of ZAP-70 from insect cells infected with a recombinant baculovirus that causes high level expression of ZAP-70. The purified recombinant ZAP-70 was enzymatically active, as evidenced by its ability to auto-phosphorylate and to phosphorylate a cytosolic fragment of erythrocyte band 3 protein, which we have previously shown is

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**Fig. 4.** Determination of ZAP-70 substrate specificity. Recombinant ZAP-70 and Lck for comparison were incubated in a kinase reaction buffer in the presence of cfb3 (0.5 \(\mu\)g), tubulin (0.5 \(\mu\)g), \(\alpha\)-casein (5 \(\mu\)g), \(\beta\)-casein (5 \(\mu\)g), endase (5 \(\mu\)g), myelin basic protein (MBP, 2 \(\mu\)g), calmodulin (2 \(\mu\)g), histone VI-S (5 \(\mu\)g), histone II-S (5 \(\mu\)g), histone VII-S (5 \(\mu\)g), or TCR\(_{cyt}\) (1 \(\mu\)g). Radioactive substrates were then resolved by electrophoresis on 12.5% gel followed by gel drying and autoradiography.

**Fig. 5.** TCR ITAM motifs are preferred substrates for Lck but not ZAP-70. A, recombinant ZAP-70 and Lck were incubated in a kinase reaction buffer in the presence of TCR\(_{cyt}\) (2 \(\mu\)g), ITAM\(_{\zeta1}\), ITAM\(_{\zeta2}\), ITAM\(_{\zeta3}\), or ITAM\(_{\epsilon}\) peptides (5 \(\mu\)g each) or cfb3 (0.5 \(\mu\)g). Radioactive peptides were then resolved on 16.5% Tris/Tricine gels followed by autoradiography. B, Jurkat T cells were either left unstimulated or stimulated with C305 mAb (anti-TCR\(_{\beta}\) chain) for 2 min at 37°C. Cells were lysed, and kinases were precipitated with anti-Lck (upper panel) or anti-ZAP-70 (lower panel) Abs. Immunoprecipitates were incubated in a kinase reaction buffer in the absence or the presence of substrates as detailed in A. Samples were then electrophoresed on 16.5% Tris/Tricine gels followed by autoradiography. ZAP-70 is the upper band seen in the lower panel.
a good exogenous substrate for ZAP-70 immunoprecipitated from activated T cells (41). The ZAP-70 purified from the insect cells was tyrosine phosphorylated, possibly due to the action of endogenous Src family PTKs, and could be detected in anti-phosphotyrosine Western blots (not shown). This degree of tyrosine phosphorylation was required for ZAP-70 activity, because removal of these phosphates by pretreatment with alkaline phosphatase inactivated the kinase (not shown). This observation is consistent with the model that ZAP-70 requires phosphorylation on certain tyrosine residues within its kinase domain for full kinase activity (23, 18–50).

The kinetic parameters of our purified recombinant ZAP-70 were determined using cb3 as a substrate. Under conditions giving linear kinase activity with respect to time, substrate concentration, and enzyme concentration, ZAP-70 was found to have a $K_m$ of $-3.0 \mu M$ for ATP. This value is in good agreement with the $K_m$ for ATP reported for other PTKs such as p60src ($-5.4(52)$, and $-8.0 \mu M$ (53)) and Csk ($-8(54)$ and $-11.5 \mu M$ (55)). The $K_m$ and $V_{max}$ values for cb3 phosphorylation by ZAP-70 were $-2.5 \mu M$ and $-7.4 nmol min^{-1} mg^{-1}$, respectively, which are consistent with what has been previously observed for the closely related Syk PTK (32, 40).

Unlike the Src family of PTKs, as represented in this study by Lck, ZAP-70 exhibited a remarkable selectivity with regards to which proteins can serve as substrates for its kinase activity. Of the numerous potential substrates tested only cb3, a-tubulin, and Poly(Glu:Tyr; 1:1) proved to be good substrates for ZAP-70. Poly(Glu:Tyr; 1:1) and $\alpha$- and $\beta$-casein were poorly phosphorylated, whereas myelin basic protein, enolase, calmodulin, angiotensin II, [Val5]-angiotensin II, cdc2p2-20, and various histone proteins did not undergo detectable phosphorylation by ZAP-70.

It is known that TCR and CD3 chains undergo tyrosine phosphorylation in activated T cells and that Lck or Fyn are the most likely enzymes to phosphorylate these sites. However, TCR has six potential tyrosine phosphorylation sites, and it is not clear whether under activation conditions some of these sites are targets for phosphorylation by ZAP-70. We found that the recombinant ZAP-70 did not phosphorylate a synthetic peptide that corresponds to the entire TCR cytoplasmic region (TCRcyt). This is despite the fact that ZAP-70 efficiently phosphorylated the cb3 under the same assay conditions. Furthermore, under similar assay conditions the TCRcyt peptide was found to be a very efficient substrate for a recombinant Lck. In addition, Lck but not ZAP-70 could in vitro phosphorylate short synthetic peptides corresponding in sequence to each of the CD3$+$ and the first, second, and third TCR$+$ ITAMs. Because of the possibility that the baculovirus-derived ZAP-70 may have undergone inappropriate post-translational modification by viral- or High Five-derived enzymes that are not found in human T cells, which could interfere with its activity toward ITAMs, we also immunoprecipitated ZAP-70 and Lck for comparison from resting and activated Jurkat T cells and tested their ability to phosphorylate TCRcyt and individual ITAM peptides. The results indicated that resting cell-derived immunoprecipitated ZAP-70 phosphorylated cb3 but not ITAM peptides. Very low levels of phosphorylation of ITAM peptides were observed with ZAP-70 immunoprecipitates from activated T cells. However, these are likely to reflect the activity of Lck or other ZAP-70 co-immunoprecipitating PTKs (42). All ITAM peptides tested were phosphorylated by Lck immunoprecipitates from resting or activated Jurkat cells supporting the assumption that tyrosine residues within the TCR ITAMs are in vivo substrates for Lck but not ZAP-70.

A recent study by Songyang et al. (56) has shown that cytosolic tyrosine kinases, such as Lck and Src, preferentially phosphorylate peptides that are recognized by their own SH2 domains. Our studies and others (3, 16, 18–21) have indicated that the ZAP-70 tandem SH2 domains exhibit high affinity of binding to tyrosine phosphorylated ITAM sequences, despite
its inability to phosphorylate them. In order to address whether ZAP-70, via its SH2 domains, can bind a protein with which it interacts via its catalytic domain, we used its preferred substrate, cb3. We found that the ZAP-70-phosphorylated cb3 does not co-immunoprecipitate with ZAP-70, despite the fact that tyrosine-phosphorylated ITAM peptides co-immunoprecipitate with ZAP-70 under similar conditions (not shown).

The mechanism of activation of ZAP-70 is not completely resolved. Recent studies with site-directed mutagenized ZAP-70 (48, 49) have shown that phosphorylation of ZAP-70 by Lck on tyrosine 493 greatly increases ZAP-70 catalytic activity. Another proposed model for ZAP-70 activation is based on studies with Syk, a ZAP-70 homologous PTK with an overall structure similar to that found in the IgDYEEEDP, where $\gamma$ is a hydrophobic residue, is the actual site of phosphorylation in the $\alpha$-tubulin peptide. Interestingly, cb3 peptide-b, which is based on a variant sequence of cb3 peptide, demonstrates amino acids of tubulin was also found to be a substrate for ZAP-70. Alignment of these two peptides based on the site of tyrosine phosphorylation in the cb3 peptide demonstrates that the sequence LXXDYEXP$, where $\Psi$ is a hydrophobic residue, is shared. It should be noted, however, that we have not ruled out the possibility that the C-terminal tyrosine is the

2 J. D. Watts and R. Aebersold, manuscript in preparation.
Purification and Characterization of Human ZAP-70 Protein-tyrosine Kinase from a Baculovirus Expression System
Noah Isakov, Ronald L. Wange, Julian D. Watts, Ruedi Aebersold and Lawrence E. Samelson

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