

Identification and characterization of a substrate specific for the T cell protein tyrosine kinase ZAP-70

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Abstract ZAP-70 is a protein tyrosine kinase (PTK) that plays a critical role in T cell activation. To study the role of ZAP-70 catalytic activity in this process, a substrate capable of distinguishing between the activities of ZAP-70 and other PTKs would be useful, especially since it has recently been shown that ZAP-70 interacts with another T cell PTK, Lck. We have thus identified a site of phosphorylation on the cytoplasmic fragment of the erythrocyte band 3 protein that is recognized by ZAP-70, but not Lck. A synthetic peptide based on this site has been demonstrated to be a good *in vitro* substrate for ZAP-70 and a poor substrate for the T cell PTKs Lck and Itk. This peptide molecule should thus prove useful to many investigators working in the field of T cell activation.

Key words: Tyrosine kinase; T cell; Band 3 protein; ZAP-70; Lck; Itk

1. Introduction

ZAP-70 is a T cell specific protein tyrosine kinase (PTK) known to play a critical role in the activation of T cells via their antigen receptor (TCR). Upon TCR engagement, a number of PTKs become activated, and a wide range of cellular substrates, including some of the TCR subunits, become tyrosine phosphorylated (for recent reviews of TCR structure, function and its associated PTKs, see [1–3]). Notable among these is the TCR ζ subunit, which contains 6 tyrosines in its cytoplasmic domain. All six of these can potentially become phosphorylated [4,5]. Collectively, these form three copies of the so-called immunoreceptor tyrosine-based activation motif (ITAM), which has the sequence Y-X-X-I/L-(X_{6–8})-Y-X-X-I/L (for recent reviews on ITAM-mediated signaling in cells of the immune system, see [1,6,7]). ZAP-70 subsequently binds specifically to the doubly phosphorylated forms of the TCR ζ and CD3 ϵ ITAMs [8,9], events which require both of its phos-

photyrosine-binding SH2 domains [10–12]. ZAP-70 subsequently becomes tyrosine phosphorylated on Y492 and Y493 [13]. These events require another T cell PTK, possibly Lck (a member of the Src PTK family), with phosphorylation of ZAP-70 on Y493 being essential its catalytic activation [14,15]. Should any of these events be blocked, or should a loss of function mutation or deletion occur in either the ZAP-70 or TCR ζ genes, T cell function is abrogated significantly [16–24]. Such gene mutations/deletions are known to cause impairment of thymic selection of T cells [16–20] as well as severe combined immune deficiency disorders [21–23] in both mice and humans. The elucidation of the roles played by ZAP-70 and TCR ζ in TCR signaling is thus an essential step towards the understanding of such diseases.

Besides ZAP-70 and Lck, two other T cell PTKs are activated via TCR or co-receptor stimulation, namely Fyn (another Src-family PTK) and Itk (reviewed in [1–3]). The analysis of changes in the catalytic activities of these enzymes is thus an important step in any investigation into the role of these molecules in this process. Typically, such analyses are performed by isolating the kinase via immunoprecipitation from cell lysates prepared under different stimulation conditions, and then assaying its activity towards an exogenous (usually non-physiological) *in vitro* substrate. Unfortunately to date, only very few *in vitro* substrates for ZAP-70 have been identified, with none being exclusively phosphorylated by this kinase [25]. Interpretation of such data is thus often complicated by the co-precipitation of other kinases. ZAP-70 has been shown to associate with Lck in an activation-dependent manner [26] and is active towards a much narrower range of substrates than Lck [25]. Indeed, it has recently been shown that ZAP-70 can also associate with Fyn *in vivo* [27]. It thus seems clear that a substrate capable of distinguishing between the activities of ZAP-70 and the other prominent T cell PTKs would be useful.

In the few studies that have been performed on ZAP-70 activation in T cells, a preferred substrate has been an expressed form of the cytoplasmic fragment of the erythrocyte band 3 ion-transport protein (cfb3) [14,15,24,28], a molecule previously identified as a substrate for the ZAP-70-related PTK, Syk [29]. Using a selection of expressed PTKs, we have here determined that while both ZAP-70 and Lck can phosphorylate cfb3 *in vitro*, they do so at different sites. We went on to identify ZAP-70 and Lck specific sites of phosphorylation, both in the N-terminal region of cfb3. Based on this information, we synthesized a peptide substrate which we have shown to be well phosphorylated by ZAP-70, but not by expressed versions of the T cell PTKs, Lck and Itk. This peptide, which we have termed ZAPstrate, should thus prove useful in the determination of the role played by ZAP-70 in the process of T cell activation.

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Abbreviations: PTK, protein tyrosine kinase; TCR, T cell antigen receptor; ITAM, immunoreceptor tyrosine-based activation motif; cfb3, cytoplasmic fragment of the erythrocyte band 3 protein; MBP, myelin basic protein; Itk-KD-GST, Itk kinase domain GST fusion protein; TFA, trifluoroacetic acid; ESI-MS, electrospray ionization mass spectrometry; EGFR, epidermal growth factor receptor.

2. Materials and methods

2.1. Materials

cfb3 protein was expressed and purified as previously described [30]. Synthetic peptides containing the Lck autophosphorylation site (Y394) and the entire cytoplasmic domain (residues 52–164) of the murine TCR ζ subunit have already been described [31], and were provided by Dr. I. Clark Lewis (University of B.C., Vancouver, Canada). Peptides corresponding to residues 2–30 and 2–16 of cfb3 (EELQDDYEDMMEENLEQEYEDPDIPESQKK and EELQDYEDMMEENLKK, respectively), both with two additional C-terminal lysine residues, were synthesized by the peptide synthesis facility in the Dept. of Immunology, University of Washington. Myelin basic protein (MBP) was purchased from Sigma.

Monoclonal antibodies were purified from the culture supernatant of 9E10 hybridoma cells (ATCC) as described in [32], using protein-G Sepharose (Pharmacia) in place of protein-A. Baculovirally expressed wild-type Lck was partially purified from Sf9 cells as described [31], and High Five cells expressing myc-tagged ZAP-70 were produced as described [25]. A GST fusion protein of the Itk kinase domain (Itk-KD-GST) was expressed in Sf9 cells according to the manufacturer's instructions, utilizing the BaculoGold baculovirus expression system (Pharmingen), and purified on a glutathione agarose column (Pharmacia) according to standard procedures.

All chemicals and solvents were purchased from J.T. Baker, unless otherwise indicated. Reagents for peptide sequencing were purchased from Applied Biosystems. Chemicals and equipment for gel electrophoresis was obtained from BioRad. Autoradiography was performed using Kodak X-OMAT film and a Kodak X-OMAT 480 RA processor. Cerenkov and liquid scintillation counting was performed on a Beckman LS 6500.

2.2. *In vitro* phosphorylation of cfb3 protein and cfb3 (2–30) with ZAP-70 and Lck

Lysate from baculovirus-infected High Five cells expressing a myc-tagged recombinant human ZAP-70 was produced as previously described [13]. 50 μ l of a cytosolic extract was diluted to 500 μ l with 25 mM Tris pH 7.5, 10% glycerol, 2 mM EDTA, 0.1% Nonidet P-40, on ice. The ZAP-70 was precipitated by the addition of 10 μ g of 9E10 (which recognizes the C-terminal myc affinity tag) for 2 h at 4°C with constant mixing, followed by 30 μ l of a 50% slurry of protein-G Sepharose for 1 h at 4°C. Precipitates were washed 3 \times with 1 ml of cold 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100.

Phosphorylation was performed by adding 50 μ l kinase assay buffer (25 mM Tris pH 7.5, 10 mM MnCl₂, 0.1% Nonidet P-40, 1 mM DTT, 1 mM ATP (Sigma), with [γ -³²P]ATP (NEN DuPont) added to 1100 dpm/pmol if required) to 25 μ g of cfb3 (2–30) peptide and either the ZAP-70 precipitates or 5 μ l of partially purified baculovirally expressed Lck [31]. Kinase reactions were incubated for 1 h at 30°C and phosphopeptides repurified by HPLC. For phosphorylation of the full-length cfb3 protein, preparation of kinases and reaction conditions were as described above, except that DTT was absent from the kinase reaction, [γ -³²P]ATP was added to 440 dpm/pmol and 10 μ g of substrate (cfb3) was added.

2.3. Substrate comparisons for T cell PTKs ZAP-70, Lck and Itk

ZAP-70 precipitates were produced using 9E10 antibodies and protein-G Sepharose as outlined above, except that only 12 μ l of lysate was used per sample. Phosphorylation assay buffer was 50 mM Tris pH 7.5, 10 mM MnCl₂, 0.1% Triton X-100, 250 μ M ATP, with [γ -³²P]ATP added to 1760 dpm/pmol. Substrates were then added to aliquots of assay buffer and kept on ice until required. TCR ζ , cfb3, Lck Y394 and cfb3 (2–16) peptides were made to a final concentration of 0.8 mg/ml, MBP was made to 0.4 mg/ml.

For ZAP-70 kinase assays, 25 μ l of each substrate was added to a separate ZAP-70 precipitate (each substrate in triplicate) with the control reaction containing no substrate (i.e. kinase and ATP only). Reactions were performed at 30°C for 10 min with frequent mixing of the beads. 22 μ l of each reaction (supernatant) was transferred to squares of p81 phosphocellulose paper (Whatman), dried in air for 1 min, and then washed extensively in 1% orthophosphoric acid. For the other kinases, the substrate/assay buffer mixtures were split into 25 μ l aliquots (three samples per kinase). 2.5 μ l of partially purified wild-type Lck or Itk-KD-GST was added to each set of substrates.

Incubation was again at 30°C for 10 min, followed by transfer to p81 paper and washing with orthophosphoric acid. Radioactive incorporation was assessed by liquid scintillation counting of the p81 paper squares [31].

2.4. Two-dimensional phosphopeptide mapping

Phosphopeptide mapping of phosphorylated cfb3 was performed on electroblotted proteins essentially as described [13] with the following modifications: Enzymatic cleavage was carried out overnight at 37°C with 1 μ g Asp-N endoprotease (Boehringer Mannheim) per sample, in 1% ammonium bicarbonate pH 7.5, and electrophoresis in the first dimension was performed on a Hunter thin-layer peptide mapping system (C.B.S. Scientific Co.) using an EC-555 power pack (E-C Apparatus Corp.) at 750 V and 8°C for 55 min.

2.5. HPLC and MS analyses

Labeled peptide samples were acidified with 1/10th volume of 10% trifluoroacetic acid (TFA) (Applied Biosystems) and injected into a Waters 600E system equipped with a 490E detector. Separation was performed on a Vydac C₄ column (4.6 \times 250 mm) at a flow rate of 0.6 ml/min. UV absorbance was recorded at 214 nm. The column was washed isocratically with buffer A (0.1% TFA). A 5 min gradient from 0 to 25% buffer B (70% acetonitrile, 0.08% TFA) was performed, followed by the main separating gradient of 25 to 45% buffer B over 30 min. The column was washed in 100% buffer B and re-equilibrated in buffer A prior to subsequent analyses. Mass spectrometry (MS) was performed on a prototype Sciex electrospray ionization (ESI) single quadrupole mass spectrometer.

2.6. Radiosequencing

Radiosequencing via solid-phase Edman degradation was performed essentially as described [33]. Briefly, following covalent cou-

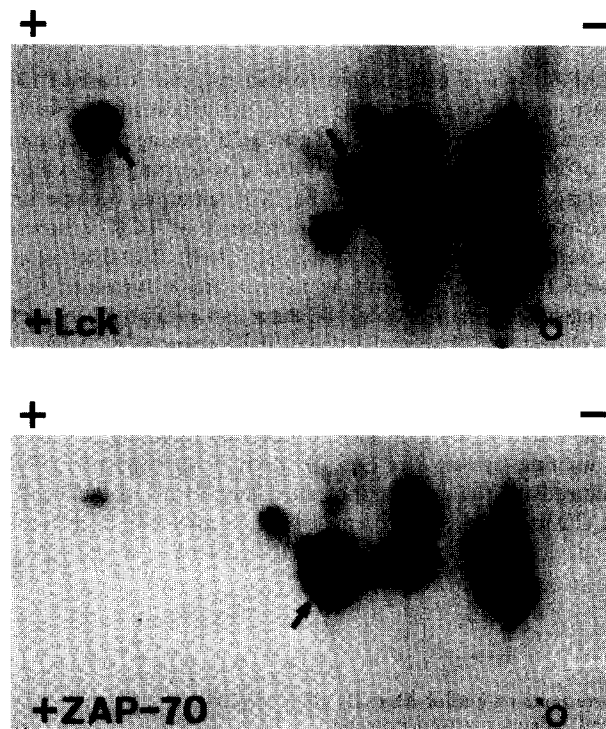


Fig. 1. 2D phosphopeptide mapping of cfb3 phosphorylated with Lck and ZAP-70. cfb3 protein was phosphorylated with either Lck or ZAP-70 (as indicated), as described under Section 2. Samples were subjected to SDS-PAGE, transferred to nitrocellulose and phospho-cfb3 identified via autoradiography. Following *in situ* cleavage with Asp-N, phosphorylated peptides were resolved on 2D phosphopeptide maps and visualized via autoradiography. The location of the sample origin (O) and the position of the positive (+) and negative (–) electrodes during electrophoresis are indicated.

pling to arylamine discs (Millipore) of peptide peaks recovered from the HPLC, the Edman degradation was performed on an Applied Biosystems 477A peptide sequencer, with eluate emerging from the sequencer following each cleavage cycle being diverted to a fraction collector, followed by analysis by liquid scintillation counting.

3. Results and discussion

3.1. Identification of a ZAP-70-specific phosphorylation site on *cfb3*

Previous observations had shown that while both ZAP-70 and Lck were capable of phosphorylating *cfb3*, it was a better substrate for ZAP-70 [25]. Thus ZAP-70 may be recognizing a site(s) on *cfb3* not recognized by Lck, and if so, its identification could form the basis of a ZAP-70-specific substrate. To investigate this possibility, *cfb3* was phosphorylated in the presence of [γ - 32 P]ATP with either recombinant ZAP-70 or Lck, and subjected to phosphopeptide mapping (Fig. 1). Since previous reports had shown that the ZAP-70-related PTK, Syk, was capable of phosphorylating *cfb3* at its N-terminus [29], and since *cfb3* has a very acidic N-terminus with no tryptic cleavage sites, we chose to perform digests with Asp-N. As can be seen in Fig. 1, the ZAP-70- and Lck-derived maps produced several spots which were either unique, or significantly more intense with one kinase over the other (arrowheads). In particular, ZAP-70 phosphorylation of *cfb3* produced one very intense spot that was only weakly apparent when using Lck.

We knew that a peptide derived from the C-terminus of α -tubulin was a good *in vitro* substrate for ZAP-70 [25]. The sequence around the phosphorylated tyrosine in this peptide (ALEKDYEEVGV) is similar to that surrounding the two N-terminal tyrosines at Y8 and Y21 of *cfb3* (ELQD-DYEDMME and LEQEEYEDPDI, respectively). Previous reports had also identified the N-terminus of *cfb3* as containing the primary phosphorylation site(s) of Syk [29]. We thus utilized a synthetic peptide corresponding to residues 2–30 of *cfb3* as a potential substrate for Lck and ZAP-70. The peptide was phosphorylated with either ZAP-70 or Lck, and phosphopeptides and unphosphorylated peptide species separated by HPLC.

As can be seen in Fig. 2A, ZAP-70 and Lck phosphorylation of *cfb3* (2–30) produced different HPLC profiles. Peak 1 had the same retention time as unphosphorylated peptide run alone (not shown), whereas peaks 2 and 3 were products of the kinase reaction. We thus subjected all three peaks to electrospray ionization mass spectrometry (ESI-MS) (Fig. 2B). Peak 1 yielded a major ion species at a mass to charge ratio (m/z) of 1284. This correlated with the expected value for the triply charged ($[M+3H]^{3+}$) ion of the non-phosphorylated peptide ($M_w=3849$). ESI-MS analyses of peaks 2 and 3 gave essentially the same result ($m/z=1311$ and 1310.5, respectively) which correlated to the expected $[M+3H]^{3+}$ value for a singly phosphorylated form of the *cfb3* (2–30) peptide ($M_w=3929$). Peaks labeled 1 and 3 had essentially identical ESI-MS spectra when generated by either kinase (not shown),

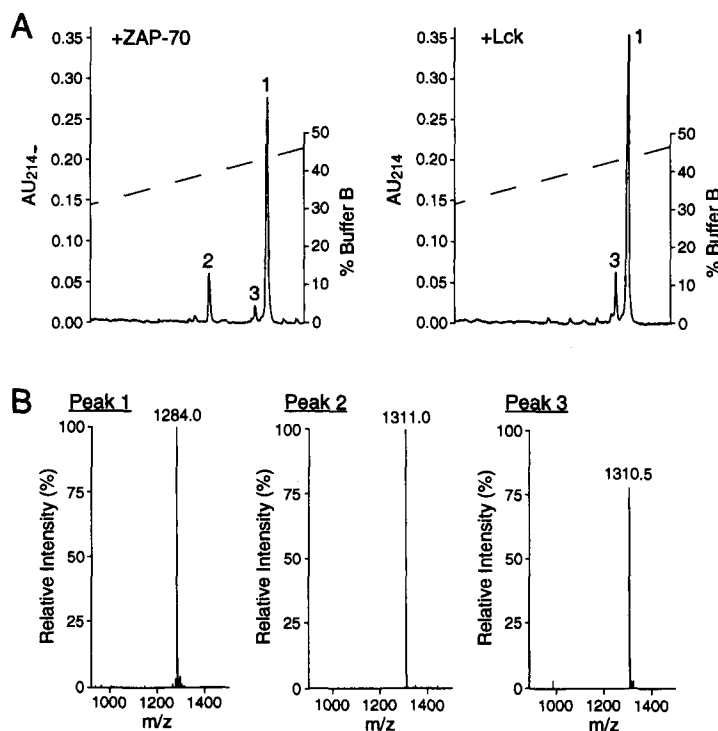


Fig. 2. HPLC and ESI-MS analyses of *cfb3* (2–30) peptide phosphorylated with Lck and ZAP-70. (A) *cfb3* (2–30) peptide was phosphorylated with either baculovirally expressed ZAP-70 or Lck and phosphopeptides were then separated from non-phosphorylated peptide via HPLC on a C4 column (4.6×250 mm) as described under Section 2. Absorbance traces (at 214 nm) for the region of the gradient where peptides eluted are shown, along with the acetonitrile buffer concentrations recorded at the pump heads. (B) ESI-MS data for the peptide peaks labeled 1–3 in (A) were recorded. Only triply charged ($[M+3H]^{3+}$) peptide species were observed. Only the region of the spectra is shown where *cfb3* (2–30) peptide peaks would be observed ($m/z=900$ –1500) and signal intensities are given relative to the largest observed signal present in the full spectrum.

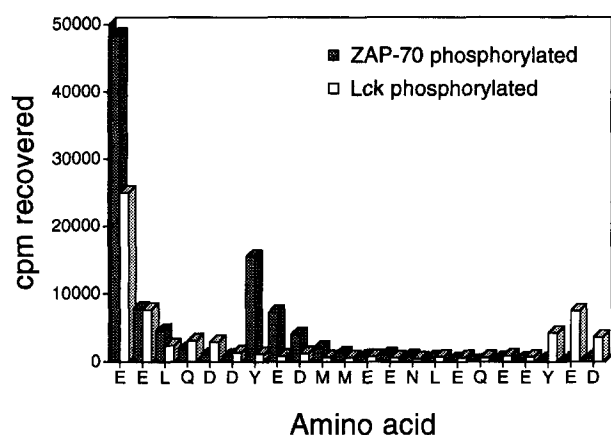


Fig. 3. Radiosequencing of cfb3 (2–30) peptide phosphorylated with Lck and ZAP-70. cfb3 (2–30) peptide was phosphorylated with either baculovirally expressed ZAP-70 or Lck and repurified by HPLC exactly as shown in Fig. 2, except for the addition of [γ - 32 P]ATP to the kinase reactions. The ZAP-70 and Lck-derived singly phosphorylated peptide species (Fig. 2, peaks 2 and 3, respectively) were covalently attached to arylamine membrane discs and subjected to radiosequencing via solid-phase Edman degradation as described [33]. Total eluate emerging from the sequencer following each cleavage cycle was diverted to a fraction collector and analyzed by liquid scintillation counting. The results are displayed graphically against the sequence of the cfb3 (2–30) peptide.

those shown in Fig. 2B being from the Lck phosphorylation reaction.

The results shown in Fig. 2 strongly suggested that ZAP-70 and Lck were selectively phosphorylating different tyrosines in cfb3 (2–30). To determine which kinase was phosphorylating which tyrosine, cfb3 (2–30) was again phosphorylated by the two PTKs, only additionally in the presence of [γ - 32 P]ATP. The singly phosphorylated peptides were again purified by

HPLC as shown in Fig. 2, and subjected to solid-phase Edman degradation. Sequencing reaction products resulting from each cycle were collected and analyzed by liquid scintillation counting (Fig. 3). The ZAP-70-phosphorylated peptide (Fig. 2, peak 2) showed a significant burst in extracted radioactive counts at cycle 7 (corresponding to Y8 of cfb3) with no counts above background extracted at cycle 20 (Y21 of cfb3). The Lck-phosphorylated peptide (Fig. 2, peak 3) yielded no radioactivity above background at cycle 7, giving an increase instead at cycle 20. Thus, it was clear that ZAP-70 can phosphorylate cfb3 at Y8, a residue not phosphorylated by Lck. These data are in agreement with previous observations that a principal site of phosphorylation on cfb3 *in vivo* is on Y8 [34,35] and is likely attributable to the ZAP-70-related Syk PTK, which is expressed in erythrocytes [29].

3.2. Evaluation of a cfb3-derived peptide as a ZAP-70-specific substrate

We already knew that the whole cfb3 protein could be phosphorylated by both ZAP-70 and Lck [25], though it was a relatively poor substrate for Lck. However, in light of recent observations that ZAP-70 can associate *in vivo* with Lck and the Lck-related PTK Fyn [26,27], cfb3 is not an ideal substrate for the evaluation of changes in the activity of ZAP-70 following T cell activation. Since such assays for ZAP-70 activity would normally involve immunoprecipitation followed by an *in vitro* kinase assay, the concurrent activation of both Lck and/or Fyn and their association with ZAP-70 could make such data misleading. Although not known to associate with either ZAP-70, Lck or Fyn, the Itk T cell PTK becomes activated [36] and might also be capable of interfering in such assays. The inability of Lck to phosphorylate Y8 of cfb3 might enable the construction of a peptide substrate specific for ZAP-70.

We thus used a synthetic peptide based around Y8 of cfb3,

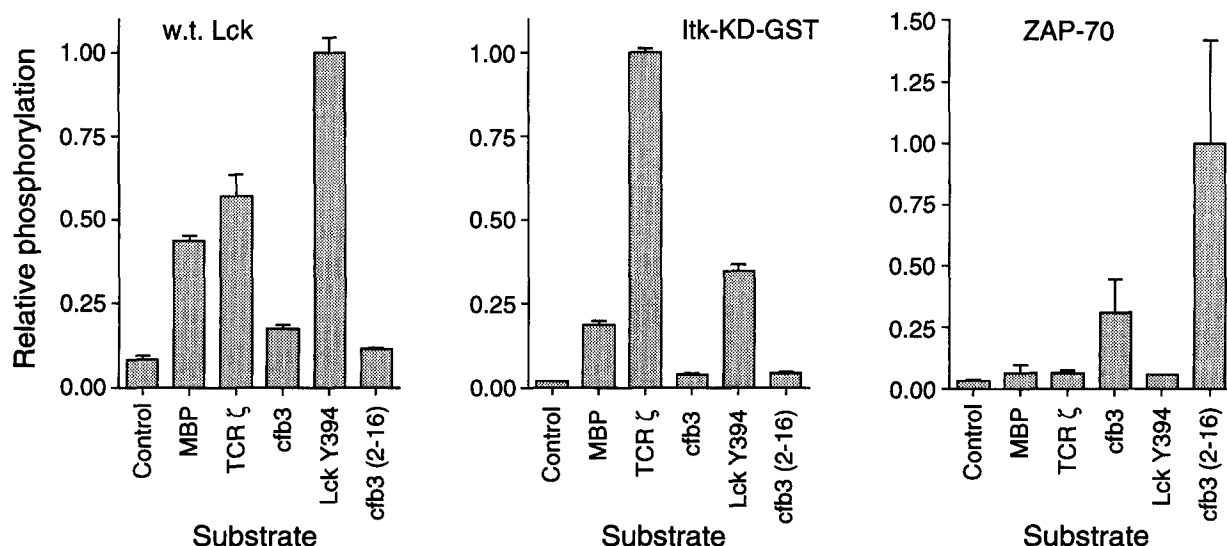


Fig. 4. Comparison of cfb3 (2–16) peptide with other PTK substrates using the T cell PTKs Lck, Itk and ZAP-70. MBP, TCR ζ , cfb3, Lck Y394 peptide and cfb3 (2–16) peptide were phosphorylated in the presence of [γ - 32 P]ATP with either wild-type Lck (w.t. Lck), a GST fusion protein of the Itk kinase domain (Itk-KD-GST), or ZAP-70 as indicated. Autophosphorylation controls (no added substrate) were also performed for each kinase. Phosphorylation was for 10 min at 30°C, reactions being stopped by spotting onto p81 paper and washing in 1% orthophosphoric acid. Radioactive incorporation into the substrate was determined via liquid scintillation counting and is represented relative to the largest peak for each kinase. Data shown are the mean of three measurements, with the standard deviation given as the error.

with two additional C-terminal lysine residues added (designated cfb3 (2–16)) to facilitate binding to the phosphocellulose paper frequently used in protein kinase assays. We tested a selection of known PTK substrates, namely MBP, TCR ζ (residues 52–164, containing all three ITAMs), cfb3, a peptide based on the Lck autophosphorylation site (Y394) and the cfb3 (2–16) peptide, as potential substrates for a selection of baculovirally expressed T cell PTK-derived molecules. We used the myc-tagged ZAP-70, wild-type Lck and a GST fusion protein containing the Itk kinase domain (Itk-KD-GST), which represent three of the major classes of non-receptor PTKs involved in T cell signaling. We screened the three kinases against each substrate, with an autophosphorylation control (no added substrate) for each (Fig. 4).

In agreement with our previous observations, MBP, TCR ζ and Lck Y394 were phosphorylated by Lck and Itk, but not ZAP-70 [25]. Also in agreement with our previous observations, cfb3 was phosphorylated relatively well by ZAP-70, weakly by Lck, and very poorly by Itk-KD-GST. However, the cfb3 (2–16) peptide was a good substrate for ZAP-70, and was barely phosphorylated at all by the other PTKs over respective controls. We observed increased variation between the individual data points for the ZAP-70 reactions, which can likely be attributed to additional experimental steps, required for the immunoprecipitation of the kinase, over the other molecules, which were used in purified form.

It is possible that the PTKs isolated from T cells will exhibit different substrate specificities to their expressed counterparts used here. However, the baculovirus system, in contrast to bacterial expression systems, has proven itself to be the superior system for the expression of active PTKs, and in the cases of ZAP-70 and Lck, the numerous studies to date on their catalytic activities have shown the *in vivo*-derived and expressed kinases to behave very similarly. It should also be noted here that while previous reports have shown that the epidermal growth factor receptor (EGFR) PTK will also phosphorylate cfb3 at Y8 [37,38], T cells do not express transmembrane PTKs of the EGFR family. Thus under typical ZAP-70 immunoprecipitation conditions, the co-precipitation of such receptor PTKs should not be a cause for concern in the use of the cfb3 (2–16) peptide as an assay for ZAP-70 activity in T cells.

With the identification of immunodeficiency disorders in both mice and humans correlated to mutations in the ZAP-70 gene or its promoter, the elucidation of ZAP-70 regulation and function is thus a critical step on the path to the determination of treatments for such disorders. Indeed, we have already shown that blocking normal ZAP-70 function with a peptide analog that disrupts its natural *in vivo* interaction with TCR ζ severely impairs TCR-mediated signaling [24]. The availability of the cfb3 (2–16) peptide, or ZAPstrate as we have named it, should thus be a useful tool in the determination of the role played by this important PTK in TCR-mediated signaling.

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