The EMBO Journal vol.14 no.11 pp.2499–2508, 1995

Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function

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ZAP-70 is a protein tyrosine kinase (PTK) required for T-cell development and T-cell antigen receptor (TCR) function. ZAP-70 is associated with the phosphorylated antigen receptor and undergoes tyrosine phosphorylation following receptor activation. We demonstrate here that tyrosine phosphorylation of ZAP-70 results in an increase in its catalytic activity and that this activation is mediated by the phosphorylation of tyrosine residue 493 by the src family of PTKs. The activity of baculoviral expressed ZAP-70 was up-regulated 10-fold when ZAP-70 was co-infected and phosphorylated by the src family PTK, lck. Mutation of Y493 alone abrogated the ability of ZAP-70 to be activated by lck. Moreover, we demonstrate that phosphorylation of Y493 and activation of ZAP-70 is required for antigen receptor-mediated induction of interleukin-2 (IL-2) secretion in lymphocytes.

Key words: protein tyrosine kinases/signal transduction/T-cell antigen receptor/ZAP-70

Introduction

Protein tyrosine kinases (PTKs) play an integral and obligate role in the activation of the T-cell antigen receptor (TCR; reviewed in Samelson and Klausner, 1992; Perlmutter *et al.*, 1993; Chan *et al.*, 1994a; Weiss and Littman, 1994). Two structurally distinct families of PTKs, the src and syk families, are required for TCR activation. While the src PTKs are membrane associated through co-translational myristylation as well as post-translational palmitylation, and contain both Src-homology 3 (SH3) and Src-homology 2 (SH2) domains, the syk PTKs (syk and ZAP-70) are characterized by the presence of two tandemly arranged SH2 domains and have no membrane localization motifs. Moreover, the two families of PTKs function in a sequential manner and cooperate to result in the induction of a variety of cellular tyrosine phosphoproteins (Chan *et al.*, 1992; Kolanus *et al.*, 1993; Couture *et al.*, 1994; Iwashima *et al.*, 1994; Kurosaki *et al.*, 1994; reviewed in Weiss and Littman, 1994). The src family of PTKs, lck and fyn, phosphorylate the two tyrosine residues within a 16 amino acid motif (YXXL X_{6-8}YXXL, termed ITAM for immunoreceptor tyrosine-based activation motif and previously designated as ARAM for antigen recognition activation motif, ARH1 for antigen receptor homology 1 motif or TAM for tyrosine-based activation motif) that is conserved in all of the signal transducing subunits of the TCR (reviewed in Samelson and Klausner, 1992; Chan *et al.*, 1994a; Weiss and Littman, 1994; Cambier, 1995). Tyrosine phosphorylation of the ITAM mediates the association of ZAP-70 with the TCR (Wange *et al.*, 1993; Gauen *et al.*, 1994; Iwashima *et al.*, 1994; Koyasu *et al.*, 1994). The association of ZAP-70 with the phosphorylated receptor facilitates its tyrosine phosphorylation that is mediated by the src PTKs (Iwashima *et al.*, 1994). The requirement for ZAP-70 in TCR function and T-cell development is underscored by the description of patients with an autosomal recessive form of severe combined immunodeficiency who lack ZAP-70 (Arpaia *et al.*, 1994; Chan *et al.*, 1994b; Elder *et al.*, 1994).

Tyrosine phosphorylation of ZAP-70 may serve two functions. First, phosphorylation may provide docking sites for SH2-containing effector molecules to interact with ZAP-70 and thereby propagate signaling. One such molecule that has been identified is lck (Duplay *et al.*, 1994). Lck associates with ZAP-70 following TCR stimulation; an interaction that is mediated through the SH2 domain of lck. However, the functional significance of this interaction remains unclear. Second, in addition to recruitment, phosphorylation of ZAP-70 may provide a mechanism for its catalytic activation.

To evaluate the role of phosphorylation in the catalytic activation of ZAP-70, we report here that ZAP-70 undergoes catalytic activation following TCR engagement and that this activation is regulated by phosphorylation of Tyr493. Moreover, we demonstrate that phosphorylation of this tyrosine residue is required for lymphocyte antigen receptor function.

Results

Tyrosine phosphorylation of ZAP-70 correlates with an increase in its catalytic activity

To examine the potential role of tyrosine phosphorylation in the regulation of ZAP-70 catalytic activity, we initially utilized the Jurkat leukemic T-cell line. Immunoprecipitates of ZAP-70 from resting and TCR-activated cells were analyzed using an *in vitro* kinase assay in the presence of an exogenous substrate, Band III. TCR stimulation resulted in an ~4-fold increase (mean of three independent experiments) in the phosphorylation of Band III (Figure 1A, top panel, lanes 1–2). Treatment of cells with pervanadate, which mimics TCR activation, further increased the catalytic activity of ZAP-70 immunoprecipitates for Band III phosphorylation by ~10-fold (Figure 1A, top panel, lane 3). These increases in Band III

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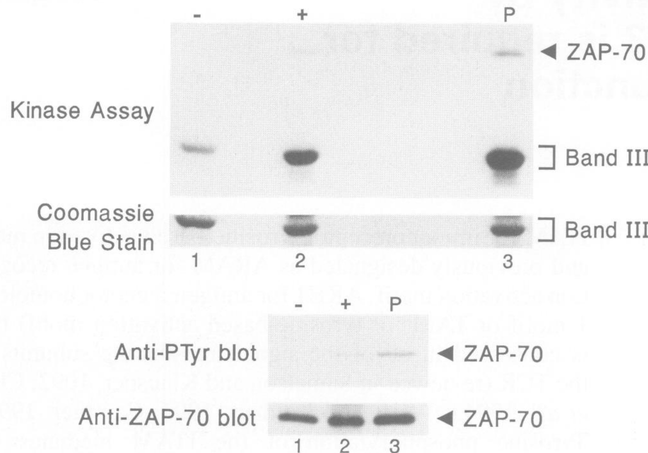


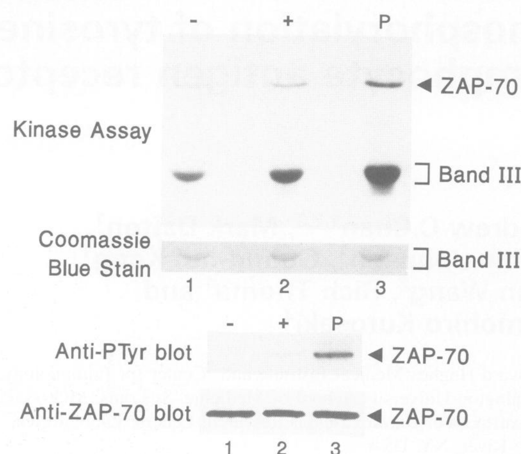


Fig. 1. Increase in ZAP-70 catalytic activity following TCR cross-linking and pervanadate treatment. (**A**) 1.5×10^7 Jurkat cells were stimulated for 2 min at 37°C with an anti-Ti mAb (1:500 dilution of C305, courtesy of A.Weiss; lane 2) or pervanadate (lane 3) and lysed in 1% NP-40-containing lysis buffer as previously described. Unstimulated cells were analyzed in lane 1. Anti-ZAP-70 immunoprecipitates (2222) were analyzed with an *in vitro* kinase assay utilizing 1 µg of Band III as an exogenous substrate. *In vitro* kinase assays were performed as previously described. Equivalent samples were analyzed in parallel by Western blotting with anti-phosphotyrosine (4G10, UBI) and anti-ZAP-70 (2F3.2, UBI) mAbs followed by [^{125}I]protein A (ICN). Quantitation of bands was performed utilizing IMAGE QUANT software after analysis on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Quantitation of exogenous substrate added was performed by staining with Coomassie Blue. (**B**) 1.5×10^7 HPB-ALL cells were stimulated for 2 min at 37°C with an anti-CD3 mAb (1:500 dilution of 235, courtesy of S.M.Fu, lane 2) or pervanadate (lane 3). Analysis of ZAP-70 *in vitro* kinase activity was then performed as described in (A).

phosphorylation were correlated with the amount of tyrosine phosphorylation of ZAP-70 (Figure 1A, bottom panel, lanes 1–3) as well as increases in ZAP-70 autophosphorylation (Figure 1A, top panel, lanes 1–3).

Analysis of ZAP-70 immunoprecipitates from another T-cell line (HPB-ALL) produced similar results (Figure 1B). TCR stimulation resulted in an ~2.5-fold increase in the phosphorylation of Band III, while treatment of HPB-ALL cells with pervanadate increased the catalytic activity of ZAP-70 immunoprecipitates by ~10-fold. Thus, phosphorylation of Band III by ZAP-70 immunoprecipitates is augmented following TCR stimulation.

Since ZAP-70 associates with other PTKs, including the fakB and lck PTKs (Duplay *et al.*, 1994; Kanner *et al.*, 1994), we could not exclude the possibility that the increase in Band III phosphorylation was due to the additive activity of these and other PTKs that associate with ZAP-70. To evaluate directly whether phosphorylation of ZAP-70 results in its catalytic activation, we expressed ZAP-70 as a fusion protein with GST in insect cells utilizing a baculoviral expression system. While expression of ZAP-70 alone resulted in a low level of tyrosine phosphorylation, co-expression of ZAP-70 with an lck molecule consisting solely of its kinase domain [designated lck(K)] resulted in a marked increase in the tyrosine phosphorylation of ZAP-70 (Figure 2, lanes 7–8). Western blotting for ZAP-70 of quantitative immunoprecipitates with an anti-phosphotyrosine monoclonal antibody (mAb) demonstrated that expression of ZAP-70 alone in SF9 cells resulted in ~3–5% of the total cellular ZAP-70 being tyrosine phosphorylated. In contrast, co-expression of ZAP-70 with lck(K) resulted in ~40% of ZAP-70 being tyrosine phosphorylated (mean of two independent experiments; data not shown).

Similar to the results derived from the Jurkat and HPB-ALL T-cell lines (Figure 1), ZAP-70 co-expressed with

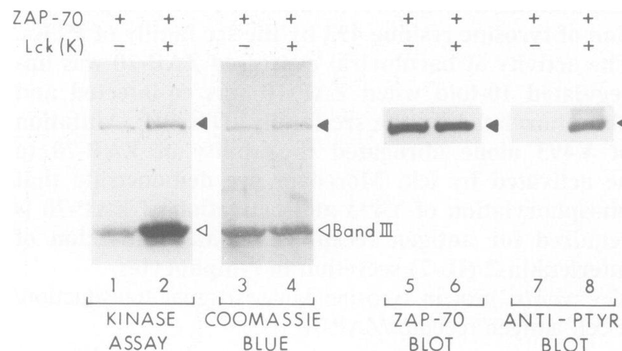


Fig. 2. Increase in ZAP-70 catalytic activity in baculoviral produced ZAP-70. ZAP-70 expressed as a chimeric protein with GST was harvested from SF9 cells co-infected in the presence (lanes 2, 4, 6 and 8) or absence (lanes 1, 3, 5, and 7) of lck(K). The fusion ZAP-70 protein was purified with glutathione–Sepharose (Pharmacia) and analyzed with an *in vitro* kinase assay in the presence of Band III (lanes 1–2), Coomassie Blue staining to ensure equivalent levels of ZAP-70 and Band III (lanes 3–4) and Western blotting with anti-ZAP-70 (lanes 5–6) and anti-phosphotyrosine (lanes 7–8) mAbs as described in Figure 1.

lck(K) demonstrated an ~10-fold increase in the phosphorylation of Band III by ZAP-70 (mean of three independent experiments; Figure 2, lanes 1–2). Comparable levels of Band III and GST–ZAP-70 were confirmed by Coomassie Blue staining (Figure 2, lanes 3–4) as well as by Western blot analysis (Figure 2, lanes 5–6). Elimination of the SH2 domain of lck was critical for measuring solely ZAP-70 activation rather than the additive effect of lck and ZAP-70, since lck associates with ZAP-70 following TCR activation, an interaction that is mediated by the SH2 domain of lck (Duplay *et al.*, 1994). Since lck(K) does not contain an SH2 domain, it should not be able to associate with ZAP-70. Consistent with this premise, lck(K) was not associated with the baculoviral produced

ZAP-70 as determined by Coomassie Blue staining, Western blotting for the hemagglutinin epitope tag or by *in vitro* kinase assays (data not shown). Thus, the increase in ZAP-70 catalytic activity truly correlated with its tyrosine phosphorylation.

Identification of Y492 and Y493 as phosphorylation sites following TCR activation

To ascertain the mechanism by which ZAP-70 catalytic activity becomes activated, we identified the tyrosine residues which undergo *in vivo* phosphorylation following receptor engagement. Phosphoamino acid analysis of ZAP-70 in the basal and activated (TCR- or pervanadate-stimulated) states demonstrated a predominance of serine phosphorylation (Figure 3A). Tyrosine phosphorylation of ZAP-70 was detected after TCR stimulation, and was markedly enhanced when cells were treated with pervanadate. A low level of threonine phosphorylation was also detected. Two-dimensional analysis of tryptic peptides derived from ZAP-70 by TLC combined with phosphoamino acid analysis of each of the peptides demonstrated a minimum of five tyrosine phosphorylated peptides (designated A–E, Figure 3B and data not shown). These peptides were not phosphorylated in unstimulated cells and the serine sites were not altered with TCR or pervanadate stimulation (data not shown). Mixing experiments indicated that peptides A–E derived from the *in vivo* ^{32}P-labeled TCR-stimulated cells were also present in pervanadate-treated cells (Figure 3B).

Manual sequencing by Edman degradation of peptide A from pervanadate-treated Jurkat cells revealed radiolabeled phosphate in cycles 2, 6, 8 and 9 (Figure 3C, bottom panel). Separation using a different electrophoretic analysis of peptide A demonstrated at least two distinct peptides with the phosphorylated residues at cycles 8 and 9 being derived from a single peptide (Figure 4B and data not shown). The differences in recovery from cycles 2 and 6 as compared with cycles 8 and 9 were also indicative that cycles 8 and 9 were derived from a peptide distinct from those giving rise to cycles 2 and 6. The presence of phosphate in cycles 8 and 9 was consistent with phosphorylation of only one tryptic peptide encoding amino acids 485–497 consisting of ALGADDSYYTAR. Analysis of a corresponding synthetic peptide phosphorylated on both Y492 and Y493 co-migrated with the radiolabeled peptide (Figure 3C, bottom panel). In addition, tryptic peptide mapping of a ZAP-70 molecule mutated on both Y492 and Y493 to F [designated ZAP-70(YYFF)] resulted in the loss of ^{32}P incorporation into peptide A (see below, Figure 4B). Together, these data indicate that both Y492 and Y493 are phosphorylated following TCR engagement.

Manual sequencing of peptide B demonstrated the presence of radiolabeled phosphate in cycle 9 only and it co-migrated with a synthetic peptide ALGADDSYY$_{PO4}$TAR that was phosphorylated only on Y493 (Figure 3C, middle panel). Thus the identity of peptide B was consistent with a singly phosphorylated peptide on Y493. As expected, the corresponding peptide phosphorylated only on Y492 also co-migrated with peptide B (data not shown). However, our inability to identify any peptide with radiolabeled phosphate in cycle 8 that co-migrated with a singly phosphorylated Y492 peptide and the quantitative recovery of *in vivo* ^{32}P-labeled peptides (>95%) suggested a sequential phosphorylation of Y493 followed by phosphorylation of Y492.

Phosphorylation of Y493 is required for catalytic activation of ZAP-70

To analyze the functional significance of Y492 and Y493 in the activation of ZAP-70 catalytic activity, both tyrosine residues were mutated to phenylalanine [ZAP-70(YYFF)] and expressed in insect cells. While co-infection of ZAP-70 with lck(K) resulted in the activation of its catalytic activity (Figure 2, lanes 1–2 and Figure 4A, lanes 1–2), co-infection of ZAP-70(YYFF) with lck(K) did not result in any increase in the phosphorylation of Band III (Figure 4A, lanes 7–8). Comparable levels of ZAP-70 were present in each sample analyzed (Figure 4A, bottom panel, lanes 1–8). Thus, phosphorylation of one or both of these tyrosine residues is required for ZAP-70 catalytic activation.

To dissect the contribution of each tyrosine to ZAP-70 activation further, the catalytic activation of single mutations, ZAP-70(Y492F) and ZAP-70(Y493F), co-infected with lck(K) were analyzed in a similar fashion. While Y492F still retained its ability to be activated by lck (Figure 4A, lanes 3–4), the activity of Y493F was minimally increased by lck (Figure 4A, lanes 5–6). Thus, phosphorylation of Y493 appeared to have a significantly greater contribution to ZAP-70 activation.

To confirm the tryptic peptide assignments made and to ensure that the baculoviral produced ZAP-70 was appropriately phosphorylated, wild-type ZAP-70 was co-infected with lck(K) in SF9 cells and labeled with [^{32}P]orthophosphate. The conditions for ascending chromatography were slightly modified to permit a better separation of the peptide giving rise to cycles 2 and 6 (peptide A2) and the doubly phosphorylated peptide ALGADDSY$_{PO4}$Y$_{PO4}$TAR (peptide A1). The tyrosine phosphorylated tryptic peptides derived from *in vivo* [^{32}P]orthophosphate-labeled ZAP-70 in pervanadate-stimulated Jurkat cells were identical to those derived from the baculoviral produced wild-type ZAP-70 (co-infected with lck; Figure 4B, panels 1–2). Mixing experiments also demonstrated that peptides A–E derived from pervanadate-stimulated Jurkat cells co-migrated with those derived from insect SF9 cells (data not shown). Thus, the tyrosine residues of ZAP-70 phosphorylated in SF9 cells by lck are identical to those phosphorylated in T cells.

Consistent with the peptide assignments made, analysis of baculoviral produced ZAP-70(YYFF) resulted in the loss of detectable phosphate in peptides A1 (the doubly phosphorylated peptide) and B (the singly phosphorylated Y493 peptide) (Figure 4B, panel 5). Only one peptide incorporated any detectable phosphate. Interestingly, phosphorylation of peptides A2, C and E was also dependent upon phosphorylation of Y492/493. Analysis of baculoviral produced ZAP-70(Y493F) showed a similar pattern to ZAP-70(YYFF) (Figure 4B, panels 4–5). Again, only one peptide was independent of the phosphorylation of Y493. Thus, activation of ZAP-70 catalytic activity, which is dependent upon Y493, is required for phosphorylation of peptides A2, C and E. Consistent with this hypothesis, analysis of baculoviral produced ZAP-70(Y492F), which can be catalytically activated by lck (Figure 4A, lanes 3–4), resulted in phosphorylation of all

peptides seen in the wild-type ZAP-70, with the exception of the doubly phosphorylated peptide A1 (Figure 4B, panel 3). Thus, phosphorylation of Y493 is required and sufficient for the catalytic activation of ZAP-70 by lck and for the phosphorylation of additional tyrosine residues on ZAP-70 (peptides A2, C and E) which may serve as docking sites for downstream effector molecules.

Y493 is required for lymphocyte antigen receptor function

To determine the biologic significance of these two tyrosine residues within ZAP-70 in lymphocyte antigen receptor function, we expressed ZAP-70(YYFF) in a syk⁻ B cell. Syk is a family member of ZAP-70 and is expressed in B cells, thymocytes, mast cells, neutrophils, platelets and monocytes (Hutchcroft *et al.*, 1992a,b; Agarwal *et al.*, 1993; Law *et al.*, 1993; Taniguchi *et al.*, 1993; Chan *et al.*, 1994c; Couture *et al.*, 1994; Minoguchi *et al.*, 1994). It has been previously demonstrated that loss of syk as a result of homologous recombination in the DT40 chicken B-cell line results in the loss of B-cell antigen receptor (BCR) function (Takata *et al.*, 1994). We have recently demonstrated that expression of ZAP-70 in syk⁻ B cells reconstitutes BCR signaling (Kong *et al.*, 1995). In particular, expression of ZAP-70 in a syk⁻ B cell results in the induction of interleukin (IL)-2 gene promoter activity when the BCR is cross-linked in the presence of ionomycin. Thus, we utilized this system to determine the functional requirement for each of these two tyrosine residues. While expression of wild-type ZAP-70 in the syk⁻ B cells resulted in an ~4.5-fold increase in the induction of IL-2 gene promoter activity in response to

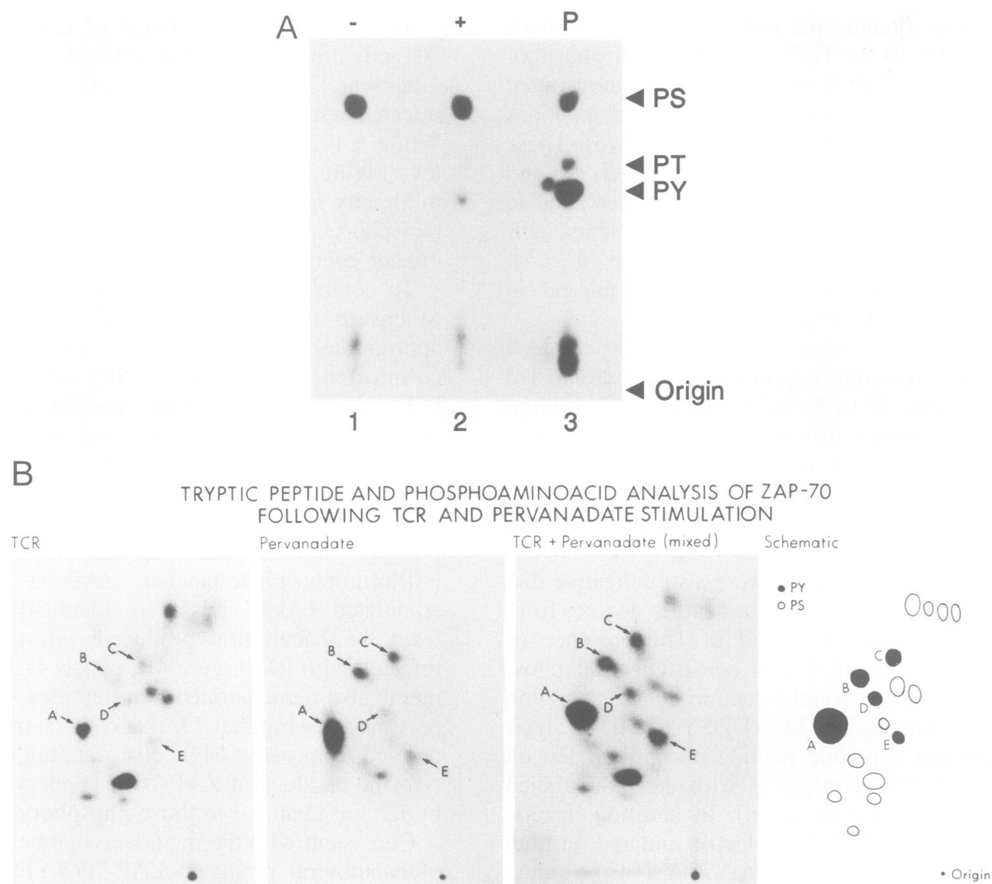
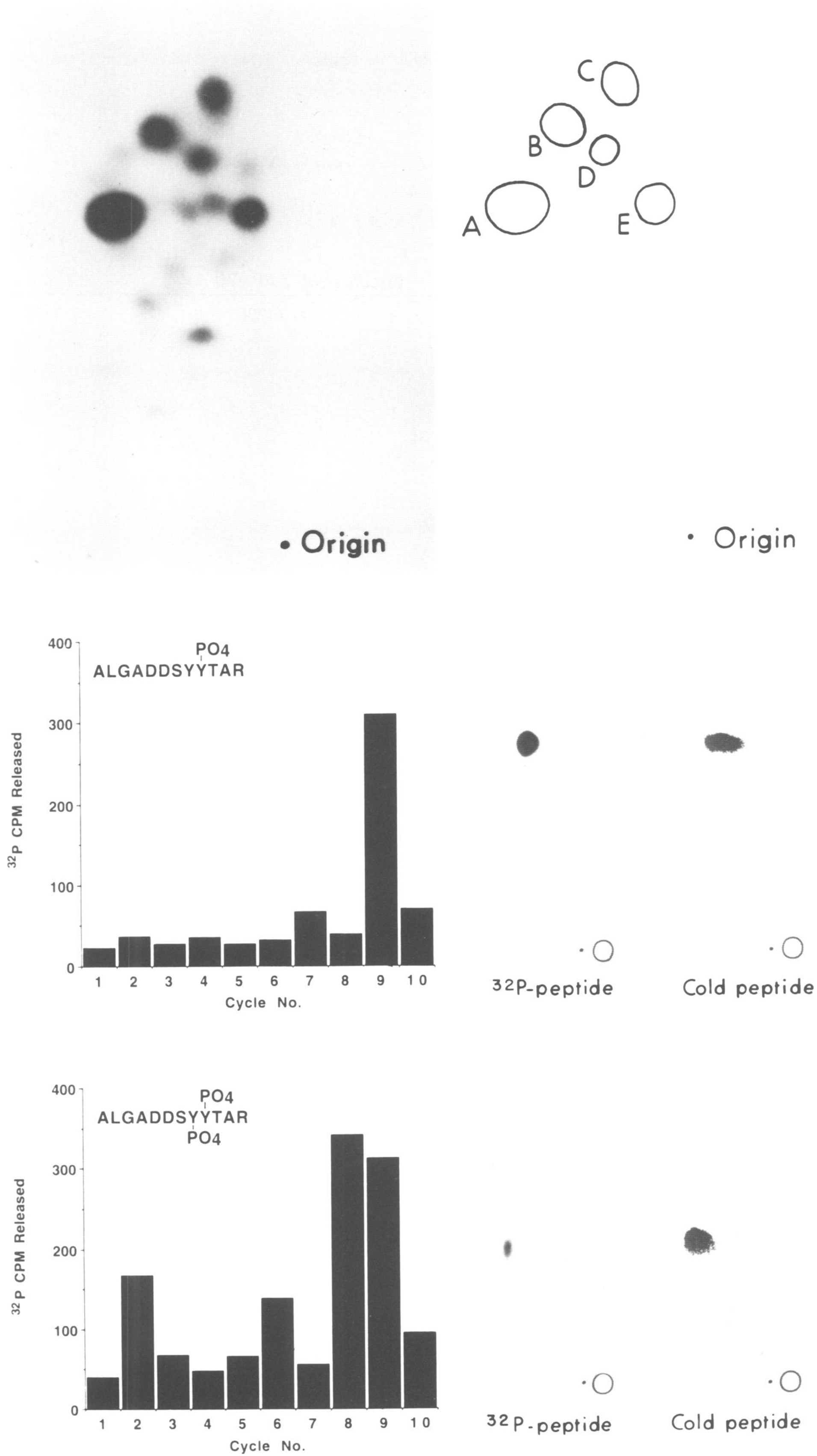


Fig. 3. (**A**) Phosphoamino acid analysis of ZAP-70 isolated from unstimulated, TCR-stimulated and pervanadate-stimulated cells. 2×10^7 Jurkat T cells/condition were labeled with 1 mCi/ml [^{32}P]orthophosphate (ICN) for 3 h in phosphate-deficient media and analyzed in the resting, TCR- or pervanadate-stimulated conditions as described in Figure 1. Proteins were transferred to nitrocellulose membranes for tryptic and phosphoamino acid analysis as previously described. Phosphoamino acid content was determined by electrophoresis on TLC plates in equal volumes of pH 1.9 and 8.9 buffers. Cold standards of phosphoserine (PS), phosphothreonine (PT) and phosphotyrosine (PY) were analyzed in parallel and their migration depicted by the designated markers. (**B**) Two-dimensional TLC analysis of tryptic peptides derived from ZAP-70. Panel 1, TCR-stimulated cells; panel 2, pervanadate-stimulated cells; panel 3, mixing of TCR-stimulated and pervanadate-stimulated cells; and panel 4, schematic of the phosphoamino acid content determined for each peptide. The filled in spots in the schematic diagram depict tyrosine phosphorylation sites and the open spots designate serine phosphorylation sites, as determined by phosphoamino acid analysis of each peptide from panel 1. Recovery of ^{32}P-labeled peptides was >95%. A comparable number of total c.p.m. was analyzed in each panel. Since the pervanadate-treated cells (panel 2) have a greater proportion of tyrosine phosphorylated peptides (A) when compared with the TCR-activated cells, a 2:1 ratio of TCR:pervanadate-stimulated samples was used for the mixing experiment to normalize for this difference. (**C**) Sequencing of ^{32}P-labeled peptides A and B. Manual Edman degradation of peptides A and B was performed as previously described. Top: the left panel depicts the 2-D TLC map of tryptic peptides of ZAP-70 derived from pervanadate-treated cells and the right panel depicts a schematic of the major tyrosine-phosphorylated peptides. Middle: the left panel depicts the ^{32}P c.p.m. released from peptide B in each sequencing cycle. The right panel demonstrates the co-migration of ^{32}P-labeled peptide B and a synthetic peptide (5 μg) encoding ALGADDSYY$_{PO4}$TAR. Bottom: the left panel depicts the ^{32}P c.p.m. released from peptide A in each sequencing cycle. The right panel demonstrates the co-migration of ^{32}P-labeled peptide A and a synthetic peptide (5 μg) encoding ALGADDSY$_{PO4}$Y$_{PO4}$TAR.

anti-BCR mAb (M4) and 1 μM ionomycin, expression of ZAP-70(YYFF) failed to induce any BCR-mediated IL-2 promoter activity (Figure 5A). Comparable levels of protein expression were confirmed by Western blotting for ZAP-70 (Figure 5B, lanes 3 and 6) and comparable levels of IL-2 promoter activity were induced with phorbol



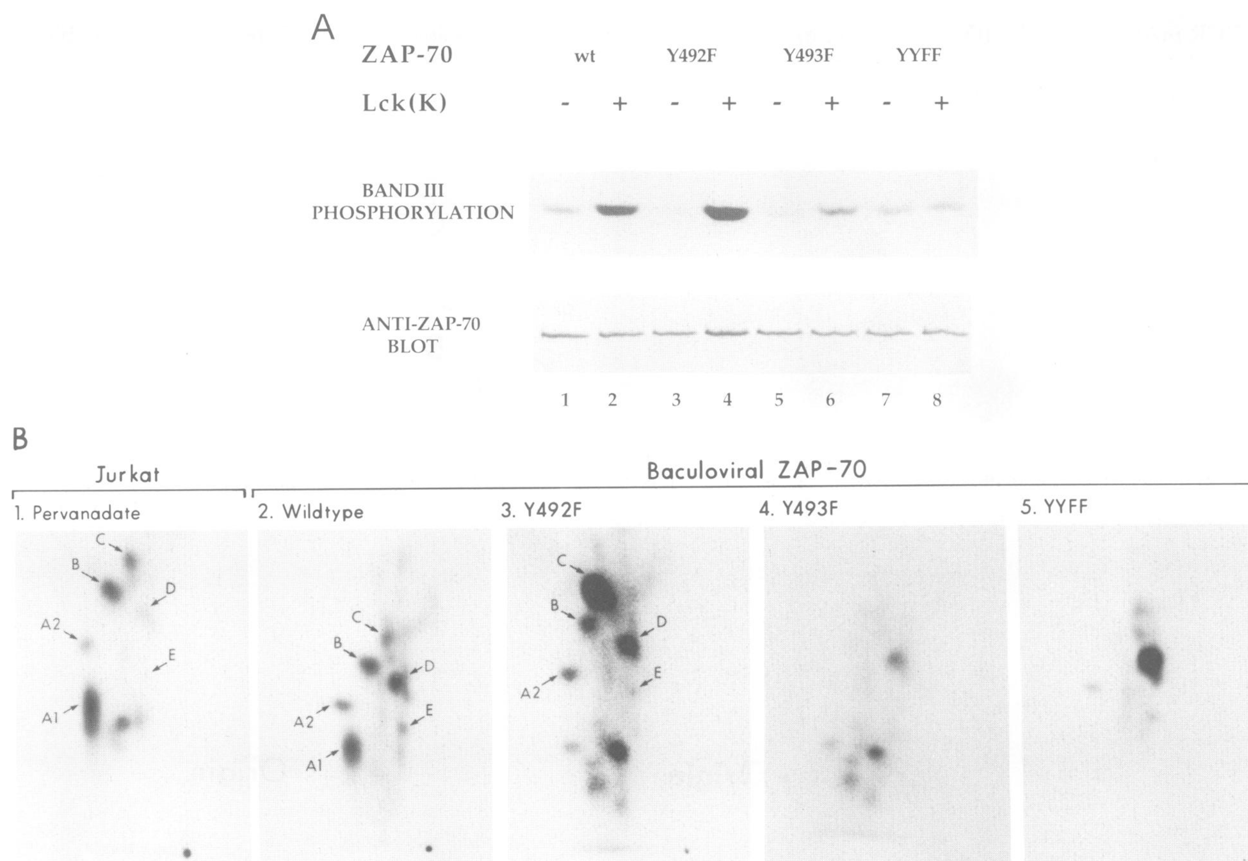


Fig. 4. (**A**) Requirement of Y493 for activation of ZAP-70 catalytic activity. ZAP-70 (wild-type), ZAP-70(Y492F), ZAP-70(Y493F) or ZAP-70(YYFF) were co-infected in the presence or absence of lck(K) as described in Figure 2. The resultant proteins were analyzed utilizing *in vitro* kinase assays with Band III (top), Coomassie Blue staining and Western blot analysis with an anti-ZAP-70 mAb 2F3.2 (bottom). ZAP-70 mutations were produced by PCR-based mutagenesis of both or either tyrosine residues to phenylalanine and confirmed by DNA sequencing as described in Materials and methods. Equivalent levels of lck(K) in all co-infections were confirmed by Western blot analysis for the hemagglutinin tag (data not shown). (**B**) Two-dimensional TLC analysis of tryptic peptides derived from *in vivo* [^{32}P]orthophosphate-labeled ZAP-70 and mutations of Y492/493. Panel 1, pervanadate-stimulated Jurkat cells; panel 2, wild-type ZAP-70; panel 3, ZAP-70(Y492F); panel 4, ZAP-70(Y493F); panel 5, ZAP-70(YYFF). Panels 2–5 represent baculoviral produced ZAP-70 molecules that were co-infected with lck(K) as described in Figure 2. The conditions for ascending chromatography were modified slightly to resolve peptides A1 and A2. Recovery of ^{32}P-labeled peptides was >95%. The total c.p.m. loaded in panels 3 and 4 were only 10% of the c.p.m. loaded in the other panels. Thus, the serine phosphorylation sites are more apparent in these two mutations compared with the other panels.

myristate (PMA) and ionomycin in each transfection (Figure 5A). Thus, Tyr492 and/or 493 are necessary for ZAP-70 function in lymphocyte activation.

To dissect the relative contributions of Y492 and Y493 further, single mutations of each tyrosine to phenylalanine were also analyzed. While ZAP-70(Y492F) reconstituted BCR-mediated induction of IL-2 promoter activity, ZAP-70(Y493F) was unable to function despite comparable levels of protein expression and transfection efficiencies (Figure 5A and B). Thus, Y493 is sufficient for ZAP-70 activation and function. Interestingly, the Y492F mutation consistently produced higher levels of IL-2 promoter activity when compared to wild-type ZAP-70. The significance of this observation is unclear at this time but raises the intriguing possibility of a negative regulatory role for Y492. Analysis of stable transfectants will provide more definitive information. The requirement for Y493 in ZAP-70 function for IL-2 secretion is also consistent with the mapping studies that suggest that Y493 is preferentially phosphorylated prior to Y492 (Figure 3B) and with the activation analysis of Y492F and Y493F mutations in the baculoviral expressed ZAP-70 (Figure 4A and B).

Discussion

Tyrosine phosphorylation has been implicated as a regulatory mechanism for the activation of PTKs. We provide evidence here that the catalytic activity of the ZAP-70 PTK can be regulated by phosphorylation of Y493. While the activity present in ZAP-70 immunoprecipitates is increased following TCR stimulation, as has been demonstrated by others (Burkhardt *et al.*, 1994; Wange *et al.*, 1995), the concern for associated PTKs, such as fakB and lck, prompted us to assess ZAP-70 activity in a heterologous system (Duplay *et al.*, 1994; Kanner *et al.*, 1994). Utilizing baculoviral produced ZAP-70, we demonstrate here that the catalytic activity of ZAP-70 is markedly enhanced when co-expressed with an lck kinase domain that does not associate with ZAP-70. Two-dimensional TLC mapping of the *in vivo* phosphorylated tryptic peptides derived from the baculoviral produced ZAP-70 (when co-infected with lck) showed that they were identical to those phosphorylated in T cells following TCR activation. Thus, the increased catalytic activity of ZAP-70 is at least in part responsible for the increased

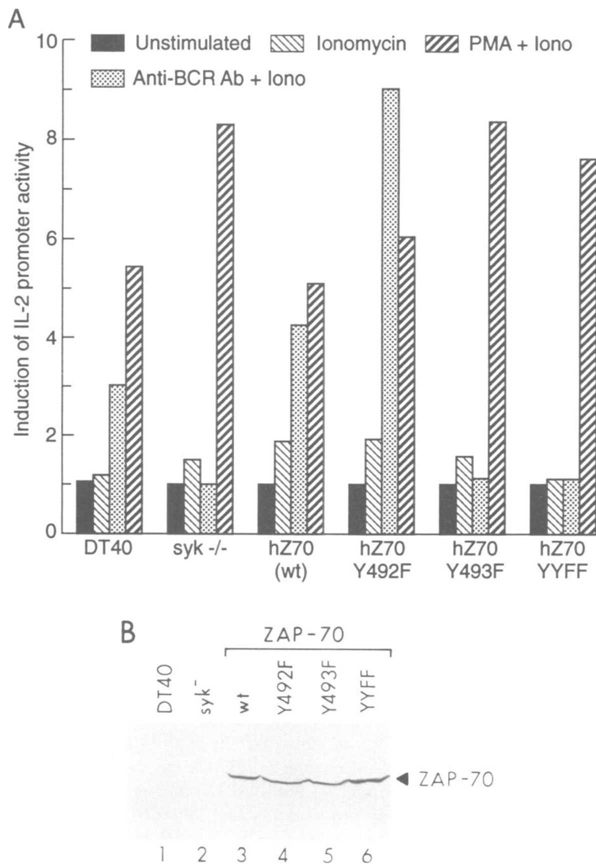


Fig. 5. Requirement of Y493 for antigen receptor function. (**A**) Induction of IL-2 promoter activity. Syk⁻ B cells were transiently transfected with 20 mg of human ZAP-70(wild-type), ZAP-70(Y492F), ZAP-70(Y493F) or ZAP-70(YYFF) subcloned in pApuro and 20 mg of IL-2–luciferase. Cells were electroporated using a BTX Electro Cell Manipulator 600 as previously described. 48 h following electroporation, cells were divided equally and treated with media, ionomycin (1 μM), ionomycin (1 μM) and anti-BCR mAb (M4, 4 μg/ml), or PMA (100 ng/ml) and ionomycin (1 μM). Cells were incubated at 37°C for 6 h, lysed and luciferase activity measured as previously described. ZAP-70 (Y492F), (Y493F) and (YYFF) mutations were produced by PCR-directed mutagenesis and confirmed by DNA sequencing. (**B**) ZAP-70 expression of ZAP-70 mutations in syk⁻ B cells. The expression of each construct was confirmed by Western blot analysis for ZAP-70 (2F3.2) of cells from each transfection. The data presented here are representative of two independent experiments.

kinase activity seen in ZAP-70 immunoprecipitates following TCR stimulation. The lower degree of activation in ZAP-70 immunoprecipitates seen in T cells (~3-fold) when compared with the baculoviral produced ZAP-70 (co-infected with lck; ~10-fold) probably indicates that a smaller number of ZAP-70 molecules undergo tyrosine phosphorylation following TCR stimulation.

While the lck SH2 domain was not required to phosphorylate ZAP-70 on the appropriate tyrosine residues in the baculoviral co-infections, this was likely to be the result of the high levels of expression achieved within this system. These results are consistent with observations in COS cells in which expression of ZAP-70 with an lck molecule in which the SH2 domain is non-functional still resulted in ZAP-70 tyrosine phosphorylation, though the sites of phosphorylation were not identified in that study (Iwashima *et al.*, 1994). In T cells, however, the SH2 domain of lck is required for efficient TCR-mediated signaling (Caron *et al.*, 1992; Straus *et al.*, manuscript in preparation). The presence of a non-functional SH2 domain in lck as the result of deletion or point mutation leads to abrogation of TCR-mediated signaling events, including markedly reduced ζ and ZAP-70 phosphorylation following TCR cross-linking (Caron *et al.*, 1992; Straus *et al.*, manuscript in preparation). Thus, the lck SH2 domain is necessary for TCR activation, though the mechanism by which it functions remains unclear.

Tyr493 of ZAP-70 corresponds to the autophosphorylation site Y416 identified within src. Phosphorylation of Y416 of src regulates both its catalytic activity and transforming ability (Cross and Hanafusa, 1983; Snyder *et al.*, 1983; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987). Mutation of Y416 to phenylalanine in v-src or its deletion leads to a 5-fold decrease in its *in vitro* kinase activity and decreases, but does not abolish, its transformation potential. Our analysis of the functional significance of Y493 demonstrates that this tyrosine residue is required for both up-regulation of ZAP-70 catalytic activity and for lymphocyte receptor function. Mutation of Y493 to phenylalanine abrogated the ability of lck to augment the catalytic activity of baculoviral produced ZAP-70. Moreover, the biological import of Y493 is demonstrated by the inability of ZAP-70(Y493F) to reconstitute the syk⁻ B cell in response to BCR activation. Thus, the activation of the catalytic activity of ZAP-70 through phosphorylation of Y493 is required for antigen receptor function.

ZAP-70′s family member, syk, also has two homologous tyrosine residues (Y518 and 519) that are likely to perform a similar function (Taniguchi *et al.*, 1991). Mutation of both tyrosines in syk abrogated its ability to undergo tyrosine phosphorylation in COS cells (Couture *et al.*, 1994). The catalytic activity of this mutation ($syk^{F518/519}$), however, was not investigated in this study, but may be similar to the findings seen here for ZAP-70. Thus, mutation of Y519 to phenylalanine may yield a syk PTK that cannot be activated by the src family of PTKs and which is non-functional in BCR signaling.

The identification of Y492 and Y493 as *in vivo* phosphorylation sites is consistent with a recent study utilizing electrospray ionization mass spectrometry (Watts *et al.*, 1994). In this study, three tyrosine sites (Y292, Y492 and Y493) were identified to be phosphorylated following TCR stimulation. Similar to our results, both a doubly phosphorylated (Y492 and Y493) and a singly phosphorylated peptide (Y492 or Y493) were identified. However, an assignment could not be made as to which tyrosine residue was phosphorylated on the singly phosphorylated peptide. Our analysis here suggests that sequential phosphorylation occurs on these two tyrosine residues. Phosphorylation of Y493 occurs first and is sufficient for ZAP-70 activation and function. The functional consequence of phosphorylating Y492, however, remains unclear. Phosphorylation of Y492 does not appear to enhance the activity of ZAP-70, since the baculoviral produced ZAP-70(Y492F) mutation can be activated as least to the same extent as wild-type ZAP-70. The phosphorylation sites of ZAP-70(Y492F) are identical to those seen with the wild-type, with the exception of the doubly phosphorylated A1 peptide. Moreover, the ZAP-70(Y492F) mutation can fully reconstitute the syk⁻ B cell

in response to activation of the BCR. Analysis of stable transfectants of the Y492F mutation and additional structural data (see below) will provide more definitive information.

The presence of two tyrosine residues within this activation domain appears to be limited to ZAP-70 and syk amongst the cytoplasmic PTKs; all of the src PTKs have only a single tyrosine within this homologous region. However, the presence of two tyrosine residues at this site is highly conserved amongst a variety of receptor PTKs. Tyr493 corresponds to Y1162 within the insulin receptor (InsR) kinase domain (Ebina, 1985). Similar to Y492 and Y493 in ZAP-70, Y1162 and Y1163 of the InsR undergo trans-phosphorylation following ligand engagement. The recently characterized crystal structure of the InsR kinase domain proposes that these two tyrosine residues reside within an activation loop (Hubbard *et al.*, 1994). Upon phosphorylation of Y1162, the P-Tyr1162 will be salt bridged to R1131 or R1155 to stablize the activated InsR. In the case of ZAP-70, phosphorylation of Y493 is dependent upon the src PTKs. Thus, phosphorylation of Y493 in ZAP-70 by lck may stabilize an activated PTK by a salt bridge to the corresponding R460 and/or K484. The structure of an activated ZAP-70 catalytic domain will provide greater insight into the stabilizing forces giving rise to the activated PTK as well as the contribution of Y492.

Our *in vivo* labeling studies here also demonstrate a greater number of tyrosine phosphorylated peptides than those recently reported (Watts *et al.*, 1994). Preliminary data suggest that peptide C represents the Y292 phosphorylated tryptic peptide (data not shown). Peptides A2, D and E may represent incompletely digested tryptic peptides derived from peptide C or additional sites that were not identified from the *in vivo* analysis utilizing electrospray ioniziation mass spectrometry. Three additional tyrosines within ZAP-70 (Y69, 126 and 178) were also identified in this recent study utilizing *in vitro* phosphorylation by lck (Watts *et al.*, 1994). Thus, peptides A2, D and E may alternatively represent these additional potential phosphorylation sites.

Finally, while phosphorylation of Y493 within ZAP-70 mediates the regulation of ZAP-70 catalytic activity, the phosphorylation of these other sites (i.e. peptides A2, C, D and E) are likely to serve as docking sites for downstream effector molecules. Tyr315 has been proposed by Katzav, Weiss and colleagues to serve as a docking site for the SH2 domain of proto-vav (Katzav *et al.*, 1994). In addition, other SH2-containing proteins, such as GTPase activating protein and abl, have been described as associating with tyrosine phosphorylated ZAP-70 (Neumeister *et al.*, 1995). Thus, tyrosine phosphorylation of ZAP-70 is likely to serve both regulatory and scaffolding functions in TCR activation. These data are consistent with a model in which ZAP-70 is initially phosphorylated by lck on Y493 resulting in an increase in ZAP-70 catalytic activity. This, in turn, may permit the activated ZAP-70 to phosphorylate additional sites within ZAP-70 (e.g. peptides A2, C and E) which may serve as docking sites for downstream effector molecules and/or to phosphorylate downstream effectors. Alternatively, but less likely, the interaction of ZAP-70 with each of the signaling subunits within a single TCR complex may permit ZAP-70 to undergo trans-phosphorylation on a specific tyrosine to recruit lck via an SH2–phosphotyrosine interaction. The association of ZAP-70 with lck may then permit phosphorylation of Y493 by lck to up-regulate ZAP-70 activity and in turn lead to the phosphorylation of additional sites within ZAP-70. Additional SH2-containing effector molecules could then be phosphorylated by either ZAP-70 or lck for downstream signaling. Analysis of additional ZAP-70 mutations within this system will permit us to dissect further the mechanisms by which this cascade of PTKs become activated.

Materials and methods

Cells, antibodies and peptides

Jurkat and HPB-ALL leukemic T cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Syk⁻ DT40 B cells were maintained as previously described (Takata *et al.*, 1994). SF9 cells were purchased from Pharmigen and maintained in suspension at 80 r.p.m. in Grace's insect cell culture medium (Gibco-BRL) supplemented with 10% FCS, penicillin, streptomycin, L-glutamine, lactalbumin hydrolysate, yeastolate and 0.01% F-68 (Sigma).

Antibodies used include: C305, an anti-Ti mAb (courtesy of A.Weiss); 235, an anti-CD3 mAb (courtesy of S.M.Fu); 4G10, an anti-phosphotyrosine mAb (UBI); 2F3.2, an anti-ZAP-70 mAb (UBI); M4, an anti-BCR mAb (courtesy of M.Cooper); 12CA5, an anti-hemagglutinin peptide mAb (BALBCO) and an anti-ZAP-70 antiserum (1222) raised against a peptide encoding amino acids (aa) 282–307 of human ZAP-70 (Chan *et al.*, 1992). This antiserum is specific for human ZAP-70 and does not cross-react with human syk (data not shown).

Peptides were purchased from QCB (Hopkinton, MA). Peptides were purified with two rounds of HPLC to >98% homogeneity. Mass spectroscopy was used to confirm their identity and ^{31}P-NMR was used to ensure that both tyrosine residues were in their appropriate phosphorylated states.

Construction of plasmids

Glutathione-S-transferase (GST)–ZAP-70 was produced as a fusion protein of GST fused to the entire coding region (bp 213–2069) of human ZAP-70 utilizing the PVIKS vector (Chan *et al.*, 1992). Lck(K) was produced by fusing an N-terminal hemagglutinin peptide tag to aa 217–509 of human lck into the PVL 1393 vector (Invitrogen) (Koga *et al.*, 1986; Perlmutter *et al.*, 1988).

Tyr492 and/or Tyr493 of ZAP-70 were mutated to phenylalanine by a PCR-directed approach. PCR products were generated using a 5′-primer encompassing bp 1645–1702, including the tyrosine (TAC) to phenylalanine (GAC) mutations (i.e. Y492F, Y493F and YYFF in which both Tyr492 and Tyr493 were changed to phenylalanine) in conjunction with a 3′-anti-sense primer encompassing bp 2067–2047. The PCR products were then exchanged with wild-type ZAP-70 utilizing the *Bst*X1 sites to generate each ZAP-70 mutation. All junctions and PCR products were confirmed by standard dideoxy DNA sequencing.

The IL-2–luciferase construct contained nucleotides –2060 to +40 of the murine IL-2 promoter in pBS-Luc and the CMV–CAT construct contained a 760 bp *Bgl*II fragment containing the immediate early promoter region from cytomegalovirus in pBS-CAT (courtesy of K.Murphy) (Szabo *et al.*, 1993).

Infection of SF9 cells

Infection of SF9 cells was accomplished by using plaque-purified viral stocks at 5 p.f.u./cell. Isolation and production of viral stocks were performed according to the manufacturer's recommendations (Davies, 1994). For protein production, cells were infected for 48–60 h and lysed in 10 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 3 mM pepstatin A, 5 mM EDTA, 0.4 mM $NaVO_3$ and 10 mM NaF (lysis buffer). Proteins were then purified using glutathione–Sepharose (Pharmacia) or glutathione–agarose (Sigma) according to the manufacturer's recommendations.

Stimulation of cells and protein analysis

Cells were resuspended at 10^8/ml in phosphate-buffered saline (PBS) for 15 min at 37°C. Jurkat, HPB-ALL and DT40 cells were cross-linked with the anti-Ti mAb C305 (1:500), anti-CD3 mAb 235 (1:500) or anti-BCR mAb M4 (1 µg/ml), respectively, for 2 min at 37°C. Cells were then quickly sedimented at 6000 r.p.m. and lysed for 15 min at 4°C in lysis buffer. Cellular debris was sedimented at 14 000 r.p.m. for 10 min at 4°C and the supernatant harvested for subsequent studies. Protocols for immunoprecipitations, SDS-PAGE, Western blotting and Edman degradation have been described previously (Sullivan and Wong, 1991; Chan *et al.*, 1992). Quantitation of proteins was performed on Western blots developed with [^{125}I]protein A (ICN) using IMAG QUANT software after analysis in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In vitro kinase assays

ZAP-70 immunoprecipitates or GST–ZAP-70 protein immobilized on glutathione–Sepharose or glutathione–agarose were washed three times with lysis buffer, twice with 10 mM Tris, pH 7.4, 0.5 M LiCl and once with kinase buffer (10 mM Tris pH 7.4, 10mM $MgCl_2$ and 10 mM MnCl). *In vitro* kinase assays were performed at 25°C for 5 min in 25 µl kinase buffer supplemented with 10 µCi [γ-^{32}P]ATP (>6000 Ci/mmol; NEN) and 1 µg Band III as an exogenous substrate. Reactions were terminated with the addition of an equal volume of SDS sample buffer and heated to 100°C for 5 min. Samples were then analyzed by SDS–PAGE, Coomassie Blue staining and autoradiography.

In vivo [^{32}P]orthophosphate labeling of ZAP-70

Jurkat cells were labeled with [^{32}P]orthophosphate for 4 h in phosphate-deficient RPMI-1640 media. Cells were washed twice with PBS and incubated for 15 min at 37°C prior to stimulation. TCR stimulation was performed by the addition of C305 (1:500) for 2 min and terminated by the addition of cold lysis buffer. Pervanadate stimulation was performed by incubating cells in a preformed mixture of 100 µM $NaVO_3$ and 0.035% H_2O_2 in PBS at room temperature for 10 min. SF9 cells were co-infected with viral stocks encoding GST–ZAP-70 and lck(K) for 48 h. Cells were then labeled with [^{32}P]orthophosphate for 6 h, cells lysed in lysis buffer and proteins purified as described above.

Analysis of ^{32}P-labeled ZAP-70 tryptic peptides

ZAP-70 was purified by incubating ^{32}P-labeled Jurkat lysates with anti-ZAP-70 antiserum immobilized on protein A–Sepharose (Pharmacia), washed three times with cold lysis buffer and eluted with SDS–PAGE loading buffer. Proteins were separated by SDS–PAGE, transferred to nitrocellulose and visualized by autoradiography as previously described (Boyle *et al.*, 1991; Luo *et al.*, 1991). In brief, the nitrocellulose containing ZAP-70 protein was excised, blocked with 1% polyvinylpyrrolidone for 30 min at room temperature, washed three times with H_2O and digested with 10 µg TPCK-trypsin (Worthington) in 50 mM NH_4CO_3 for 2 h at 37°C followed by an additional 5 µg TPCK-trypsin for 2 h to ensure complete cleavage. Peptides were then dried, washed three times with H_2O to remove the NH_4CO_3 and resuspended in 5 µl H_2O for analysis by TLC.

Tryptic peptides were separated in the first dimension at pH 8.9 by TLC utilizing a HTLE-7000 electrophoretic apparatus (CBS, Del Mar, CA) (Boyle *et al.*, 1991; Luo *et al.*, 1991). Electrophoresis was performed for 25 min at 1000 at 4°C. Separation in the second dimension was performed by ascending chromatography in isobutyric acid:butanol: pyridine:acetic acid (62.5%:1.9%:4.8%:2.9%) for 12–16 h. Plates were dried and ^{32}P-labeled peptides detected by autoradiography. Marker dyes consisting of orange G, acid fuchin, ε-dinitrophenyl-lysine and xylene cyanol FF were used to monitor migration in both dimensions. Cold peptides were visualized by ninhydrin (0.25% in acetone) at 60°C. For co-migration and sequencing analysis, labeled peptides were eluted in pH 1.9 buffer as previously described (Boyle *et al.*, 1991; Lu, *et al.*, 1991).

Phosphoamino acid analysis

Tryptic peptides of ^{32}P-labeled proteins were hydrolyzed in 6 M boiling HCl (Pierce) at 110°C for 90 min under vacuum. Samples were concentrated and resuspended in 10 µl H_2O and analyzed by one-dimensional phosphoamino acid analysis utilizing a HTLE-7000 apparatus with equal volumes of pH 1.9 and pH 3.5 buffers. Cold phosphoserine, phosphothreonine and phosphotyrosine were added to the samples and visualized by ninhydrin staining at 60°C. The ^{32}P-labeled amino acids were detected by autoradiography.

Transfection of cells

Transient transfections were performed by electroporating 10^7 syk^- DT40 B cells with 20 µg of pApuro containing wild-type ZAP-70 or ZAP-70 mutations, 20 µg of IL-2-luciferase and 20 µg CMV–CAT as previously described (Kong *et al.*, 1995). Cells were harvested at 48 h and analyzed as described in the text. Luciferase assays were performed using a OptocompII automated luminometer (MGM, Hamden, Conn) as previously described (Kong *et al.*, 1995). CAT assays were performed as previously described (Kong *et al.*, 1995).

Acknowledgements

The authors thank A.Shaw, M.Thomas, R.Schreiber, H.Piwnica-Worms, E.Unanue, C.Fu and J.J.Bu for critical reading of this manuscript and Philip Low (Purdue University) for providing the Band III construct. A.C.C. is the recipient of an ACR Investigator Award.

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Received on January 16, 1995; revised on March 10, 1995