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Distinct Tyrosine Phosphorylation Sites in ZAP-70 Mediate Activation and Negative Regulation of Antigen Receptor Function

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Biochemical and genetic evidence has implicated two families of protein tyrosine kinases (PTKs), the Src- and Syk-PTKs, in T- and B-cell antigen receptor signaling. ZAP-70 is a member of the Syk-PTKs that associates with the T-cell antigen receptor and undergoes tyrosine phosphorylation following receptor activation. Three tyrosine residues, Tyr-292, -492, and -493, have been identified as sites of phosphorylation following T-cell antigen receptor engagement. Utilizing ZAP-70- and Syk-deficient lymphocytes (Syk$^-$ DT40 cells), we provide biochemical and functional evidence that heterologous *trans*-phosphorylation of Tyr-493 by a Src-PTK is required for antigen receptor-mediated activation of both the calcium and ras pathways. In contrast, cells expressing mutations at Tyr-292 or -492 demonstrate hyperactive T- and B-cell antigen receptor phenotypes. Thus, phosphorylation of ZAP-70 mediates both activation and inactivation of antigen receptor signaling.

Stimulation of T and B lymphocytes through the T-cell receptor (TCR) and B-cell receptor (BCR), respectively, activates a cascade of protein tyrosine kinases (PTKs) that is required for lymphocyte cellular responses (5, 43). T and B cells appear to utilize analogous proximal signal transduction pathways in mediating these responses. Two distinct families of cytoplasmic PTKs, the Src- and Syk-PTK families, have been implicated in the proximal activation events for both BCRs and TCRs (6). In T cells, the Src-PTKs Lck and/or Fyn are thought to phosphorylate the two tyrosine residues within a 16-amino-acid motif (YXXLX$_{6-8}$YXXL, the immunoreceptor tyrosine-based activation motif [ITAM]) located in the cytoplasmic domains of CD3 and ζ chains. In B cells, the Src-PTKs Lyn, Fyn, and/or Blk are thought to phosphorylate the immunoglobulin α chain (Igα) and Igβ ITAM sequences. Phosphorylation of the ITAM provides docking sites for the two SH2 domains within the Syk-PTKs (ZAP-70 and Syk) which associate with the phosphorylated TCR and BCR complexes. The phosphorylation and activation of ZAP-70 and Syk following TCR and BCR engagement, respectively, are in part mediated by the Src-PTKs. Coexpression of Lck or Fyn with ZAP-70 or Syk in insect and COS cells results in catalytic activation of the Syk-PTKs (7, 9, 10, 12, 19, 24, 38, 41). Recent evidence also suggests that binding of Syk to the Igα, Igβ, or FcεRIγ ITAMs can mediate Syk activation and phosphorylation presumably by an allosteric mechanism (33, 34).

Expression of dominant negative forms or deletion of Src or Syk-PTKs abrogates thymocyte development and/or antigen receptor function (2, 3, 8, 11, 14, 21, 28, 29, 35, 36, 39). Activation of both families of PTKs is thought to be required to activate at least two distinct pathways, ras and calcium, to mediate transcriptional activation of cytokine genes. Recent evidence in anergic T cells has demonstrated that activation of these two pathways may be differentially regulated (15, 25). We examine here the role of ZAP-70 phosphorylation and activation in regulating the divergent pathways activated by the lymphocyte antigen receptor (20). In addition, we provide evidence that the catalytic activation of ZAP-70 and the phosphorylation of downstream effector molecules is initiated by the heterologous *trans*-phosphorylation of ZAP-70 at Tyr-493 by Lck. Finally, we provide evidence that phosphorylation of ZAP-70 at Tyr-492 and -292 mediates two distinct mechanisms for down regulation of antigen receptor function.

MATERIALS AND METHODS

Cells and antibodies. DT40 Syk$^-$ B cells, Jurkat T cells, and Sf9 cells (Pharmingen) were maintained as previously described (10, 23, 38). The M4 monoclonal antibody (MAb) directed against the chicken BCR was provided courtesy of M. Cooper, 2F3.2 is an anti-ZAP-70 MAb (Upstate Biotechnology Inc.), 9E10 is an anti-myc epitope MAb, 12CA5 is an anti-hemagglutinin epitope MAb (Boehringer Mannheim), and 4G10 is an antiphosphotyrosine MAb (Upstate Biotechnology Inc.). The anti-ZAP-70 immunoprecipitating antiserum used was raised against a peptide encoding amino acids 282 to 307 of human ZAP-70 as previously described (9). Fluorescein isothiocyanate-conjugated anti-mouse IgM and IgG were purchased from Sigma.

Construction of plasmids. ZAP-70 mutants Y292F, Y492F, and Y493F were constructed as previously described (10). A kinase-inactive version of ZAP-70 [designated ZAP-70(KD)] was produced by PCR-directed mutagenesis of Lys-369 to Arg. All constructs were subcloned into the *Eco*RI and *Sal*I sites of a modified version of the pApuro vector to generate hemagglutinin-tagged versions of human ZAP-70 (23). In addition, myc-tagged versions of wild-type ZAP-70 and ZAP-70(Y292F) were also subcloned into the p409 vector for tetracycline-regulated gene expression (16). Glutathione *S*-transferase (GST)–ZAP-70(KD) was constructed in the PVIKS vector (10). All constructs were confirmed by standard DNA sequencing methods. The interleukin 2 (IL-2)-luciferase and cytomegalovirus-chloramphenicol acetyltransferase (CMV-CAT) reporter genes were gifts from K. Murphy (Washington University, St. Louis, Mo.); the AP-1–luciferase reporter gene was a gift from T. Chatila (Washington University).

Cellular expression and biochemical analysis. Protocols for the generation of stable clones and the conditions for transient and stable transfections have been previously described (16, 23). For expression of genes under the control of the

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tetracycline-regulated promoter (16), a parental Jurkat T-cell line (449-3) that expressed a tetracycline-controlled transactivator was established. This parental line was then transfected with ZAP-70 mutants under the control of a tetracycline-dependent promoter. Clones were selected in G418 (1 mg/ml; Gibco), hygromycin (1 mg/ml), and tetracycline (1 μg/ml). For analysis, stable clones were washed and grown in the absence of tetracycline for 24 to 48 h prior to analysis.

For luciferase assays, cells were electroporated with 20 μg of IL-2– or AP-1–luciferase cDNA and 5 μg of CMV-CAT cDNA as previously described (23). Luciferase activity was determined with an OptocompII automated luminometer (MGM Instruments, Hamden, Conn.) and normalized for transfection efficiency with CMV-CAT. Generation of baculovirus-encoded proteins was performed as previously described (10). Stimulation of B cells, in vitro kinase assays, flow cytometric analysis, and calcium fluorimetry were performed as previously described (10, 23).

Analysis of ^{32}P-labeled ZAP-70(KD) tryptic peptides. GST–ZAP-70(KD) and GST-Lck(K) were immobilized on glutathione-Sepharose and incubated with [γ-^{32}P]ATP for 5 min in the presence of 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, and 10 mM $MnCl_2$. The in vitro-labeled proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and visualized by autoradiography. ^{32}P-labeled GST–ZAP-70 was digested with trypsin, and the resultant peptides were separated by two-dimensional thin-layer chromatography as previously described (10). Extraction of radiolabeled peptides and manual sequencing of separated peptides were performed as previously described (4, 26, 37).

Measurement of inositol phosphate generation. Incorporation of [^{3}H]inositol into phospholipids was achieved by incubating cells (10^6/ml) with 10 μCi of myo-[^{3}H]inositol (37 MBq/ml; Amersham) per ml for 18 h in inositol-free RPMI 1640 supplemented with 10% dialyzed fetal calf serum at 37°C. The cells were then washed extensively, and 5×10^6 cells were stimulated with an anti-BCR MAb (M4; 4 μg/ml) at 37°C in the presence of 10 mM LiCl. Extraction and separation of inositol phosphates were performed as previously described (18). Briefly, cells were extracted with chloroform-methanol, and soluble fractions were applied to a 0.6-ml AG 1-X8 (formate form) ion-exchange column (Bio-Rad) that was preequilibrated with 0.1 M formic acid. The columns were washed with 60 mM sodium formate and eluted with 0.2, 0.4, and 1 M ammonium formate to yield the IP_1, IP_2, and IP_3 fractions, respectively.

RESULTS

Positive and negative regulation of ZAP-70 by tyrosine phosphorylation. Peptide analysis of ZAP-70 has demonstrated that tyrosine residues 292, 492, and 493 are phosphorylated following TCR stimulation (10, 42). We have previously demonstrated that phosphorylation of Tyr-493 is required for catalytic activation of ZAP-70 and for efficient induction of cytokine release (10). To further explore the role of these three tyrosine residues in antigen receptor function, we analyzed a Syk$^-$ chicken B-cell line (DT40) that had been transfected with ZAP-70 cDNAs in which tyrosine 292, 492, or 493 had been mutated to phenylalanine, [designated ZAP-70(Y292F), ZAP-70(Y492F), or ZAP-70(Y493F), respectively]. While loss of Syk in these cells abrogates BCR-mediated signaling (38), expression of transfected wild-type ZAP-70 reconstitutes BCR-mediated signaling (23). Expression of wild-type ZAP-70 in Syk$^-$ DT40 cells reconstitutes the ability of the BCR to induce cellular tyrosine phosphoproteins, mobilize the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and induce IL-2 promoter activity following receptor cross-linking. A minimum of two clones expressing equivalent levels of ZAP-70 protein and BCR surface expression (Fig. 1) for each ZAP-70 mutation was analyzed.

To confirm our previous result that Tyr-493 is required for ZAP-70 activation, ZAP-70(Y493F) was expressed in DT40 Syk$^-$ cells. Cell lysates from resting or BCR-stimulated cells were analyzed by immunoblotting with an antiphosphotyrosine MAb (4G10). Analysis of a representative clone expressing ZAP-70(Y493F) demonstrated a reduction in tyrosine phosphorylation of proteins with M_rs of 170,000, 150,000, 120,000, and 80,000 compared with that with wild-type ZAP-70 (Fig. 2A, lanes 1 to 4, 9, and 10). A similar reduction in the cellular tyrosine phosphoproteins induced was also observed in cells expressing ZAP-70(KD) (Fig. 2A, lanes 11 to 14). Thus, the induction of these cellular tyrosine phosphoproteins is dependent on Tyr-493 as well as the catalytic activity of ZAP-70.

In contrast to Tyr-493, Tyr-292 and -492 serve to attentuate antigen receptor function. In two representative clones expressing ZAP-70(Y492F) or ZAP-70(Y292F), a qualitatively similar pattern of cellular tyrosine phosphoproteins was induced following BCR cross-linking (Fig. 2A, lanes 5 to 8, and 2B). Quantitatively, however, BCR cross-linking of cells expressing ZAP-70(Y492F) and ZAP-70(Y292F) consistently resulted in a small increase in tyrosine phosphorylation of cellular proteins compared with that in cells expressing wild-type ZAP-70. In view of these small differences, we analyzed more quantitative measures of the two major signaling pathways activated by antigen receptor cross-linking, calcium and ras.

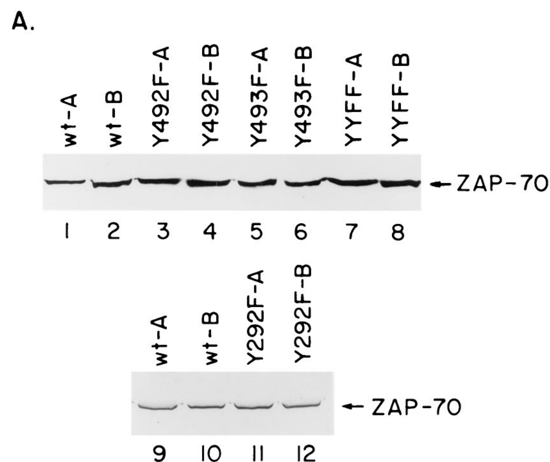
Consistent with the ability of ZAP-70(Y492F) to induce cellular tyrosine phosphoproteins, expression of ZAP-70(Y492F) also induced increases in $[Ca^{2+}]_i$ following BCR cross-linking (Fig. 3A). However, with lower concentrations of anti-BCR MAb, the increase in $[Ca^{2+}]_i$ flux was greater in cells expressing ZAP-70(Y492F) than in cells expressing wild-type ZAP-70 (Fig. 3B). Expression of ZAP-70(Y292F) also induced increases in $[Ca^{2+}]_i$ following BCR cross-linking (Fig. 3A), though no dosage differences in the stimulating antibody were found compared with cells expressing wild-type ZAP-70 (data not shown). Notably, cells expressing ZAP-70(Y493F) were unable to exhibit a $[Ca^{2+}]_i$ response.

Since mobilization of $[Ca^{2+}]_i$ is the result of activation of the phosphoinositide pathway, we measured the accumulation of phosphoinositide metabolites following BCR cross-linking (Fig. 4). Cells expressing ZAP-70(Y492F) or ZAP-70(Y292F) accumulated, respectively, approximately three- or fivefold more IP_1, IP_2, and IP_3 over the 20-min course of BCR stimulation compared with cells expressing wild-type ZAP-70. As expected, the phosphoinositide pathway was not activated in cells expressing ZAP-70(Y493F). Together, these data support the notion that phosphorylation of Tyr-292 and -492 serves to down regulate antigen receptor function.

These negative regulatory functions were also reflected in the ras pathway as measured by AP-1 transcriptional activation (Fig. 5). BCR cross-linking of cells expressing ZAP-70(Y492F) resulted in an approximately twofold greater induction of AP-1 transcriptional activation compared with that in cells expressing wild-type ZAP-70 (Fig. 5) [33% of phorbol myristate acetate (PMA) and ionomycin for the wild type versus 63% for ZAP-70(Y492F)]. As expected, cells expressing ZAP-70(Y493F) were unresponsive. The AP-1 measurements extend and are consistent with our previous observations utilizing the IL-2 promoter wherein cells expressing ZAP-70(Y492F) had approximately twofold greater IL-2 promoter activity in the presence of ionomycin and anti-BCR MAbs (10). However, in this study, the AP-1 measurements required only BCR cross-linking and did not have a corequirement for ionophore. Thus, the degree of AP-1 transcriptional activation can be ascribed solely to the true sensitivity of BCR-mediated signaling events without the contribution of exogenous pharmacologic agents.

Cells expressing ZAP-70(Y292F) had an even greater degree of both AP-1 and IL-2 promoter transcriptional activation compared with cells expressing wild-type ZAP-70 or ZAP-70(492F) (Fig. 5 and data not shown). An ~3.5-fold greater induction of AP-1 transcriptional activity was found following BCR activation in cells expressing ZAP-70(Y292F) compared with that in cells expressing wild-type ZAP-70 [33% for the wild type versus 112% for ZAP-70(Y292F)]. Therefore, the negative regulatory functions of Tyr-292 and -492 are reflected in both calcium and ras signaling pathways.

To determine the mechanisms by which Tyr-292 and -492



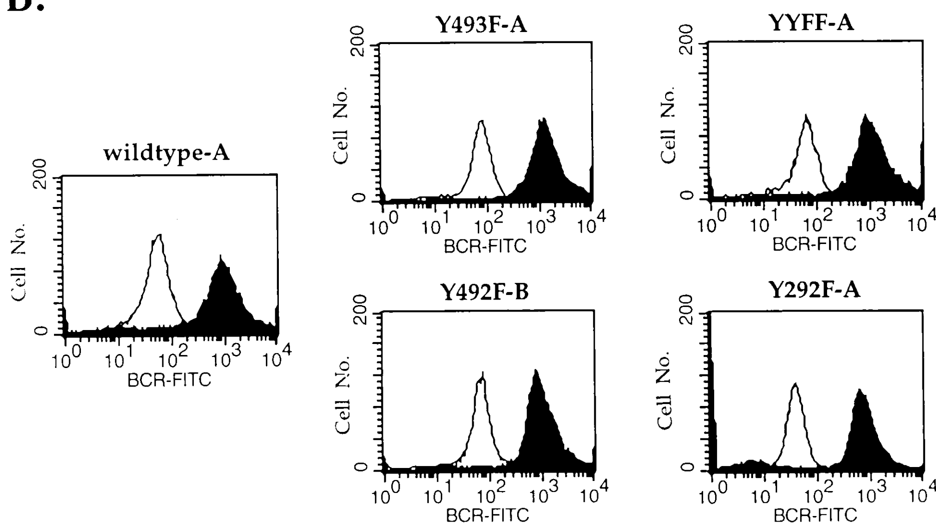


FIG. 1. (A) Expression of human ZAP-70 mutants (Y492F, Y493F, and YYFF) in Syk⁻ B cells. Syk⁻ cells expressing ZAP-70(Y492F), ZAP-70(Y493F), ZAP-70(YYFF), or ZAP-70(Y292F) were generated as described in Materials and Methods. Multiple clones were analyzed for ZAP-70 expression with two representative clones of each mutation shown here. Cell lysates (35 μg of total protein) were separated on a 9% polyacrylamide gel and analyzed by immunoblotting with an anti-ZAP-70 MAb (2F3.2). wt, wild type. The phenotype of the ZAP-70(YYFF) clones was indistinguishable from that of clones expressing ZAP-70(Y493F). (B) Flow cytometric analysis of BCR surface expression. All clones in panel A were analyzed by flow cytometric analysis, and the results from a representative clone from each transfection are shown. Open areas represent cells stained with a control antibody (anti-mouse IgM), and shaded areas represent cells stained with an anti-chicken IgM MAb (M4). FITC, fluorescein isothiocyanate.

may regulate antigen receptor function, we analyzed the catalytic activation of ZAP-70 in cells expressing ZAP-70(Y292F) or ZAP-70(Y492F) utilizing in vitro kinase assays (Fig. 6). While wild-type ZAP-70 activity was induced ~3.5-fold following BCR cross-linking, the catalytic activity of ZAP-70(Y492F) was induced ~6-fold compared with that of wild-type ZAP-70. However, the fold increases in ZAP-70 activity between resting and stimulated cells were comparable in cells expressing wild-type ZAP-70 and those expressing ZAP-70(Y492F). The basal activity of ZAP-70(Y492F) was also significantly higher than that of wild-type ZAP-70, consistent with observations in COS cells (41). In fact, the basal activity of ZAP-70(Y492F) in lymphocytes was almost comparable to the activity of the wild-type ZAP-70 following BCR cross-linking. In contrast, the catalytic activity of ZAP-70(Y292F) in lymphocytes (Fig. 6) and in insect cells (data not shown) was induced to approximately the same level as that of wild-type ZAP-70. Thus, phosphorylation of Tyr-292 does not appear to significantly alter the enzymatic activity of ZAP-70. Consistent with our observations in insect cells (10), no induction of enzymatic activity was seen with ZAP-70(Y493F) isolated from the Syk⁻ DT40 cells (Fig. 6). Together, these data indicate that the differential biochemical and functional effects of ZAP-70 phosphorylation can be measured in both calcium and ras effector pathways and support the notion that ZAP-70 is critically positioned to mediate both ras and calcium pathways following receptor engagement.

Since the receptor hypersensitivity seen with the ZAP-70(Y292F) mutation was analyzed in a B-cell line, we determined if a similar phenotype was observed in T cells. Jurkat T cells expressing wild-type ZAP-70 or ZAP-70(Y292F) under a tetracycline-regulated promoter were selected and analyzed for transcriptional activation of the IL-2 promoter. Jurkat T cells overexpressing wild-type ZAP-70 had an approximately ninefold induction of IL-2 transcriptional activity following TCR cross-linking (Fig. 7A). Consistent with the observations in the DT40 B-cell line, Jurkat T cells expressing ZAP-70 (Y292F) had an ~20-fold induction of TCR-induced IL-2 transcriptional activity (Fig. 7A). Equivalent levels of expression of wild-type ZAP-70 and ZAP-70(Y292F) was confirmed by Western blot (immunoblot) analysis with an anti-myc epitope MAb (9E10) (Fig. 7B). In addition, equivalent levels of TCR expression in these clones were confirmed by fluorescence-activated cell sorter analysis (data not shown). Thus, the negative regulatory mechanisms mediated through phosphorylation of Tyr-292 must exist in both T and B cells.

***trans*-Phosphorylation of ZAP-70 by Lck at Tyr-493.** Both ZAP-70 and the Src family of PTKs can contribute to the

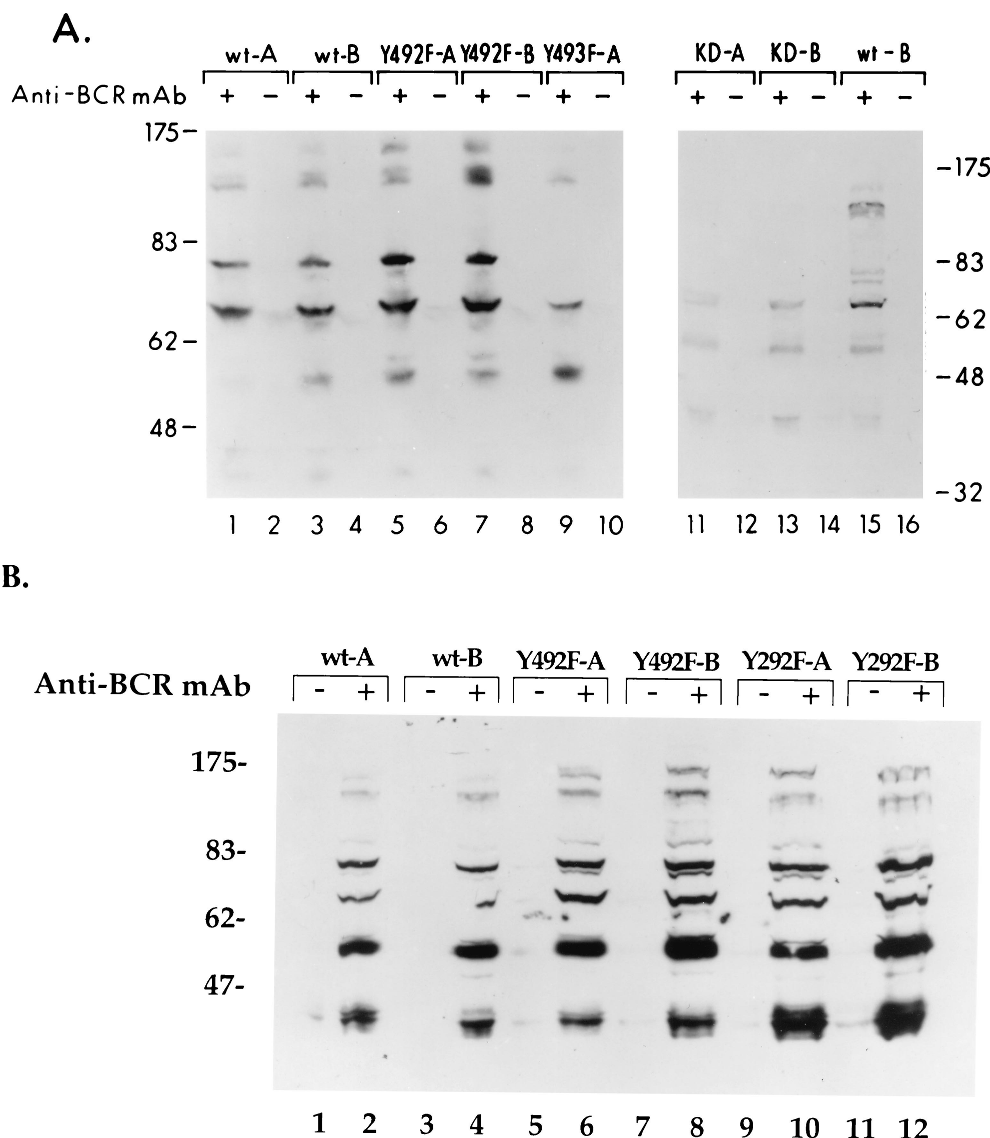
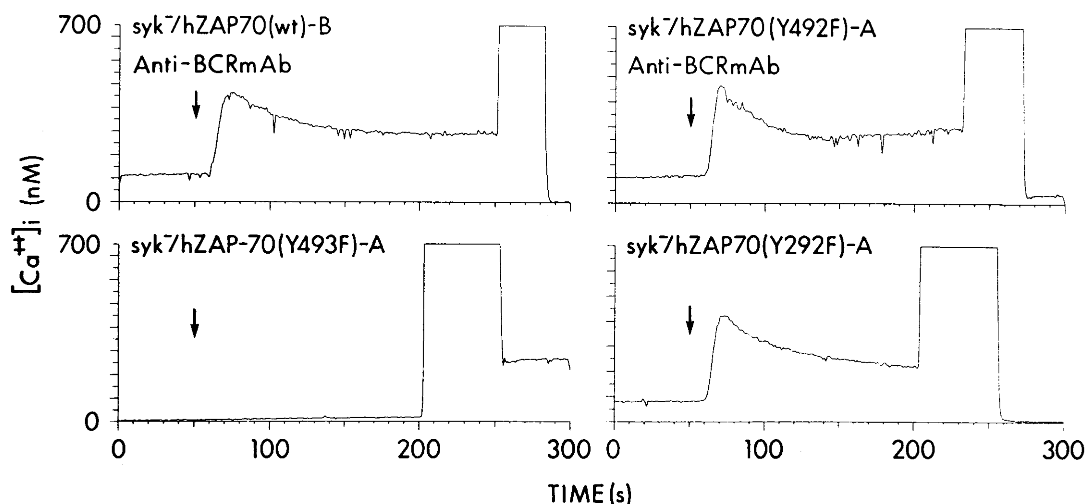


FIG. 2. (A) Induction of cellular tyrosine phosphoproteins following BCR cross-linking. Cellular tyrosine phosphorylation from cells expressing wild-type, Y492F, Y493F, or kinase-inactive (KD) forms of ZAP-70 (two clones for the wild type [wt], Y492F, and KD and one for Y493F) was analyzed in resting cells (even-numbered lanes) or following anti-BCR MAb (M4, 4 μg/ml for 2 min) stimulation (odd-numbered lanes). Cell lysates (5×10^6 cells) were analyzed by Western blotting with an antiphosphotyrosine MAb (4G10) and developed by chemiluminescence. The ~50-kDa protein does not represent the stimulating anti-BCR MAb, as cross-linking of the BCR on parental Syk$^-$ cells results in tyrosine phosphorylation of a subset of cellular proteins (23). (B) Increased tyrosine phosphorylation of cellular tyrosine phosphoproteins in cells expressing ZAP-70(Y292F) and ZAP-70(Y492F). Tyrosine phosphoproteins from cells expressing wild-type ZAP-70, ZAP-70(Y492F), and ZAP-70(Y292F) under resting or BCR-stimulated conditions were analyzed as described above. Sizes are indicated on the sides of both gels in kilodaltons.

phosphorylation and activation of ZAP-70. To dissect the contribution of each to ZAP-70 phosphorylation, we coinfected a kinase-inactive version of ZAP-70 [designated ZAP-70(KD)] with the Lck catalytic domain [designated Lck(K)] in insect cells. In the absence of Lck, no tyrosine phosphorylation of ZAP-70(KD) was detected by Western blotting with an antiphosphotyrosine MAb and no in vitro catalytic activity of ZAP-70(KD) was detected, as measured by band III phosphorylation (Fig. 8, lane 9). Interestingly, coinfection of GST–ZAP-70(KD) and Lck(K) also resulted in tyrosine phosphorylation of GST–ZAP-70(KD), but only at 1/10 of the level with wild-type ZAP-70 (Fig. 8, top panel, lanes 8 and 10). These data suggest that while Lck initiates phosphorylation of ZAP-70, autophosphorylation of ZAP-70 is required for maximal modification.

We also analyzed a kinase-inactive version of ZAP-70 in Syk$^-$ DT40 cells. Similar to the results obtained in insect cells, tyrosine phosphorylation of two representative Syk$^-$ DT40 clones expressing ZAP-70(KD) demonstrated decreased phosphorylation of ZAP-70 (Fig. 8, lanes 2, 4, and 6). Together, these data support the notion that ZAP-70 contributes to its own autophosphorylation following receptor engagement and that autophosphorylation likely follows phosphorylation of Tyr-493 by Src-PTKs (see below).

To determine if a specific tyrosine residue was preferentially phosphorylated by Lck, ZAP-70(KD) purified from insect cells was phosphorylated in vitro in the presence of GST-Lck(K) and [γ-^{32}P]ATP and analyzed by tryptic peptide analysis. One major peptide was preferentially phosphorylated (Fig. 9) and comigrated with a synthetic peptide, ALGADDSYY$_{PO_4}$TAR



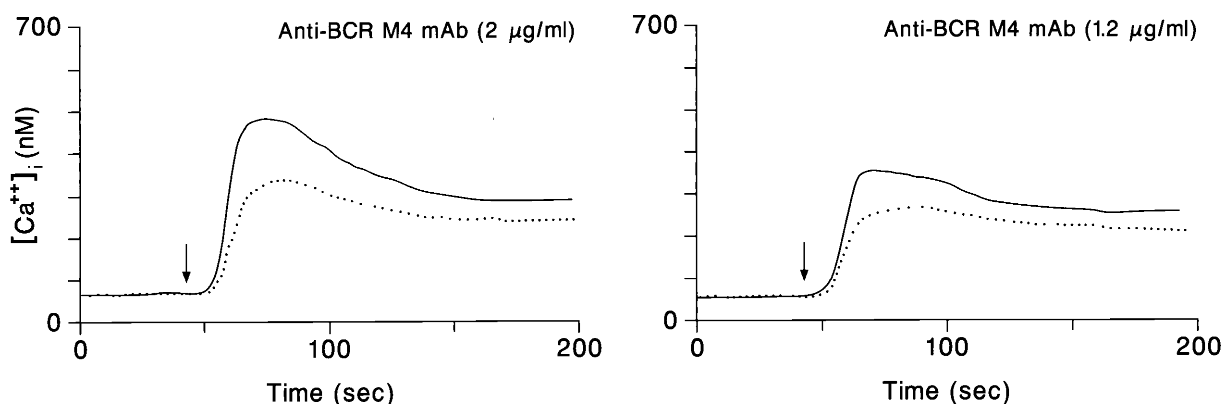


FIG. 3. (A) Calcium mobilization following BCR cross-linking. Cells were loaded with 3 μM Fura-2 for 40 min and analyzed as described in Materials and Methods. Cells (6×10^6) were stimulated with 4 μg of an anti-BCR MAb (M4; arrow) per ml and analyzed by spectrofluorimetry. Ionomycin was added at the end of each experiment to ensure proper loading of cells. The lower basal $[Ca^{2+}]_i$ seen in the ZAP-70(Y493F)-expressing clone was not consistently observed in other experiments. (B) Increased calcium mobilization in cells expressing ZAP-70(Y492F). Cells expressing wild-type ZAP-70 (. . .) or ZAP-70(Y492F) (——) were stimulated with 2 μg (left) or 1.2 μg (right) of an anti-BCR MAb (M4; arrow) per ml and analyzed as described above.

(data not shown). Additional peptides were also detected with prolonged exposures, but the major peptide was preferentially phosphorylated at least 10-fold in comparison with the other peptides. Manual sequencing of the major peptide revealed radiolabeled phosphate only in cycle 9, consistent with the sequence predicted by the cold peptide. Thus, Tyr-493 within ZAP-70 appears to be the preferential phosphorylation site for Lck. Since no major phosphopeptides corresponding to ALG ADDSY$_{PO_4}$YTAR or ALGADDSY$_{PO_4}$Y$_{PO_4}$TAR were identified, Tyr-492 is not a favored phosphorylation site for Lck relative to Tyr-493. In addition, since ZAP-70 is not phosphorylated in lymphocytes prior to TCR engagement (40), these data suggest that phosphorylation of ZAP-70 is initiated by a heterologous *trans*-phosphorylation of ZAP-70 by Lck on Tyr-493. This *trans*-phosphorylation in turn upregulates ZAP-70 catalytic activity, which then mediates ZAP-70 autophosphorylation and phosphorylation of downstream effector molecules.

DISCUSSION

Our data indicate that tyrosine phosphorylation plays a key role in ZAP-70 function. Phosphorylation of ZAP-70 on Tyr-493 is essential for antigen receptor-mediated activation of calcium and ras pathways. The data further support a model in which ZAP-70 is first phosphorylated by Lck at Tyr-493 to upregulate the catalytic activity of ZAP-70. This in turn permits additional phosphorylation of ZAP-70 mediated, in part, by autophosphorylation at sites including Tyr-292 and -492 (41). Phosphorylation of Tyr-493 and the catalytic activity of ZAP-70 itself are both required to trigger downstream signaling events, as indicated by the absence of antigen receptor-induced $[Ca^{2+}]_i$ flux or IL-2 gene synthesis in cells expressing ZAP-70(Y493F) or ZAP-70(KD) (references 10 and 23 and this manuscript). Consistent with this model, ZAP-70(KD) expressed in Syk$^-$ DT40 cells resulted in a markedly lower level

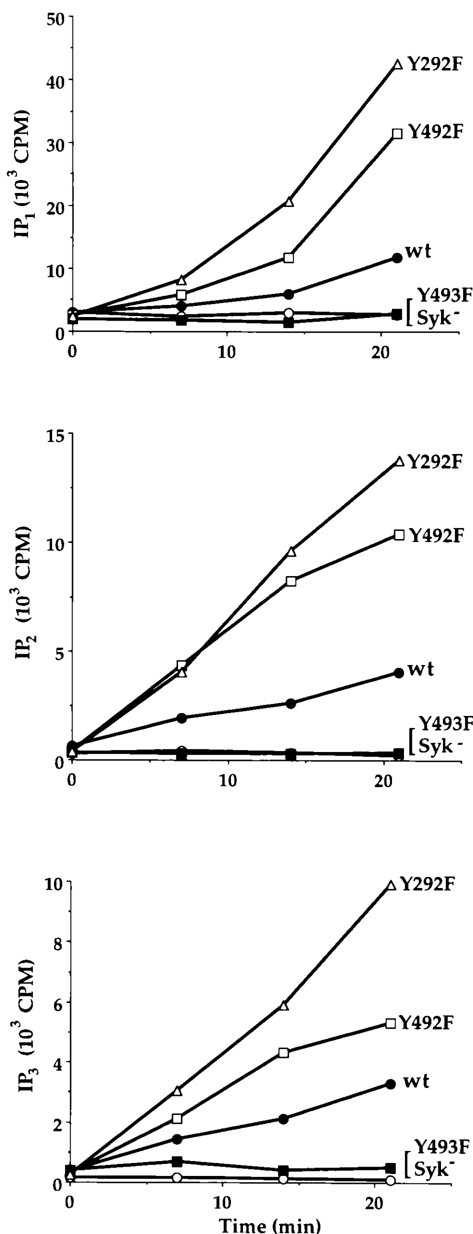


FIG. 4. BCR-mediated inositol phosphate generation. Syk$^-$, wild-type (wt), Y492F, Y493F, and Y292F cells were labeled with myo-[^{3}H]inositol as described in Materials and Methods and stimulated with M4 (4 μg/ml) for the indicated times. Soluble fractions of IP_1, IP_2, and IP_3 were separated by AG 1-X8 ion-exchange chromatography and quantitated by scintillography. Shown are results of one of four representative experiments with at least two independent clones from each stable transfection.

of ZAP-70 phosphorylation. Thus, ZAP-70 autophosphorylation represents a major contribution to the tyrosine phosphorylation of ZAP-70. This model of heterologous *trans*-phosphorylation of ZAP-70 at its activation loop by the Src-PTKs likely will serve as a paradigm for regulation of many receptor systems in which two or more PTKs are involved. In addition, the cascading effect of multiple PTKs provides additional levels for regulation and amplification of receptor activation.

Inference of ZAP-70 kinase domain structure from the structure of the insulin receptor (InsR) catalytic domain has provided some intriguing insights into the regulation by Tyr-492 and -493 (17). We and others have previously proposed (10, 41) that Tyr-1162 within the InsR is likely to be structurally homologous to Tyr-493 within ZAP-70. Tyr-1162 of the InsR, in the unphosphorylated state, is thought to be engaged in the active site and thus to block both substrate and ATP accessibility. Tyr-1162 becometo s phosphorylated via a *trans*-autophosphorylation mechanism following insulin binding. It has been proposed that phosphorylation then engages Tyr-1162 into an activating conformation and thereby permits both Mg-ATP and substrate accessibility to the catalytic site. This conformation is then further stabilized by salt bridges to Arg-1131 and/or Arg-1155 to provide the noninhibitory or activating conformation. Correspondingly, Tyr-493 of ZAP-70 appears to function in a similar fashion. In the unphosphorylated state, the *trans*-activation loop may reside in an inhibitory conformation which then is displaced upon phosphorylation of Tyr-493. However, in contrast to the InsR *trans*-autophosphorylation, the phosphorylation of Tyr-493 within ZAP-70 occurs through a heterologous *trans*-phosphorylation by the Src-PTKs. Thus, this heterologous *trans*-phosphorylation of ZAP-70 would then shift the activation loop into a noninhibitory conformation and upregulate ZAP-70 catalytic activity.

In contrast to the activating function of Tyr-493, both our previous biochemical and our present functional data indicate that mutation of Tyr-492 to Phe results in a hyperactive antigen receptor. In both insect and COS cells, ZAP-70(Y492F) exhibits a greater catalytic activity than wild-type ZAP-70 (10, 41). Our functional analysis of cells expressing ZAP-70(Y492F) supports these in vitro observations. Cells expressing ZAP-70 (Y492F) displayed a more active BCR-induced response. These cells demonstrated a higher degree of BCR-induced phosphoinositide turnover and calcium mobilization (Fig. 3 and 4), transcriptional activation of AP-1 promoter elements (Fig. 5), and IL-2 promoter activity (10). At least two mechanisms may account for this phenotype. Since ZAP-70(Y492F) exhibits a greater level of catalytic activity without being significantly tyrosine phosphorylated in COS cells (41) as well as in resting lymphocytes (Fig. 6 and data not shown), Wange and colleagues have proposed that mutation of Tyr-492 to Phe may result in the loss of hydrogen bonding, making the active site more accessible to substrate (41). A similar phenotype of increased basal activity is also seen in the InsR in which Tyr-1162 and -1163 are mutated to Phe (44). This then would explain the increased enzymatic activity of ZAP-70(Y492F) in the basal state. However, our functional analysis of Syk$^-$ DT40 cells expressing ZAP-70(Y492F) indicates that there is no increased basal activity of the antigen receptor as measured by increases in $[Ca^{2+}]_i$, phosphoinositide turnover, mitogen-activated protein kinase activation, or IL-2 or AP-1 promoter transcriptional activity (Fig. 3 to 5 and data not shown). In contrast, cells expressing an activated Src (i.e., v-src) or activated Lck (i.e., Tyr-505 to Phe) demonstrate constitutive induction of cellular tyrosine phosphoproteins, cytoplasmic calcium levels, and cytokine secretion (27, 31, 32), though this basal hyperactivity appears to be cell type specific (1). Thus, the mechanism by which Tyr-492 modulates ZAP-70 activity and receptor-mediated function may be distinct from that served by the homologous tyrosines in the InsR. For example, the two tyrosine residues within the *trans*-activation loop of the fibroblast growth factor 1 receptor are positioned differently compared with that of the InsR and may well be regulated in a different manner. These issues will require additional structural and biophysical analysis of the ZAP-70 catalytic domain.

While phosphorylation of Tyr-493 and -492 within the *trans*-activation loop of ZAP-70 can modulate antigen receptor function through regulation of ZAP-70 catalytic activity, phosphor-

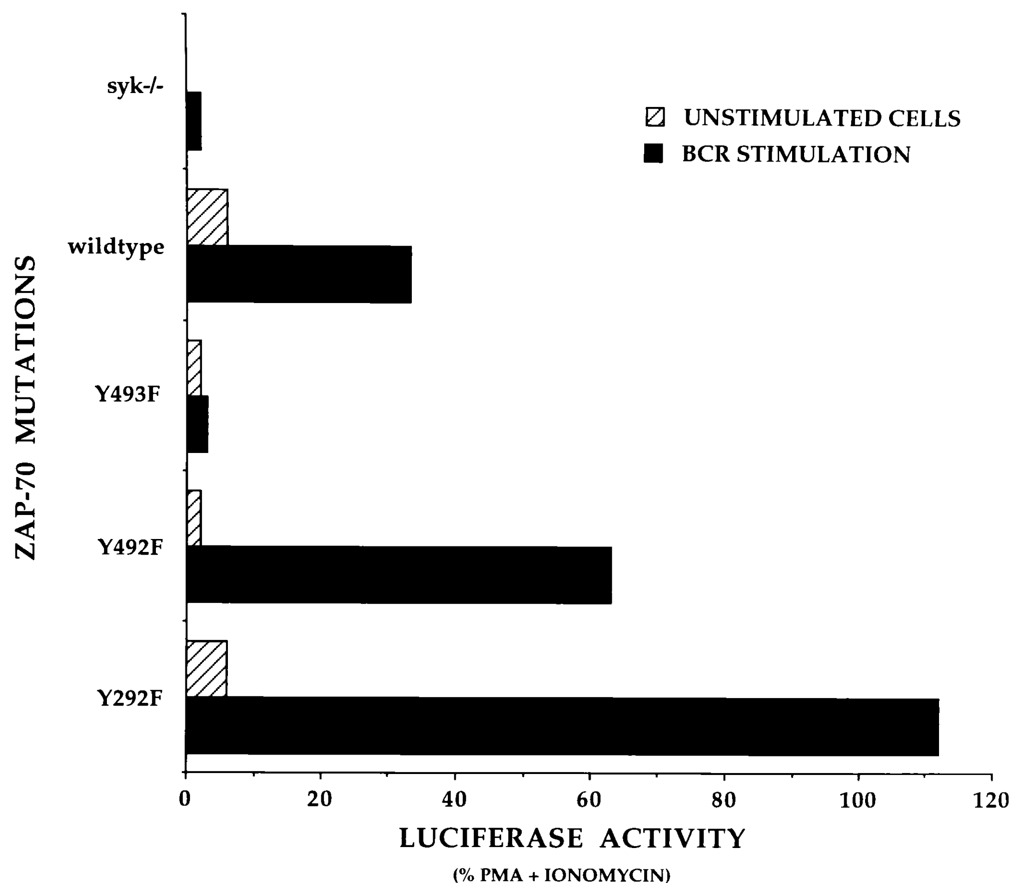


FIG. 5. AP-1 transcriptional activation. Cells transfected with AP-1 luciferase were analyzed as described in Materials and Methods. Cells were divided equally 48 h following electroporation and incubated with medium, anti-BCR MAb (4 μg/ml), or a combination of PMA (500 ng/ml) and ionomycin (1 μM). Cells were incubated at 37°C for 6 h and lysed, and luciferase activity was measured as described in Materials and Methods. The luciferase activity induced by PMA and ionomycin ranged from ~2,400 to 2,700 light units. Shown are results of one of three representative experiments.

ylation of Tyr-292 down regulates antigen receptor function through mechanisms independent of ZAP-70 activity. The mechanisms by which Tyr-292 and -492 regulate antigen receptor function are reflected not only in quantitative differences with cells expressing ZAP-70(Y292F) exhibiting a greater accumulation of inositol phospholipid metabolites and a greater degree of transactivation of cytokine promoter elements but also in subtle qualitative differences in their signaling phenotypes. Surprisingly, while cells expressing ZAP-70(Y492F) mobilize higher levels of calcium in response to receptor stimulation than do cells expressing wild-type ZAP-70, this difference is not seen in cells expressing ZAP-70(Y292F). Thus, additional mechanisms may exist to regulate the degree of calcium mobilization independent of phosphoinositide metabolism.

Tyrosine 292 lies in the linker region between the carboxyl-terminal SH2 and catalytic domains. Phosphorylation of Tyr-292 may induce a conformational change in ZAP-70 to stabilize its enzymatic activity following antigen receptor cross-linking. However, we did not detect gross differences in the enzymatic activities of wild-type ZAP-70 and ZAP-70(Y292F) in both insect cells and lymphocytes (Fig. 7 and data not shown). Rather, phosphorylation of Tyr-292 more likely mediates the recruitment and/or activation of negative regulators of antigen receptor function.

Together, these studies suggest that tyrosine phosphorylation of ZAP-70 mediates a multitude of regulatory and signaling functions. Evidence provided here indicates not only that

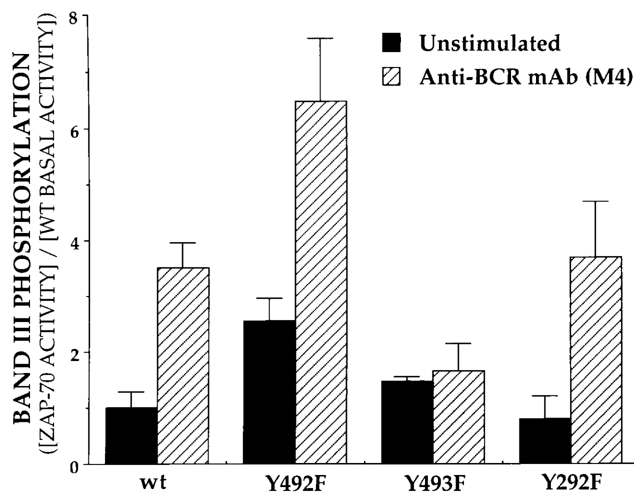


FIG. 6. Catalytic activation of ZAP-70. ZAP-70 immunoprecipitates (as described in the legend to Fig. 2B) were used to phosphorylate an exogenous substrate, band III, as described in Materials and Methods. The incorporation of [γ-^{32}P]phosphate in band III was quantitated with a PhosphorImager, and the results were normalized to the level of ZAP-70 protein in each reaction (as determined by densitometric analysis of an anti-ZAP-70 immunoblot). Standard deviations are depicted by the error bars.

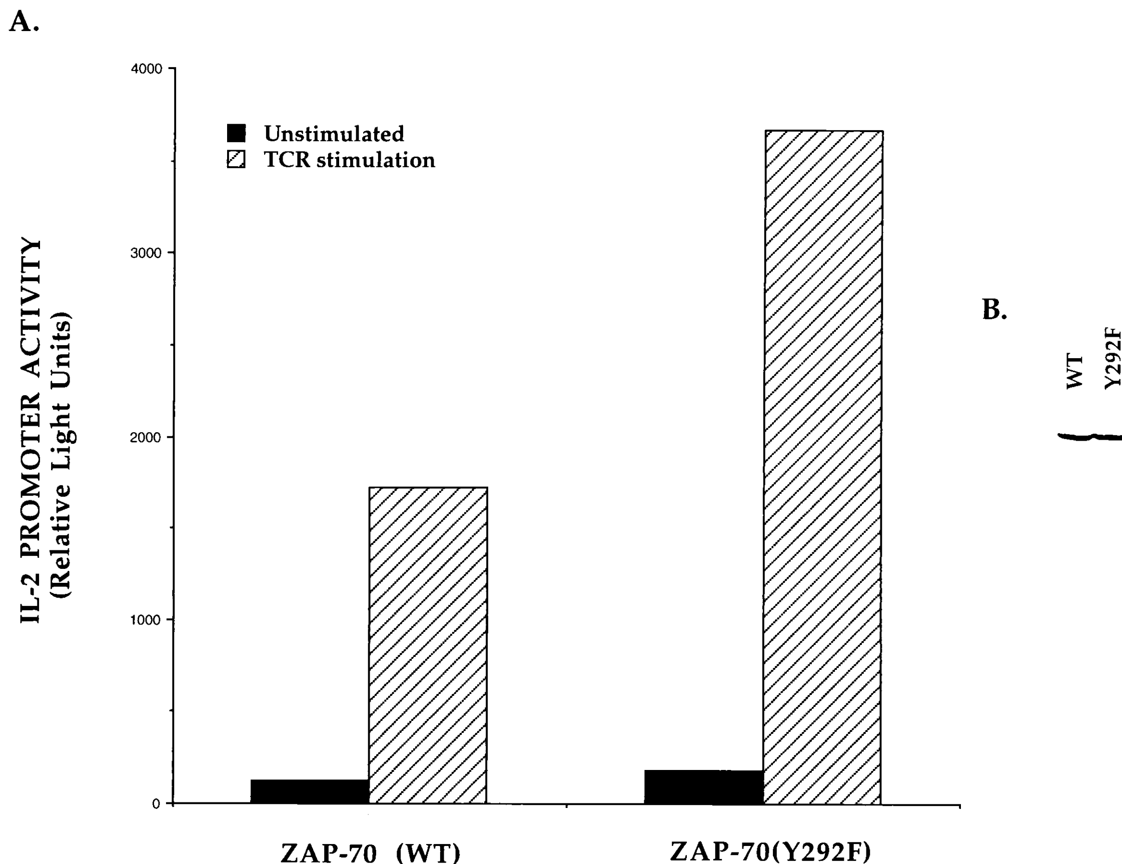


FIG. 7. (A) Increased IL-2 promoter transcriptional activation of T cells expressing ZAP-70(Y292F). Jurkat T cells expressing wild-type ZAP-70 (WT) or ZAP-70(Y292F) were transfected with IL-2–luciferase (20 μg) and CMV-CAT (5 μg) reporter genes. Cells were divided equally 48 h following electroporation and incubated with medium or with anti-TCR MAb (C305, 1:1,000) in the presence of PMA (500 ng/ml). Cells were incubated at 37°C for 6 h and lysed, and luciferase activity was measured as described in Materials and Methods. CAT activity was also determined for each transfection, and luciferase activity was normalized for transfection efficiency. Shown are results of one of two representative experiments from two independent clones. (B) Expression of the induced wild-type ZAP-70 or ZAP-70(Y292F). Equivalent levels of induced ZAP-70 protein were confirmed by Western blotting with an anti-myc epitope MAb (9E10). Protein expression of the exogenous genes was approximately fivefold greater than the level of endogenous ZAP-70 in these cells. In addition, these clones express equivalent levels of TCR and endogenous ZAP-70 (data not shown).

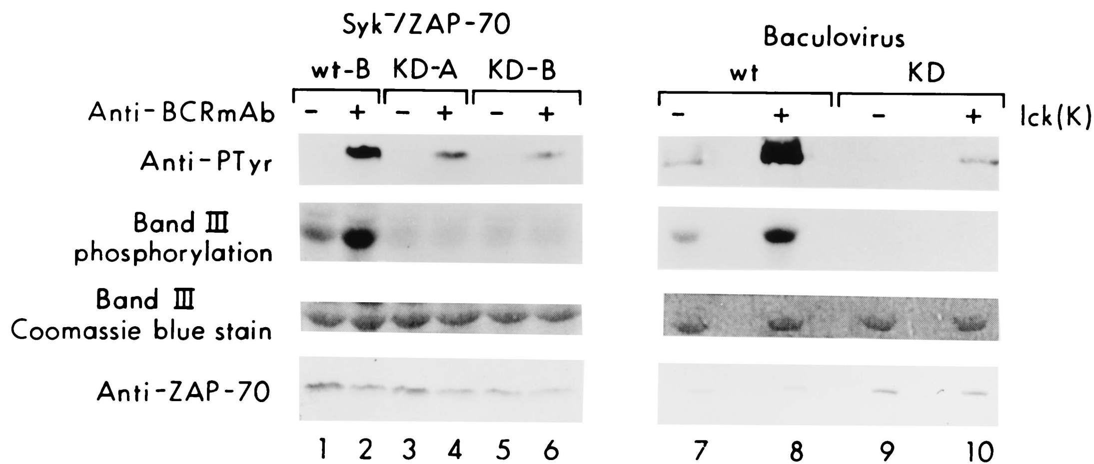


FIG. 8. Tyrosine phosphorylation of a kinase-inactive version of ZAP-70 is reduced. ZAP-70 immunoprecipitates from cells expressing the wild type (wt) or ZAP-70(KD) clones A and B were analyzed by antiphosphotyrosine MAb immunoblotting (top row) and by in vitro kinase assays using band III as an exogenous substrate (second row). Cells were analyzed both in resting conditions (lanes 1, 3, and 5) and following BCR (M4 MAb, 4 μg/ml) cross-linking (lanes 2, 4, and 6). Coomassie blue staining of band III (third row) and ZAP-70 immunoblotting (bottom row) were performed to ensure that comparable levels of protein were analyzed. Baculovirus-infected insect cells producing GST-fusion proteins encoding wild-type ZAP-70 and ZAP-70(KD) were coinfected with Lck(K) (lanes 8 and 10) and analyzed in a similar fashion.

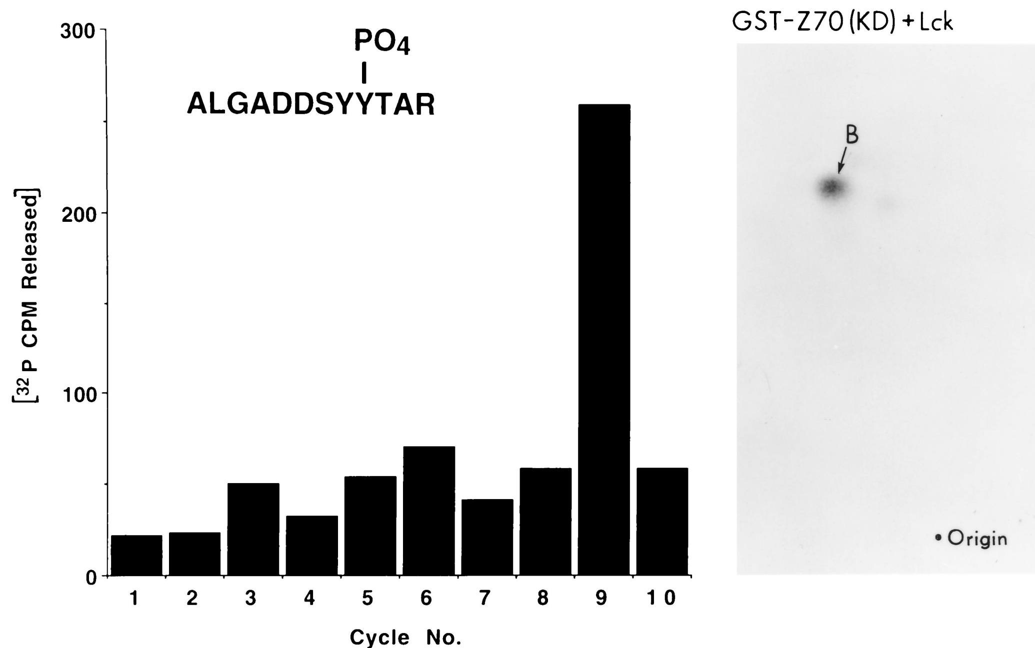


FIG. 9. Lck preferentially phosphorylates Y-493 of ZAP-70. Baculoviral GST–ZAP-70(KD) and GST-Lck(K) were produced separately in Sf9 cells and immobilized on glutathione beads. The fractions were then combined following extensive washing with lysis buffer and incubated with [γ-^{32}P]ATP for 15 min. The phosphorylated ZAP-70(KD) was then analyzed by tryptic peptide analysis and separated by thin-layer chromatography (right). The major phosphorylated peptide (B) was then analyzed by manual sequencing, and the radioactivity released from each sequencing cycle was quantitated (left). The arrow indicates the favored ^{32}P-labeled peptide B which comigrated with the synthetic peptide ALGADDSYY_{PO_4}TAR.

phosphorylation of ZAP-70 is required for upregulation of ZAP-70 activity and for antigen receptor function but also that phosphorylation of ZAP-70 mediates down regulation of antigen receptor function through multiple distinct mechanisms. In addition, phosphorylation of ZAP-70 provides docking sites for downstream SH2-containing effector molecules which themselves may serve as substrates for the activated enzyme (13, 22, 30). Thus, the complexities of ZAP-70 tyrosine phosphorylation warrant further investigation into how this critical enzyme effects its signaling and regulatory roles in lymphocyte function.

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