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Enhancement of Lymphocyte Responsiveness by a Gain-of-Function Mutation of ZAP-70

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The protein tyrosine kinase ZAP-70 plays an essential role in T-cell activation and development. After T-cell receptor stimulation, ZAP-70 is associated with the receptor and is phosphorylated on many tyrosine residues, including tyrosine 292 (Y-292), in the region between the C-terminal SH2 domain and the kinase domain (interdomain B). Here we show that a mutation of Y-292 (292F) or deletion of interdomain B enhanced the ability of ZAP-70 to reconstitute B-cell receptor stimulation-dependent NF-AT induction in a B-cell line deficient in Syk. In contrast, in a T-cell line, expression of 292F led to basal NF-AT induction independent of T-cell receptor stimulation. These results demonstrate that the role of Y-292 is to negatively regulate the function of ZAP-70 in lymphocytes. This appears to be a dominant function of interdomain B because deletion of most of interdomain B also resulted in a mutant of ZAP-70 with enhanced ability to reconstitute Syk-deficient DT-40 B cells. Since our biochemical studies did not reveal an effect of the 292F mutation on either the kinase activity of ZAP-70 or on the ability of ZAP-70 to bind to the receptor, we propose a model in which Y-292 interacts with an inhibitory protein to negatively regulate ZAP-70 function.

The T-cell receptor (TCR) and B-cell receptor (BCR) are multimeric protein complexes consisting of antigen binding subunits (αβTi for the TCR and membrane immunoglobulin [mIg] for the BCR) and the signal transducing invariant subunits (γ, δ, and ε subunits of CD3 complex and ζ proteins for the TCR; Igα and Igβ for the BCR) (reviewed in reference 48). Engagement of the TCR and BCR initiates a cascade of intracellular processes that lead to cellular response. One of the earliest detectable biochemical events after TCR and BCR stimulation is the tyrosine phosphorylation of multiple cellular protein substrates, including the invariant chains of the TCR and BCR (reviewed in references 3, 4, 12, 29, 35, and 48).

At least two families of protein tyrosine kinases (PTKs), the Src family and the Syk/ZAP-70 family, are implicated in regulating this tyrosine phosphorylation process (reviewed in reference 48). Both biochemical and genetic studies have demonstrated that Src family PTKs activated by TCR and BCR stimulation are required to phosphorylate the signal transducing subunits of these receptors (7, 19, 34, 39). This phosphorylation occurs on the two tyrosine residues present in a common signaling motif which exists as one copy in CD3γ, CD3δ, CD3ε, Igα, and Igβ and as three copies in TCR ζ (33). This motif, the immunoreceptor tyrosine-based activation motif (ITAM), consists of pairs of tyrosine and leucine residues arranged in the consensus sequence $YxxL(x)_{6-8}YxxL$, where x is variable. After TCR stimulation, ZAP-70 is recruited to the receptor complex through the interaction of its two SH2 domains with the doubly phosphorylated ITAMs (19, 46). This interaction is believed to be critical for TCR signaling, since ζ phosphopeptides that block the interaction of ZAP-70 with the ζ chain also inhibit TCR signaling events (45). The association of ZAP-70 with the TCR ITAMs facilitates its autophosphorylation and the tyrosine phosphorylation of ZAP-70 mediated by Src family PTKs (19, 27). The critical role for ZAP-70 in T-cell, not B-cell, activation and development has been demonstrated in patients with severe combined immunodeficiency who were deficient in ZAP-70 (2, 8, 15) and in mice which had been made deficient in ZAP-70 (26). Similarly, a critical role for Syk in B-cell, not αβ T-cell, activation and development has been shown both in chicken B cells (39) and in mice which had been made deficient in Syk (9, 41).

Like Syk, ZAP-70 is composed of three easily identifiable domains, a tyrosine kinase domain and two tandemly arranged SH2 domains (N terminal and C terminal) which mediate the association of ZAP-70 with the TCR after its stimulation (7, 19, 46). Between the two SH2 domains exists a region (60 amino acids [aa] in length; interdomain A) which forms a coiled-coil structure and is likely involved in bringing together the two SH2 domains which bind to the receptor ITAMs (18). Between the second SH2 domain and the kinase domain lies an additional region (84 aa in size; interdomain B) whose structure and function are unclear. Interdomain B contains a proline-rich sequence and several potential and documented tyrosine phosphorylation sites, including a putative vav binding site (Y-315) and an autophosphorylation site (Y-292). Recently, both electrospray ionization mass spectrometry and biochemical approaches have been used to identify tyrosine residues in ZAP-70 which are phosphorylated in vivo in Jurkat T cells after TCR stimulation (5, 47). These include tyrosine 292 (Y-292), which is located within interdomain B, and tyrosines 492 and 493 (Y-492 and Y-493, respectively), which are in the activation loop of the kinase domain. Mutation of Y-493 to phenylalanine (493F) significantly reduced both the intrinsic kinase activity and the ability of ZAP-70 to be activated catalytically by Src family PTKs in COS-7 cells and insect cells (5, 44). Moreover, 493F rendered ZAP-70 unable to reconstitute BCR-mediated signaling in Syk-deficient B cells (5). In contrast, mutation of Y-492 to phenylalanine (492F) increased the intrinsic kinase activity of ZAP-70 by fourfold in COS-7 cells (44) and enhanced the ability of ZAP-70 to reconstitute BCR-mediated signaling in Syk-deficient DT-40 B cells (5). Although Y-292 has been identified as one of the in vivo phosphorylation sites and also as the primary in vitro auto-

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phosphorylation site, its role in regulating ZAP-70 function remains unknown.

To determine the role of Y-292 in regulating ZAP-70 function, we mutated this residue to either phenylalanine (292F) to mimic the size of a tyrosine or to glutamic acid (292E) to mimic the charge of a phosphorylated tyrosine. By expressing the two mutants both in Syk-deficient DT-40 B cells and in T antigen (TAg)-Jurkat T cells, we examined their effects on NF-AT (nuclear factor of activated T cells) induction in the basal state or after antigen receptor stimulation in both cell types. In this study, expression of either 292F or 292E enhanced the ability of ZAP-70 to reconstitute BCR stimulation-dependent NF-AT activation in Syk-deficient DT-40 B cells. In contrast, in TAg-Jurkat cells, expression of either 292F or 292E led to basal activation of NF-AT, which was independent of TCR stimulation. Therefore, we conclude that the role of Y-292 is to negatively regulate the function of ZAP-70. This appears to be a dominant function of interdomain B because deletion of most of interdomain B also resulted in a mutant of ZAP-70 with an enhanced ability to reconstitute Syk-deficient DT-40 B cells. Since our biochemical studies did not reveal an effect of 292F on either the kinase activity of ZAP-70 or on the ability of ZAP-70 to bind to the receptor, we propose that Y-292 negatively regulates ZAP-70 function by interacting with an inhibitory protein.

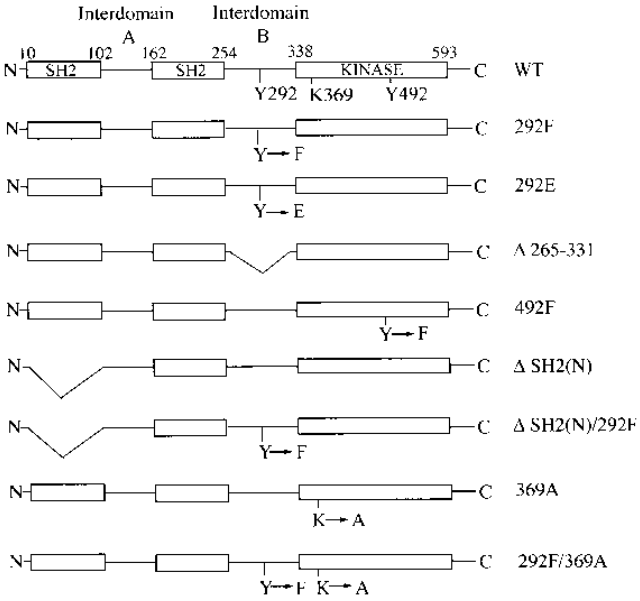

FIG. 1. Schematic representations of WT ZAP-70 and its mutant derivatives. Note that all the cDNAs encoding WT ZAP-70 and its mutant derivatives are myc epitope-tagged at the C terminus.

MATERIALS AND METHODS

Cells, antibodies, and peptides. Syk-deficient DT-40 B cells (provided by Tomohiro Kurosaki, Kansai Medical School, Moriguchi, Japan) were maintained as previously described (39). TAg-Jurkat T cells (provided by G. Crabtree, Stanford University, Stanford, Calif.), human leukemia Jurkat T cells stably transfected with simian virus 40 large T antigen, were maintained in RPMI 1640 supplemented with 5% fetal calf serum (FCS), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM L-glutamine. COS-7 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FCS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM L-glutamine. The antibodies used in this study included M4 (provided by M. Cooper and C. L. Chen, University of Alabama, Birmingham), an anti-chicken BCR μ chain monoclonal antibody (MAb); C305, an anti-Jurkat Ti chain MAb; 2F3.2, an anti-ZAP-70 MAb; 4G10 (Upstate Biotechnology, Inc., Lake Placid, N.Y.), an anti-phosphotyrosine MAb; and 9E10 (provided by J. M. Bishop, University of California, San Francisco), a MAb recognizing epitopic tag SMEQKLISEEDLN, derived from c-myc. The peptides used in this study represented biotinylated unphosphorylated and phosphorylated versions of the second ITAM of the TCR ζ chain as described previously (19).

Construction of plasmids. The parental plasmid for all the mutants used in this study was pSXSRα-ZAP-70-myc (provided by L. Samelson, National Institutes of Health, Bethesda, Md.). This form of ZAP-70 was created by subcloning into the pSXSRα vector ZAP-70 cDNA tagged with a myc epitope at the C terminus (44). All the mutants (292F, 292E, Δ265-331, and 492F) shown schematically in Fig. 1 were created by M13-based, oligonucleotide-directed, site-specific mutagenesis procedures, as previously described (53). The template used to make these mutations was full-length ZAP-70 cDNA subcloned into the double-stranded form of m13mp19. The oligonucleotides used for making these mutants were (mutated nucleotides are underlined) 5′ GGC TCA GGG GTG AAT CCA TCT GAG TTG AGG 3′ (ZAP-70 nucleotides [nt] 1096 to 1066; for 292F), 5′ GCT GGC TCA GGG GTC TCT CCA TCT GAG TTG AAG 3′ (ZAP-70 nt 1099 to 1066; for 292E), and 5′ GCG GGC AGT GTA GAA GCT GTC GTC GGC ACC 3′ (ZAP-70 nt 1697 to 1667; for 492F). The oligonucleotide used for making Δ265-331 spanned the ZAP-70 sequence from nt 966 to 1235 with an internal deletion from nt 1001 to 1199. After mutagenesis, the *Xho*I-*Bst*EII fragment containing each mutation was subcloned into pSXSRα-ZAP-70-myc. ΔSH2(N), with the entire N-terminal SH2 domain (aa 10 to 102) deleted, was created by PCR as described previously (19) and subcloned into pSM-ZAP-70 (7). ΔSH2(N) was subcloned into pSXSRα-ZAP-70-myc through the *Xba*I-*Xho*I fragment. ΔSH2(N)/292F was created by subcloning the *Xba*I-*Xho*I fragment containing the ΔSH2(N) mutation from ΔSH2(N) into pSXSRα-292F-myc. 369A, which changes the ATP binding site of ZAP-70 from lysine to alanine, was created as previously described (19, 31) and subcloned into pSXSRα-ZAP-70-myc through the *Xho*I-*Bst*EII fragment of ZAP-70 cDNA. The *Xmn*I-*Bst*EII fragment containing the 369A mutation was subcloned into pSXSRα-292F-myc to create 292F/369A. Each mutant was sequenced in the affected area to ensure the absence of additional mutations. The NF-AT luciferase reporter construct, in which the expression of luciferase is driven by three copies of the NF-AT DNA binding element, was provided by G. Crabtree. To create glutathione *S*-transferase (GST)–band III, two overlapping oligonucleotides representing band III aa 1 to 14 were annealed with *Bam*HI and *Eco*RI present at the ends. The annealed duplex was ligated to the pGEX-2TK vector through *Bam*HI and *Eco*RI. GST-band III was induced and affinity purified as previously described (37).

Electroporation, stimulation, luciferase assay, and solubilization for biochemical analysis. For Syk-deficient DT-40 B cells, cells (10^7) were washed with phosphate-buffered saline (PBS) once and transferred into a 0.4-cm-diameter cuvette (Bio-Rad Laboratories, Hercules, Calif.). Twenty micrograms of the NF-AT luciferase construct and the indicated amounts of plasmids containing wild-type (WT) ZAP-70 or its mutant derivatives were added to the cuvette containing cells. The transfection suspension was mixed and left on ice for 10 min. The mixture was electroporated with a Gene Pulser (Bio-Rad Laboratories) at 500 μF and 350 V and then left on ice for another 10 min. The electroporated cells were transferred to the medium used to maintain Syk-deficient DT-40 B cells, as described previously (39). For TAg-Jurkat T cells, cells (10^7) were transiently transfected by electroporation with a Bio-Rad Gene Pulser as previously described (51). Twenty-four to forty hours posttransfection, 2×10^5 transfected cells (either Syk-deficient DT-40 B cells or TAg-Jurkat T cells) were aliquoted into a U-bottom 96-well plate and cultured with various stimuli (medium alone, 1:1,000 dilution of C305 ascites for TAg-Jurkat cells, 1:40 dilution of M4 supernatant MAb for Syk-deficient DT-40 B cells, or 50 ng of phorbol myristate acetate (PMA) per ml and 1 μM ionomycin) in medium in a final volume of 100 μl. Six hours after stimulation at 37°C, cells were lysed in a buffer containing 1% Triton X-100, 100 mM KPO_4 (pH 7.8), and 1.0 mM dithiothreitol. Lysates (100 μl) were mixed with 100 μl of assay buffer (200 mM KPO_4 [pH 7.8], 10 mM ATP, and 20 mM $MgCl_2$) and 100 μl of 1.0 mM luciferin. Luciferase activity was measured immediately in a MONOLIGHT 2001 luminometer (Analytical Luminescence Laboratory Inc., San Diego, Calif.). For protein expression analysis, 5×10^5 transfected cells were lysed in 30 μl of a buffer containing 1% Nonidet P-40 (NP-40), 10 mM Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, and protease and phosphatase inhibitors, as described previously (31). For biochemical analyses, transfected TAg-Jurkat T cells were washed once with PBS, resuspended in PBS, equilibrated at 37°C for 15 to 20 minutes, stimulated with a 1:500 dilution of C305 ascites for 2 min, and lysed immediately in 1% NP-40-containing lysis buffer as described previously (31, 32).

DEAE-dextran transfection of COS-7 cells. COS-7 cells were transfected with DEAE-dextran as described previously (7). Twenty-four hours before transfection, cells were divided to 25% confluence on 100-mm-diameter plastic tissue culture dishes. Each dish was washed twice with prewarmed Opti-MEM prior to transfection. The transfection of 5 μg of DNA per plate was carried out in 2 ml of Opti-MEM supplemented with 250 μg of DEAE-dextran (Pharmacia) per ml and 100 μM chloroquine (Calbiochem). Four to five hours after transfection, the transfection mix was removed and the plates were washed once with DMEM supplemented with 5% FCS. Transfected cells were cultured for 60 to 70 h in DMEM supplemented with 10% FCS, harvested, and lysed in 1% NP-40-containing lysis buffer as described previously (7) for further analysis.

Immunoprecipitations, immunoblotting, and peptide binding. Immunoprecipitations, immunoblotting, and peptide binding were carried out as described previously (19, 31, 32).

In vitro immune complex kinase assay. For in vitro kinase assays, WT ZAP-70 and mutant ZAP-70 were immunoprecipitated and washed once with 1% NP-40-containing buffer as described previously (6), twice with 10 mM Tris (pH 7.4)–0.5 M LiCl, and once with kinase assay buffer (10 mM Tris [pH 7.4], 10 mM $MnCl_2$). In vitro kinase assays were performed at 25°C for 10 min in 30 μl of kinase assay buffer supplemented with 20 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; NEN) and 6 μg of GST-band III as an exogenous substrate. Reactions were stopped by the addition of sample buffer and heated to 100°C for 5 min. Then samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride membrane, and treated with 1 M KOH for 1 h. The treated membrane was then subjected to autoradiography for in vitro phosphorylation and to immunoblotting for protein expression.

RESULTS

BCR stimulation-mediated NF-AT induction was reconstituted by ZAP-70 in Syk-deficient DT-40 B cells. To study the structure-function relationship of ZAP-70, we constructed several ZAP-70 mutants and introduced them into a chicken B-cell line which is deficient in Syk expression. It has been previously shown that ZAP-70 can compensate for the defect in Syk, as determined by the induction of cellular tyrosine phosphoproteins, the mobilization of cytoplasmic calcium, and the induction of interleukin-2 (IL-2) gene expression (22). To establish a transient-transfection system in this cell line, we first optimized the transfection conditions and the luciferase reporter system in its parental line (WT DT-40 cells). Using the optimized transfection conditions and reporter system, we found that NF-AT luciferase activity can be induced more than 100-fold after BCR stimulation in this cell line (data not shown), which is consistent with previous reports that certain B-cell lines can secrete IL-2 and induce NF-AT binding activity after BCR stimulation (10, 43). The NF-AT luciferase reporter construct, in which the luciferase gene is driven by three copies of NF-AT binding sites, has been used as a reporter system of TCR-mediated signaling (14). When the NF-AT luciferase construct was introduced into Syk-deficient cells, no luciferase activity was induced after BCR stimulation. However, when ZAP-70 and the NF-AT luciferase construct were cotransfected, the induction of luciferase activity reached more than 15-fold above that of control vector-transfected cells after BCR stimulation (Fig. 2A). This confirmed a previous report that the expression of ZAP-70 is both necessary and sufficient to reconstitute BCR-mediated signaling in Syk-deficient B cells (22) and allowed us to study the structure-function relationship of ZAP-70.

Mutation of Y-292 enhanced the ability of ZAP-70 to reconstitute BCR stimulation-dependent NF-AT induction in Syk-deficient DT-40 B cells. Although Y-292 is phosphorylated in vivo and autophosphorylated in vitro, its functional implications remain unknown. To examine the role of this residue in regulating the function of ZAP-70, we mutated it to phenylalanine (292F) to mimic the size of a tyrosine or to glutamic acid (292E) to mimic the charge of a phosphorylated tyrosine. Expression of either mutant at a level comparable to that of WT ZAP-70 resulted in four- to eightfold-higher reconstitution of NF-AT induction than that of WT ZAP-70 in Syk-deficient DT-40 B cells after BCR stimulation (Fig. 2A and B). NF-AT induction mediated by stimulation with PMA plus ionomycin was not significantly affected in cells transfected with WT ZAP-70, 292F, or 292E, indicating that the effect is specific to BCR stimulation (data not shown). The enhanced effects of Y-292 mutants appear to be dose dependent (Fig. 2C). These results demonstrate that Y-292 plays an inhibitory role in regulating ZAP-70 function.

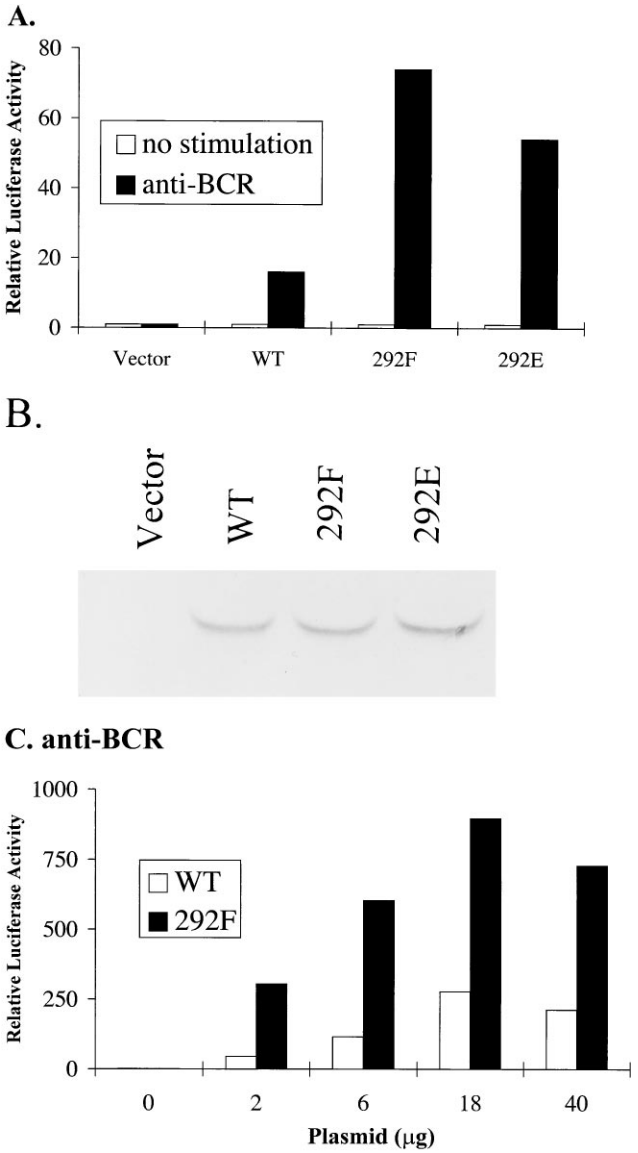

FIG. 2. Effects of WT ZAP-70 and its mutant derivatives on BCR-mediated NF-AT induction in Syk-deficient DT-40 B cells. (A) Syk-deficient DT-40 cells were cotransfected with 20 μg of the NF-AT luciferase reporter construct together with either 40 μg of empty expression vector (Vector) or expression plasmids containing cDNAs encoding WT ZAP-70 and its indicated mutant derivatives. Twenty-four to forty hours later, transfected cells were stimulated with culture medium (no stimulation) or anti-BCR. Six hours poststimulation, cells were lysed and the luciferase activity was determined. Relative luciferase activity is the luciferase activity produced in cells transfected with WT ZAP-70 and its mutant derivatives divided by the activity produced in the presence of an empty expression vector in the presence of medium. The PMA-plus-ionomycin responses in cells transfected with WT ZAP-70 and its mutant derivatives were similar (not shown). Data are representative of at least five independent experiments. (B) Expression of WT ZAP-70 and its mutant derivatives in Syk-deficient DT-40 cells. The expression of each construct was examined by Western blot (immunoblot) analysis for ZAP-70 (2F3.2) in cells from each transfection. Data are representative of at least five independent experiments. (C) Syk-deficient DT-40 cells were cotransfected with the indicated amounts of WT ZAP-70 and 292F together with 20 μg of the NF-AT luciferase construct. The amount of DNA was adjusted to 60 μg with the empty expression vector. Twenty-four to forty hours later, cells were stimulated with medium (not shown), anti-BCR, or PMA plus ionomycin. The relative luciferase activity was determined as described for panel A. The PMA-plus-ionomycin responses in cells transfected with WT ZAP-70 and 292F were similar (not shown). Data are representative of two separate experiments.

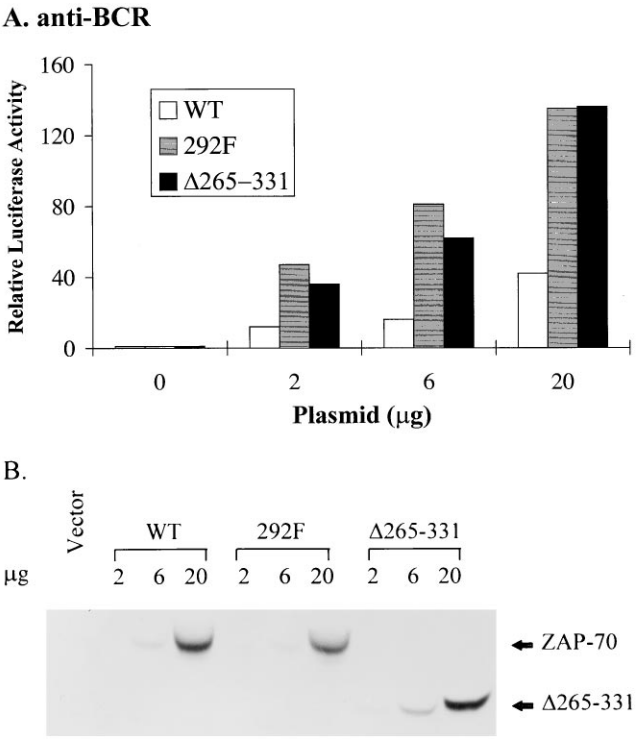

FIG. 3. Effects of the Δ265-331 mutation on BCR-mediated NF-AT induction in Syk-deficient DT-40 cells. (A) Syk-deficient DT-40 cells were cotransfected with the indicated amounts of WT ZAP-70, 292F, or Δ265-331 with 20 μg of the NF-AT luciferase reporter. The amount of DNA was adjusted to 40 μg with the empty vector. Twenty-four to forty hours later, cells were stimulated with medium (not shown), anti-BCR, or PMA plus ionomycin. The relative luciferase activity was determined as described in the legend to Fig. 2A. The PMA-plus-ionomycin responses in cells transfected with WT ZAP-70 and its mutant derivatives were similar (not shown). (B) Expression of WT ZAP-70 and its mutant derivatives was determined as described in the legend to Fig. 2B. Data are representative of two independent experiments.

Deletion of interdomain B encompassing Y-292 also enhanced the ability of ZAP-70 to reconstitute NF-AT induction in Syk-deficient DT-40 B cells. In contrast to other families of PTKs, ZAP-70 contains a relatively large region (84 aa; interdomain B) between the second SH2 domain and the kinase domain. Interdomain B consists of several putative structural motifs, including a proline-rich sequence and several potential tyrosine phosphorylation sites, such as the putative vav binding site (Y-315), in addition to Y292. Surprisingly, deletion of 80% of interdomain B (Δ265-331) still permitted ZAP-70 to reconstitute NF-AT induction in Syk-deficient DT-40 B cells in a dose-dependent manner (Fig. 3). Moreover, the reconstituting ability of Δ265-331 ZAP-70 was very similar to the effect mediated by the 292F point mutation in these cells (Fig. 3). These results suggest that the overall function of interdomain B, likely dominated by the function of Y-292, is to serve a regulatory function in ZAP-70.

Expression of 292F led to TCR stimulation-independent induction of NF-AT in TAg-Jurkat cells. To examine the effects of 292F or 292E on TCR signaling, we introduced these two mutants into TAg-Jurkat T cells. Interestingly, unlike the effects observed in Syk-deficient DT-40 B cells, expression of 292F or 292E led to constitutive activation of NF-AT in TAg-Jurkat cells (30- to 60-fold NF-AT activity), whereas comparable expression of WT ZAP-70 resulted only in minimal, if any, basal induction of NF-AT (Fig. 4A and C). The constitutive induction of NF-AT by 292F was dose dependent (Fig. 4D). NF-AT induction mediated by 292F could be further increased upon TCR stimulation (Fig. 4B and C); this effect was also dose dependent (Fig. 4E). TCR stimulation induced only twice the NF-AT activity in 292F-transfected cells compared with that of WT ZAP-70-transfected cells even though WT ZAP-70 and 292F were expressed at comparable levels (Fig. 4B; also data not shown). These results demonstrate that expression of the Y-292 mutant leads to TCR stimulation-independent activation of NF-AT in TAg-Jurkat cells and that TCR stimulation can further increase this response.

Expression of 492F failed to lead to TCR stimulation-independent NF-AT induction in TAg-Jurkat cells. Tyrosine 492 (Y-492) is another tyrosine of ZAP-70 that is phosphorylated in vivo after TCR stimulation (5, 47). It has been previously reported that a mutation of Y-492 (492F) can increase the intrinsic kinase activity by fourfold in COS-7 cells (44). Moreover, 492F enhanced the ability of ZAP-70 to reconstitute NF-AT induction in Syk-deficient B cells after BCR stimulation (5). These results indicated that Y-492 also functions to negatively regulate ZAP-70 function. To determine whether the negative regulatory functions of Y-292 and Y-492 operate in the same manner, we compared the effects of mutations of ZAP-70 at these sites upon NF-AT induction in both Syk-deficient DT-40 B cells and TAg-Jurkat cells. Comparable levels of expression of either 292F or 492F led to similar dose-dependent enhancement of NF-AT induction in Syk-deficient B cells after BCR stimulation (Fig. 5A and B). However, 492F failed to induce activation of NF-AT in TAg-Jurkat cells in the absence of TCR stimulation (Fig. 5C). These results suggest that these two sites use different mechanisms to negatively regulate ZAP-70 function. Since Y-492 is located in the putative activation loop of the kinase domain and 492-F has been shown to upregulate the intrinsic kinase activity (44) (see Fig. 6C), we suggest that Y-292 uses different mechanisms to negatively regulate ZAP-70 function.

292F failed to affect the kinase activity of ZAP-70. After TCR stimulation, the kinase activity of ZAP-70 is increased by three- to fourfold, which results, at least in part, from its tyrosine phosphorylation. This catalytic activation is critical for antigen receptor-mediated signal transduction. Therefore, one possible mechanism by which Y-292 acts to negatively regulate ZAP-70 function is to affect the kinase activity of ZAP-70. To examine this possibility, we performed in vitro kinase assays on the anti-myc immunoprecipitates isolated from lysates of COS-7 cells transfected with myc epitope-tagged WT ZAP-70 and 292F. Both autophosphorylation of these two proteins and the phosphorylation of the exogenous substrate band III were examined. Band III has been used as a relatively specific substrate for Syk/ZAP-70 PTKs (5, 17, 44). To ensure that the two proteins had comparable levels of expression, we titrated the concentrations of lysates expressing WT ZAP-70 and 292F (Fig. 6B). Although Y-292 has been identified as one of the sites for in vitro autophosphorylation, 292F showed similar kinase activities when compared with those of WT ZAP-70 toward itself and band III (Fig. 6A and B). Under the same assay conditions, 492F upregulated the kinase activity of ZAP-70 by approximately threefold above that of 292F (Fig. 6C). Furthermore, we reproducibly failed to show any difference in activated kinase activity between WT ZAP-70 and 292F when Lck was cotransfected into COS-7 cells (data not shown). Moreover, in vitro kinase assays performed with anti-myc immunoprecipitates of WT ZAP-70 and 292F from lysates of either Syk-deficient B cells or TAg-Jurkat cells transfected with myc epitope-tagged WT ZAP-70 and 292F before and after antigen receptor stimulation did not reveal any differ-

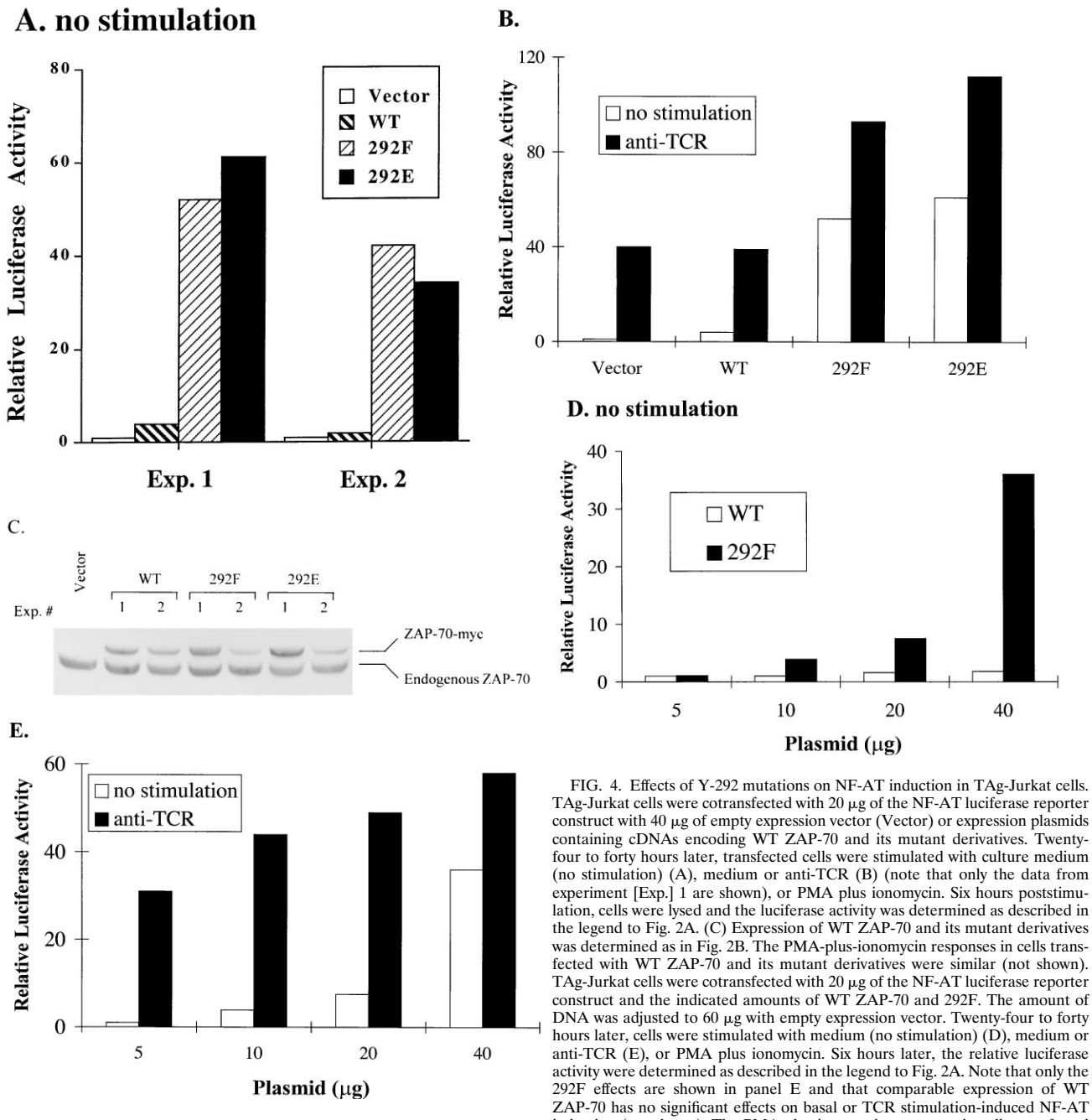

FIG. 4. Effects of Y-292 mutations on NF-AT induction in TAg-Jurkat cells. TAg-Jurkat cells were cotransfected with 20 μg of the NF-AT luciferase reporter construct with 40 μg of empty expression vector (Vector) or expression plasmids containing cDNAs encoding WT ZAP-70 and its mutant derivatives. Twenty-four to forty hours later, transfected cells were stimulated with culture medium (no stimulation) (A), medium or anti-TCR (B) (note that only the data from experiment [Exp.] 1 are shown), or PMA plus ionomycin. Six hours poststimulation, cells were lysed and the luciferase activity was determined as described in the legend to Fig. 2A. (C) Expression of WT ZAP-70 and its mutant derivatives was determined as in Fig. 2B. The PMA-plus-ionomycin responses in cells transfected with WT ZAP-70 and its mutant derivatives were similar (not shown). TAg-Jurkat cells were cotransfected with 20 μg of the NF-AT luciferase reporter construct and the indicated amounts of WT ZAP-70 and 292F. The amount of DNA was adjusted to 60 μg with empty expression vector. Twenty-four to forty hours later, cells were stimulated with medium (no stimulation) (D), medium or anti-TCR (E), or PMA plus ionomycin. Six hours later, the relative luciferase activity were determined as described in the legend to Fig. 2A. Note that only the 292F effects are shown in panel E and that comparable expression of WT ZAP-70 has no significant effects on basal or TCR stimulation-induced NF-AT induction (not shown). The PMA-plus-ionomycin responses in cells transfected with WT ZAP-70 and 292F were similar (not shown). Data are representative of three independent experiments.

ences in the kinase activities of these two proteins toward themselves (autophosphorylation) and band III (data not shown). Consistent with our results is a recent observation that 292F does not affect the basal and BCR-stimulated kinase activities of ZAP-70 in Syk-deficient DT-40 cells (23). These results suggest that 292F does not affect the catalytic activity of ZAP-70.

292F failed to affect the ability of ZAP-70 to bind to the receptor ITAMs. After TCR stimulation, ZAP-70 is recruited to the antigen receptor complex via its interaction with receptor ITAMs, a step critical for ZAP-70 activation. Therefore, one possible mechanism by which Y-292 negatively regulates ZAP-70 function is by affecting the binding of ZAP-70 to the receptor ITAM. To examine this possibility, we conducted two types of experiments. First, we blotted with an anti-phosphotyrosine antibody the anti-myc immunoprecipitates from lysates of TAg-Jurkat cells transfected with myc epitope-tagged WT ZAP-70 and 292F. No difference in binding to tyrosine-phosphorylated TCR ζ chain either in the basal state or after TCR stimulation was observed (Fig. 7A), suggesting that 292F did not affect the accessibility of ZAP-70 to the receptor ITAM; nor could we detect an increase in ZAP-70 tyrosine phosphorylation in cells transfected with myc-tagged 292F in the basal state or after TCR stimulation. Secondly, we used a biotinylated doubly phosphorylated peptide representing the

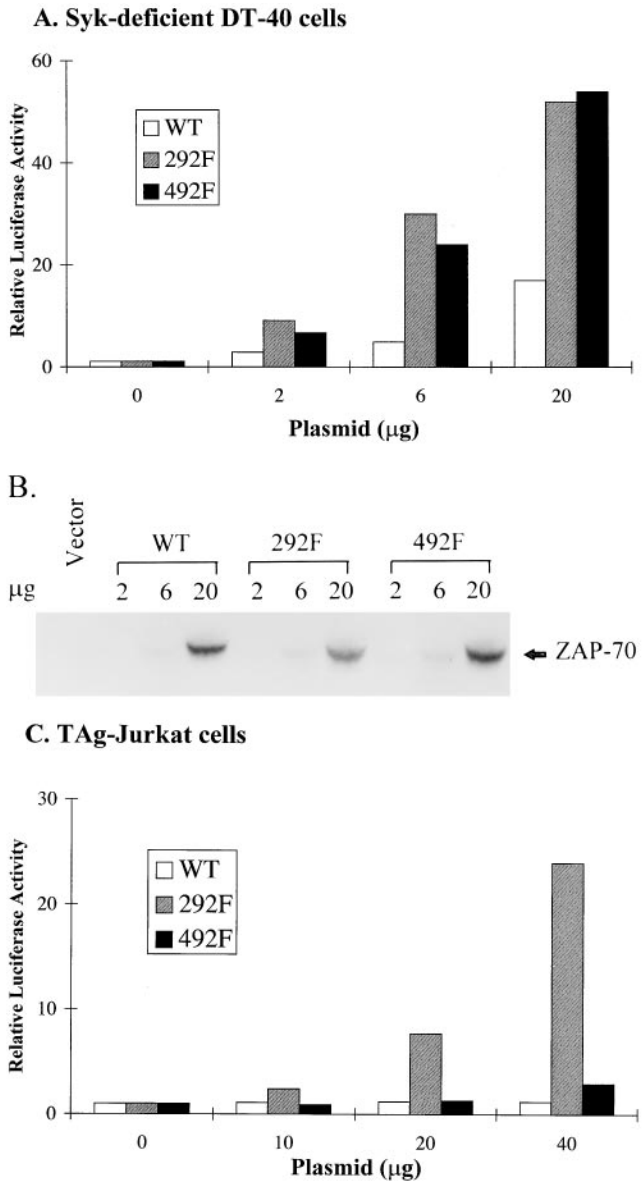

FIG. 5. Comparison of the effects of 292F and 492F on NF-AT induction in Syk-deficient DT-40 cells and in TAg-Jurkat cells. (A) Syk-deficient DT-40 B cells were cotransfected with 20 μg of the NF-AT luciferase reporter construct and the indicated amounts of WT ZAP-70 and its mutant derivatives. The amount of DNA was adjusted to 40 μg with the empty expression vector. Twenty-four to forty hours later, cells were stimulated with medium (not shown), anti-BCR, or PMA plus ionomycin. The relative luciferase activity was determined as described in the legend to Fig. 2A. The PMA-plus-ionomycin responses in cells transfected with WT ZAP-70 and its mutant derivatives were similar (not shown). (B) The expression of each construct at different concentrations was determined as described in the legend to Fig. 2B. Data in panels A and B are representative of two separate experiments. (C) TAg-Jurkat cells were transfected with 20 μg of the NF-AT luciferase reporter construct and the indicated amounts of WT ZAP-70 and its mutant derivatives. The amount of DNA for each transfection was adjusted to 60 μg with the empty expression vector. Twenty-four to forty hours later, cells were stimulated with medium. Six hours later, cells were lysed and the relative luciferase activity was determined as described in the legend to Fig. 2A. Data in panel C are representative of three independent experiments.

second ITAM of TCR ζ chain to precipitate ZAP-70 proteins from lysates of COS-7 cells transfected with WT ZAP-70 or 292F. No difference in binding to the doubly phosphorylated ITAM peptide was seen between WT ZAP-70 and 292F (Fig. 7B). Also, when titration studies were conducted with the same peptide, comparable amounts of WT ZAP-70 and 292F were bound by the peptide at each concentration (data not shown). These data suggested that 292F failed to affect the affinity of ZAP-70 to bind to the receptor ITAM. Taken together, these results suggest that 292F does not affect the interaction of ZAP-70 with the receptor or its intrinsic phosphorylation status.

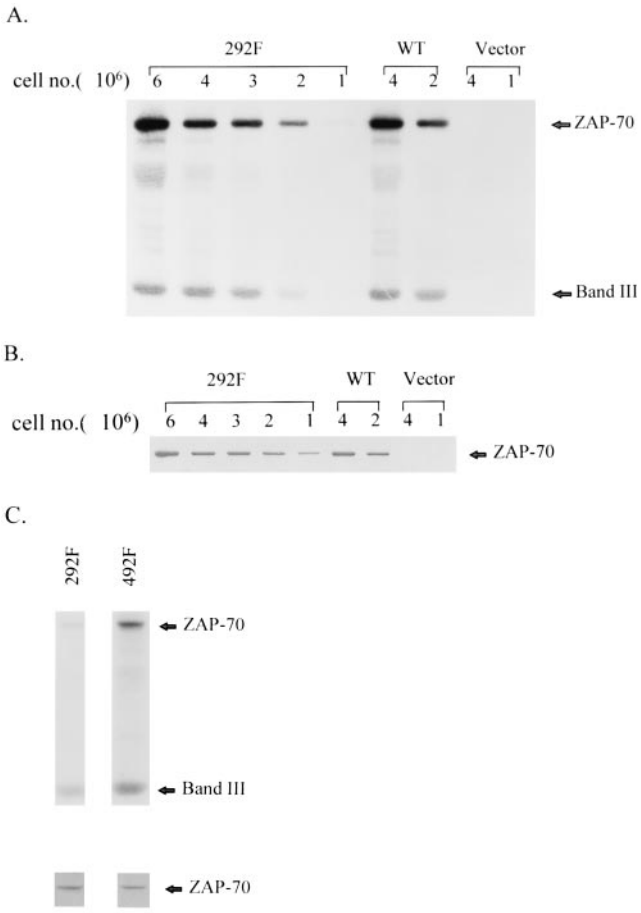

FIG. 6. Effects of 292F on the intrinsic kinase activity of ZAP-70. COS-7 cells were transiently transfected with 5 μg of vector, WT ZAP-70, or 292F. Sixty to seventy hours later, anti-epitope immunoprecipitates of WT ZAP-70 and 292F from different cell numbers of cell lysates were prepared and subjected to an in vitro kinase assay. The products were separated on an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The membrane was subjected to KOH treatment. In vitro-phosphorylated proteins were detected by autoradiography (A). (B) Expression of ZAP-70 was detected by immunoblotting with an anti-ZAP-70 MAb. (C) Comparison of the effects of 292F and 492F on the intrinsic kinase activity of ZAP-70. The assay of the in vitro kinase activity of anti-epitope immunoprecipitates of 292F and 492F was performed as described for panel A. 292F and 492F were expressed at comparable levels (lower panels).

Both the SH2 domain and the kinase activity of ZAP-70 are required for the increased activity of 292F in Syk-deficient DT-40 cells and TAg-Jurkat cells. To determine whether the SH2 domain and/or kinase activity of ZAP-70 is required for the activity of 292F, we constructed the double mutants ΔSH2(N)/292F and 292F/369A (Fig. 1). Since it had been shown that a mutation of either SH2 domain of ZAP-70 abolished its binding to the receptor (19), we combined the N-terminal SH2 domain deletion with the 292F mutation to create ΔSH2(N)/292F. Like that of ΔSH2(N) or 369A, expression

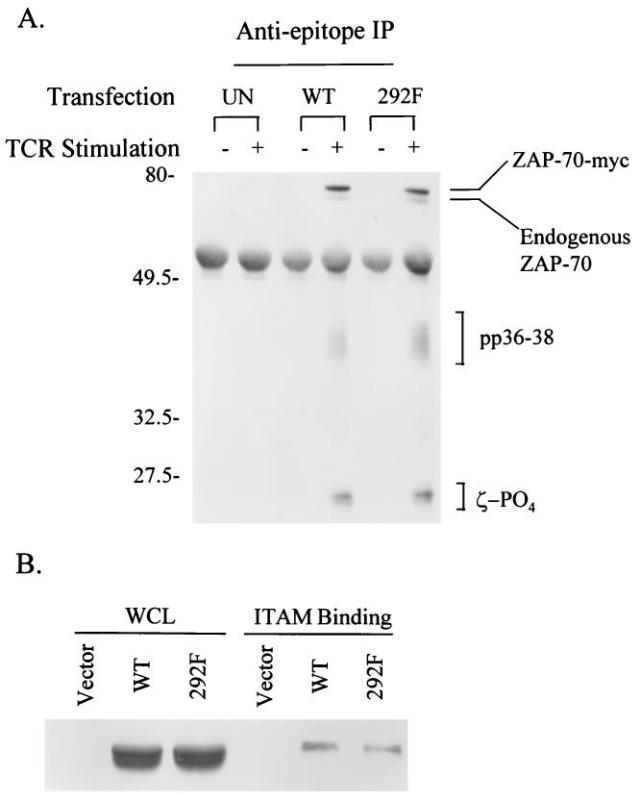

FIG. 7. Effects of 292F on the ability of ZAP-70 to bind to the receptor ITAM. (A) TAg-Jurkat cells were transfected with 40 μg of WT ZAP-70 or 292F. Forty hours later, untransfected (UN) or transfected cells were either left unstimulated (−) or stimulated (+) for 2 min with anti-TCR MAb. Cells were lysed, and anti-epitope immunoprecipitates (IP) of WT ZAP-70 and 292F were prepared and blotted with anti-phosphotyrosine MAb 4G10. Comparable levels of WT ZAP-70 and 292F expression were confirmed by stripping and reprobing with anti-ZAP-70 MAb 2F3.2 (not shown). Positions of molecular mass markers are shown in kilodaltons on the left. (B) COS-7 cells were transiently transfected with 5 μg of the empty expression vector (Vector), WT ZAP-70, or 292F. Sixty to seventy hours later, lysates were prepared and mixed with 1 μg of doubly phosphorylated peptide representing the second ITAM of TCR ζ chain, with the subsequent addition of avidin beads to collect complexes. The complexes were separated on an SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane, and blotted with anti-ZAP-70 MAb 2F3.2. Unphosphorylated ITAM did not bind to either WT ZAP-70 or 292F (not shown). The levels of WT ZAP-70 and 292F expression vectors were determined by immunoblotting of whole-cell lysates (WCL) with anti-ZAP-70 MAb 2F3.2 and are shown in the left half of the panel.

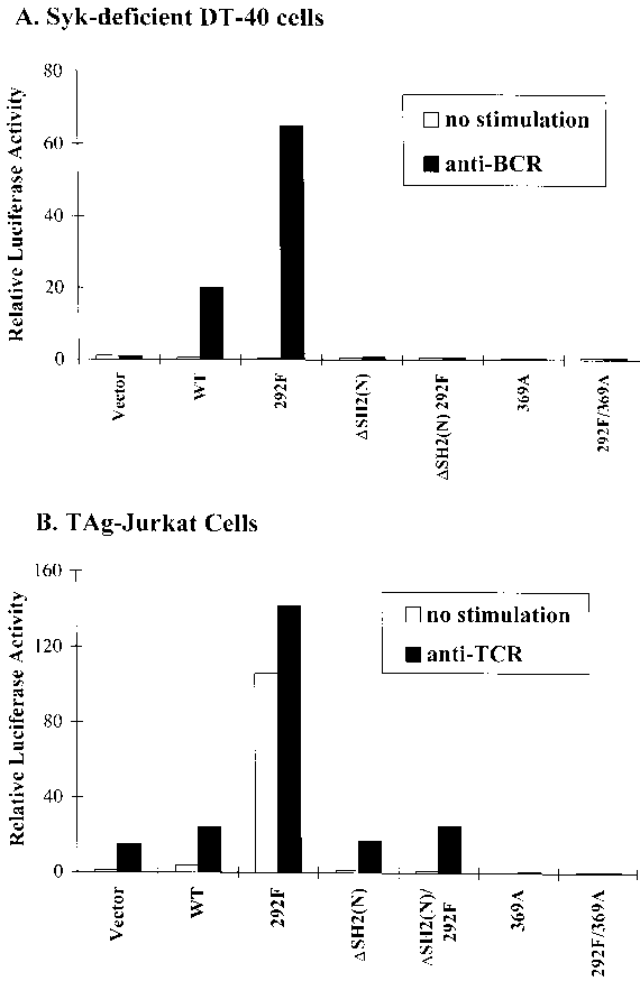

FIG. 8. Both the SH2 domain and kinase activity are required for the effects of 292F. (A) Syk-deficient DT-40 cells were transfected with 20 μg of WT ZAP-70 or its mutant derivatives and 20 μg of the NF-AT luciferase reporter construct. Twenty-four to forty hours later, cells were stimulated with medium, anti-BCR antibody (M4), or PMA plus ionomycin. The relative luciferase activity was determined as described in the legend to Fig. 2A. The PMA-plus-ionomycin responses in cells transfected with WT ZAP-70 and its mutant derivatives were similar (not shown). The expression levels of all the constructs were comparable (not shown). Data are representative of at least two independent experiments. (B) TAg-Jurkat cells were transfected with 40 μg of WT ZAP-70 or its mutant derivatives and 20 μg of the NF-AT luciferase reporter construct. Twenty-four to forty hours later, cells were stimulated with medium, anti-TCR antibody (C305), or PMA plus ionomycin. The relative luciferase activity was determined as described in the legend to Fig. 2A. The PMA-plus-ionomycin responses in cells transfected with WT ZAP-70 and its mutant derivatives were similar (not shown). The levels of protein expression of all DNA constructs were comparable (not shown). Data are representative of at least three separate experiments.

of either ΔSH2(N)/292F or 292F/369A failed to reconstitute BCR stimulation-dependent NF-AT induction in Syk-deficient DT-40 cells (Fig. 8A). The levels of protein expression from all the DNA constructs were comparable when they were blotted with anti-myc epitope antibody (data not shown). Similarly, like that of ΔSH2(N) or 369A, expression of ΔSH2(N)/292F or 292F/369A in TAg-Jurkat cells failed to induce basal NF-AT activation. However, expression of 292F/369A further inhibited TCR stimulation-dependent NF-AT induction, which is similar to the dominant negative effect of 369A, as previously shown (31). The levels of protein expression from all the constructs were comparable (data not shown). These results demonstrate that both the SH2 domain and kinase activity are required for the gain-of-function effects of 292F on NF-AT activation in lymphocytes.

DISCUSSION

In this study, we examined the role of Y-292 in regulating the function of ZAP-70 by expressing mutants of Y-292 in two types of lymphocytes. In Syk-deficient DT-40 B cells, Y-292 mutants enhanced the ability of ZAP-70 to reconstitute BCR stimulation-dependent NF-AT induction (Fig. 2). In contrast, in TAg-Jurkat T cells, the expression of 292F led to constitutive NF-AT induction independent of TCR stimulation (Fig. 4A and C). TCR stimulation further potentiated 292F-mediated NF-AT induction in these cells (Fig. 4B and D). The constitutive activation of NF-AT mediated by 292F in TAg-Jurkat T cells was specific, as WT ZAP-70 produced only little

or no basal NF-AT induction (Fig. 4A and C). Furthermore, unlike 292F, 492F failed to lead to constitutive activation of NF-AT in TAg-Jurkat cells, although both 292F and 492F enhanced the ability of ZAP-70 to reconstitute BCR stimulation-dependent NF-AT induction to a similar degree in Syk-deficient DT-40 B cells (Fig. 5). Since both the SH2 domain and kinase activity are required for the 292F effect in Syk-deficient DT-40 cells and TAg-Jurkat cells (Fig. 8), it is suggested that 292F functions through normal antigen receptor-mediated signaling pathways, leading to NF-AT activation. These results demonstrate that the role of Y-292 is to negatively regulate ZAP-70 function in lymphocytes. Furthermore, constitutive induction of NF-AT by 292F in TAg-Jurkat cells offers a useful system to study the function of ZAP-70 in T cells.

One current model for ZAP-70 activation in a T-cell line is that TCR stimulation induces tyrosine phosphorylation of the receptor ITAMs which is mediated by Src family of PTKs (reviewed in reference 48). ZAP-70 is activated after both its interaction with the phosphorylated ITAMs and its tyrosine phosphorylation. The association of ZAP-70 with tyrosine-phosphorylated antigen receptor ITAMs is likely to be critical, as the introduction of a phosphatase-resistant tyrosine-phosphorylated peptide representing the C-terminal ITAM of the TCR ζ chain into permeabilized T cells prevented TCR-stimulated tyrosine phosphorylation and activation of ZAP-70 (45). Mutations of the SH2 domain of ZAP-70 that disrupt its association with the receptor ITAM also prevent reconstitution of BCR stimulation-dependent signaling in Syk-deficient DT-40 B cells (22). However, the association of ZAP-70 with the receptor ITAMs is not sufficient to activate ZAP-70. In freshly isolated lymph node T cells and thymocytes, ZAP-70 was constitutively associated with tyrosine-phosphorylated TCR ζ chain, yet there was no activation (42). TCR stimulation is required for a large increase in tyrosine phosphorylation of ZAP-70 and for cellular activation. In antagonist-induced anergic T cells, ZAP-70 also associated with one isoform of tyrosine-phosphorylated TCR but was not tyrosine phosphorylated nor activated upon antagonist peptide stimulation (25, 36). Tyrosine phosphorylation of ZAP-70 has been shown to be required for its activation (5). Tyrosine phosphorylation of ZAP-70 may serve two purposes. First, tyrosine phosphorylation of ZAP-70 may provide one mechanism for its catalytic activation. Second, phosphorylated tyrosine residues may serve as docking sites for other regulators and/or effector molecules; Lck, a positive regulator and/or effector for TCR signaling, has been shown to associate with ZAP-70 through the Lck SH2 domain, although the significance of this association has yet to be determined (13, 38).

At least three tyrosines (Y-292, Y-492, and Y-493) have been identified as in vivo phosphorylation sites upon TCR stimulation (5, 47). Y-492 and Y-493 are located in the activation loop of the kinase domain. Both genetic and biochemical studies have indicated that Y-492 and Y-493 are involved in regulating the catalytic activation of ZAP-70 upon antigen receptor stimulation (5, 44). Y-292 is located in interdomain B between the C-terminal SH2 domain and the kinase domain. It is identified not only as one of the in vivo phosphorylation sites after TCR stimulation but also as the primary in vitro autophosphorylation site. In this study, we have shown that the role of Y-292 is to negatively regulate the function of ZAP-70 in both Syk-deficient DT-40 B cells and TAg-Jurkat cells. Our results are consistent with those of a recent report which also show that the role of Y-292 is to negatively regulate ZAP-70 function (23). However, the mechanism of this negatively regulated function is unclear. First, we have provided evidence to argue against the possibility that 292F affects the kinase activity of ZAP-70. In vitro kinase assays performed with WT ZAP-70 and 292F isolated from COS-7 cells in the absence or presence of cotransfected Lck failed to reveal any difference in the intrinsic or activated kinase activity between WT ZAP-70 and 292F (Fig. 6; also data not shown). In addition, when we performed in vitro kinase assays with WT ZAP-70 and 292F isolated from TAg-Jurkat T cells or from Syk-deficient DT-40 B cells transfected with these kinases, no significant difference in the intrinsic or TCR- or BCR stimulation-induced kinase activity between WT ZAP-70 and 292F was detected (data not shown). This possibility is further argued against by the finding that 492F, which has increased kinase activity (44) (Fig. 6C), did not function like the 292F mutant in constitutively activating NF-AT in TAg-Jurkat cells (Fig. 5C). Consistent with our results is a recent observation that 292F does not affect the basal or BCR-stimulated kinase activity of ZAP-70 in Syk-deficient DT-40 cells (23). These results strongly argue that the negative regulatory function of Y-292 is not mediated by directly influencing ZAP-70 kinase activity. We have also provided evidence to argue against the possibility that 292F affects the interaction between ZAP-70 and the receptor ITAMs by two approaches. First, we did not detect a difference between WT ZAP-70 and 292F in binding to the tyrosine-phosphorylated ζ chain either in the basal state or after TCR stimulation (Fig. 7A). In the second approach, we did not detect any significant difference between WT ZAP-70 and 292F in binding to the doubly phosphorylated ITAM peptide (Fig. 7B). Taken together, these results argue against the possibility that Y-292 functions by affecting the binding of ZAP-70 to the receptor ITAMs.

Therefore, we propose two models to explain the mechanisms by which Y-292 negatively regulates ZAP-70 function. First, Y-292 may function to sequester ZAP-70 in a cellular compartment. TCR stimulation induces phosphorylation of Y-292, releasing the sequestered ZAP-70 to allow for its association with the receptor ITAMs or with its substrates. This seems unlikely because our preliminary studies of subcellular fractionation did not reveal any significant difference in the relative localization of WT ZAP-70 and 292F (data not shown). Secondly, Y-292 may function to negatively regulate ZAP-70 function by interacting with an inhibitory protein. Consistent with this, the mutation of Y-292 to either phenylalanine (mimicking the size of a tyrosine) or to glutamic acid (mimicking the charge of a phosphorylated tyrosine) and mutations of the residues at positions 291, 293, and 294 surrounding Y-292 all enhanced the ability of ZAP-70 to reconstitute BCR-mediated signaling in Syk-deficient DT-40 B cells (Fig. 2; also data not shown), implying that the sequence surrounding Y-292 is a protein binding site. Hence, TCR stimulation induces phosphorylation of ZAP-70 at position 493 in the activation loop of ZAP-70 by a Src family kinase to allow for an increase in its catalytic activity (5). Subsequently, phosphorylation at Y-492 and Y-292 serves as a feedback mechanism to suppress ZAP-70 function. In the case of phosphorylated Y-292, the binding of a putative inhibitor negatively regulates ZAP-70 activation. Candidates for such an inhibitory protein include tyrosine kinases (i.e., Csk or Ctk) or tyrosine phosphatases. Although Plas et al. have recently shown that PTP1C associate with ZAP-70 to negatively regulate the function of ZAP-70 (30), we failed to detect this interaction in multiple experiments. Work is in progress to identify this putative inhibitor.

One of our most surprising results is that deletion of interdomain B (Δ265-331) also enhanced the ability of ZAP-70 to reconstitute BCR-mediated signaling in Syk-deficient DT-40

cells (Fig. 3). This result is surprising since interdomain B contains a proline-rich sequence and other potential tyrosine phosphorylation sites which could serve as putative protein binding motifs for downstream effector molecules. For example, Y-315 within interdomain B has been proposed to be a putative binding site for vav, a positive regulator and/or effector for TCR-mediated signaling (16, 21, 40, 51, 52). However, the results obtained here with the deletion mutant suggest that the function of interdomain B is likely to be dominated by the function of Y-292. This interpretation must be tempered by the fact that we failed to observe an enhanced response in TAg-Jurkat cells transfected with Δ265-331 either in the basal state or after TCR stimulation. This may be due to differences in the cell context (see below).

The 292F mutation enhances the ability of ZAP-70 to reconstitute BCR stimulation-dependent NF-AT induction in Syk-deficient DT-40 B cells (Fig. 2). In contrast, in TAg-Jurkat cells, the expression of 292F led to constitutive TCR stimulation-independent NF-AT induction (Fig. 4). However, we have not been able to detect any biochemical changes in the immunoprecipitates of 292F from transfected TAg-Jurkat cells with anti-phosphotyrosine antibody in the basal state, although we have consistently seen significant TCR stimulation-independent NF-AT induction. It is possible that since we examined cells only 24 to 40 h posttransfection, the biochemical changes induced by 292F may have already been compensated for. Therefore, more detailed kinetic and biochemical analyses will be critical in addressing this issue. The difference in the effects of the 292F mutant in these two cell types might be explained by differences in the cell context between Syk-deficient B cells and TAg-Jurkat T cells. The cell context difference might be the presence or absence of positive or negative signaling molecules or alternatively the higher or lower abundance levels of certain signaling molecules. There is a precedent for this. For example, an activating mutation of p56Lck (LckF505) also shows cell context-dependent constitutive activation. In beef insulin-specific CD4-negative class II-restricted helper T-cell hybridoma cells, LckF505 upregulates only TCR stimulation-dependent IL-2 secretion (1); in contrast, LckF505 stimulates IL-2 secretion independent of TCR stimulation in three different CD4-positive and CD4-negative T-cell hybridomas specific for chicken ovalbumin and I-A^d (24). The lack of Syk expression in Syk-deficient DT-40 cells cannot explain the functional difference of 292F in Syk-deficient DT-40 cells versus TAg-Jurkat cells because overexpression of 292F in Syk-expressing WT DT-40 cells failed to lead to constitutive activation of NF-AT in the absence of BCR stimulation (data not shown). Identification of the components that are responsible for the different functional consequences of the 292F mutant in Syk-deficient cells versus TAg-Jurkat T cells will help not only in resolving how Y-292 functions to negatively regulate ZAP-70 function but also in dissecting the antigen receptor-mediated signal transduction pathway.

The study of gain-of-function mutations has been a useful approach to dissect signal transduction pathways. Gain-of-function mutants of the Src family PTK lck, the Ser/Thr kinase Raf, the small GTP-binding protein Ras, and the calcium-dependent Ser/Thr phosphatase calcineurin have all been successfully used to dissect signaling pathways in T cells (11, 20, 28, 49, 50). Constitutive activation of NF-AT mediated by 292F in a T-cell line demonstrates a critical role for Y-292 in regulating ZAP-70 function. Resolving how Y-292 functions to negatively regulate ZAP-70 function and analyzing the biochemical consequences induced by Y-292 mutations should provide useful insights into the mechanisms of how ZAP-70 regulates TCR-mediated signal transduction in T cells.

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