The protein-tyrosine kinase ZAP-70 is implicated, together with the Src kinase p56\textsuperscript{lck}, in controlling the early steps of the T-cell antigen receptor (TCR) signaling cascade. To help elucidate further the mechanism by which ZAP-70 regulates these initial events, we used a dominant-negative mutant approach. We overexpressed in the Jurkat T-cell line ZAP-70 mutant on Tyr-492 and Tyr-493 in the putative regulatory loop of its kinase domain. This mutant inhibited TCR-induced activation of nuclear factor of activated T cells by interfering with both intracellular calcium increase and Ras-regulated activation of extracellular signal-regulated kinases. Moreover, TCR-induced phosphorylation of pp36-38, thought to play a role upstream of these pathways, was found to be reduced. In contrast, overexpression of wild-type ZAP-70 induced constitutive activation of nuclear factor of activated T cells. The ZAP-70 mutant studied here could be phosphorylated on tyrosine when associated to the TCR \(\zeta\) chain and was able to bind p56\textsuperscript{lck}. This result demonstrates that Tyr-492 and Tyr-493 are not responsible for the Src homology domain 2-mediated association of p56\textsuperscript{lck} with ZAP-70. Our data are most consistent with a model in which recruitment to the TCR allows ZAP-70 autophosphorylation and binding to p56\textsuperscript{lck}, which in turn phosphorylates Tyr-492 and/or Tyr-493 with consequent up-regulation of the ZAP-70 kinase activity. ZAP-70 will then be able to effectively control phosphorylation of its substrates and lead to gene activation.

Biochemical and genetic studies have indicated that cytoplasmic protein-tyrosine kinases (PTKs)\textsuperscript{1} of the Src and Syk families control the early steps of the signaling cascade initiated by T-cell antigen receptor (TCR) triggering (1). The TCR signal-transducing subunits (\(\gamma, \delta, \) and \(\epsilon\) chains of the CD3 complex and the \(\zeta\) homodimer) do not possess intrinsic PTK activity, yet, TCR ligation by antigen/major histocompatibility complex or by anti-TCR mAbs induces rapid tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) present in their cytoplasmic tails (2, 3), an event essential for activation of lymphokine genes (4, 5). Several lines of evidence suggest that the Src family PTKs p56\textsuperscript{lck} and p59\textsuperscript{ lyn} mediate phosphorylation of the ITAM (6–9), although the former may have a prominent role (10). ITAM phosphorylation allows recruitment of the Syk-related PTK ZAP-70 to the TCR (11) via binding of the two tandemly arranged ZAP-70 SH2 domains to the two phosphoryrosines of ITAMs (8, 12, 13). As a consequence, ZAP-70 itself is phosphorylated on tyrosine residues, a modification contributing to its catalytic activation (14–16). The importance of ZAP-70 in TCR signaling has been established by studies of a rare human immunodeficiency caused by the absence of ZAP-70 (17, 18). In these individuals, only CD4\textsuperscript{+} T cells develop but fail to proliferate in response to TCR ligation and show a largely compromised activation-induced tyrosine phosphorylation of intracellular proteins and calcium increase.

The mechanism whereby ZAP-70, once recruited to the ITAMs, becomes tyrosine-phosphorylated and the functional consequences of this event on TCR-controlled signaling pathways remain partly unclear. Biochemical studies have shown that Tyr-492 and Tyr-493 within the kinase domain of ZAP-70 are phosphorylated after TCR stimulation (15, 19). Homologous tyrosines are found in a number of PTKs (20), and their phosphorylation is thought to regulate the enzymatic activity (21). Although \textit{in vitro} ZAP-70 is essentially unable to auto-phosphorylate Tyr-492 and Tyr-493 (19), studies in heterologous cell systems and \textit{in vitro} indicated that p56\textsuperscript{lck} efficiently phosphorylates ZAP-70 on Tyr-492 and Tyr-493 (14, 15), suggesting a regulatory role of p56\textsuperscript{lck} on ZAP-70. In these studies, mutational analysis of ZAP-70 suggested that the kinase activity was positively regulated by phosphorylation of Tyr-493, whereas a negative regulatory function was proposed for Tyr-492. Consistently, it was shown that a ZAP-70 Y493F mutant failed to complement, whereas a Y492F mutant enhanced BCR-stimulated IL-2 gene promoter activity in a Syk-null chicken B-cell line (15). However, the double mutant Y492F/Y493F was ineffective in activating the IL-2 promoter.

We have reported previously that after TCR triggering, p56\textsuperscript{lck} associates via its SH2 domain to tyrosine-phosphorylated ZAP-70 bound to \(\zeta\) (22, 23) and proposed that p56\textsuperscript{lck} recruitment may be important for regulating ZAP-70 activity and/or for phosphorylating other substrates. \textit{In vitro} studies have shown that binding of ZAP-70 to phosphorylated \(\zeta\) chain, in the absence of Src kinases, resulted in enhanced ZAP-70
autophosphorylation (24). ZAP-70 autophosphorylated under those conditions was able to bind to the SH2 domains of Ab1 and ras-GAP, and an association in Jurkat cells with these proteins was detected after TCR activation (24). Collectively, these results showed that tyrosine phosphorylation of ZAP-70 is also important for recruiting signaling proteins to the activated TCR, although the functional consequences of these associations remain to be elucidated.

In this work, we sought to further understand the regulation of ZAP-70 by tyrosine phosphorylation following TCR stimulation and the consequences of these modifications on the ensuing signaling cascade. To this end, we analyzed in the Jurkat T-cell line the effect of overexpressing ZAP-70 mutated on both Tyr-492 and Tyr-493 on early TCR signaling events leading to the activation of nuclear factor of activated T cells (NFAT).

ZAP-70 Regulation of T-cell Activation

Cell Lines and Antibodies—The human leukemia Jurkat T-cell line was maintained in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin (Life Technologies, Inc.). The following mouse mAbs were used: 101.5.2 (anti-human TCR Vβ8, IgM, kindly provided by J. Ravetch, Memorial Sloan-Kettering Institute, New York, NY); anti-zap-70 antiserum (4.06) directed against a synthetic peptide corresponding to amino acids 106–117 of the human sequence was produced in our laboratory using keyhole limpet hemocyanin as a carrier; anti-VSV-G epitope antiserum (kindly provided by M. Arpin, Institut Curie, Paris, France) was raised against an 11-amino acid determinant derived from a vesicular stomatitis virus glycoprotein (VSV-G) (25). Anti-TCR mAb 101.5.2 (anti-human TCR Vβ8) at 1:200 dilution of ascites was used, blotswere blocked with 5% skimmed milk in 10m M Tris-HCl, pH 7.5, 0.1% Tween 20. ZAP-70 antiserum (kindly provided by C. Baldari, University of Siena, Siena, Italy) derived pUBT-luc plasmid (26), contained the luciferase gene under the control of the human IL-2 promoter NFAT binding site (27). pSV-bgal vector (Promega Corp., Madison, WI) contained the β-galactosidase gene driven by the SV40 promoter/enhancer. The full-length EcoRI fragment encoding the human ZAP-70 cDNA (11) in pBluescript (pBS) derivative from a vesicular stomatitis virus glycoprotein (VSV-G) (25). Plasmids and Constructs—NFAT-luciferase reporter construct was kindly provided by C. Baldari, University of Siena, Siena, Italy, derived from pUBT-luc plasmid (26), contained the luciferase gene under the control of the human IL-2 promoter NFAT binding site (27). pSV-βgal vector (Promega Corp., Madison, WI) contained the β-galactosidase gene driven by the SV40 promoter/enhancer. The full-length EcoRI fragment encoding the human ZAP-70 cDNA (11) in pBluescript (pBS) vector (a kind gift of R. Sekaly, Institut de Recherches Cliniques de Montreal, Montreal, Canada), which is a derivative of the pSR vector (17), was used, blotswere blocked with 5% skimmed milk in 10m M Tris-HCl, pH 7.5, 0.1% Tween 20. The final PCR product was digested with SacI and NsiI at 37 °C for 2 h with 101.5.2 anti-TCR mAb precoated to wells at 1:1000 dilution of ascites or with PMA (50 ng/ml) (Sigma) and the calcium ionophore A23187 (2 μg/ml) (Sigma). β-Galactosidase and luciferase assays were performed according to the manufacturer’s instructions (Promega Corp., Madison, WI). Luciferase activity was determined in duplicate samples using an automated luminometer (Lumat LB 9501, EG&G Berthold, Wildbad, Germany), was expressed in arbitrary units (AU) after normalization to the β-galactosidase values to correct for variation in transfection efficiency. For stable transfections, Jurkat cells were washed twice and resuspended at 107 cells/ml in HeBS buffer, pH 7.05 (0.8m M of cold ATP, 157 mM NaCl, 250 mM Na2HPO4, 5m M NaHCO3, 1m M p-glucose), mixed with 30 μg of plasmid DNA, and electroporated at 250 V, 960 μF in a Gene Pulse cuvette. After 72 h in growth medium, cells were selected at 106 cells/well in flat-bottomed, 96-well plates and placed under selection in the presence of 10 μg/ml puromycin (Sigma). Puromycin-resistant clones expressing ZAP-70-tag were analyzed by FACScan for the expression of CD3.

Immunoprecipitations and Immunoblotting—Jurkat cell transfectants were preincubated for 5 min at 37 °C and then stimulated with anti-TCR mAb 101.5.2 at 1:200 dilution of ascites for 2–3 min. Cells were immediately centrifuged and solubilized at 106 cells/ml for 15 min in ice-cold lysis buffer containing 1% Nonidet P-40, 20m M Tris-HCl, pH 7.5, 7.5, 150 m M NaCl, 1 m M MgCl2, and 1 m M EDTA in the presence of inhibitors of proteases and phosphatases (10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 m M Pefabloc-sc, 50 m M NaF, 10 m M Na3P2O7, and 1 m M NaVO4). Postnuclear lysates were precleared and subjected to immunoprecipitation with the indicated anti sera preadsorbed to protein A-Sepharose as described (22). For precipitation of p56lck SH2-bound proteins, maltose-binding protein-p56lck SH2 fusion protein covalently coupled to amylose-agarose (Pharmacia, Uppsala, Sweden) was used (22). Immunoprecipitates were washed twice in 1% Nonidet P-40 lysis buffer, twice with 0.5% Nonidet P-40 lysis buffer, and boiled in sample buffer before SDS-polyacrylamide gel electrophoresis. Immunoblotting and detection of proteins by enhanced chemiluminescence (Amersham Corp.) were performed as described previously (22). In the experiments where 125I-labeled protein A (2–5 μg) (Amersham Corp.) was used, blots were blocked with 5% skimmed milk in 10m M Tris-HCl, pH 7.5, 25 μg NaCl, 2.5 m M EDTA, pH 7.5, buffer containing 0.1% Tween 20. Quantitation of 125I-labeled proteins was performed using Image Quant software after scanning in a PhosphorImager (Molecular Dynamics).

In Vitro Kinase Assays—Anti-TCR-G V-tag immunoprecipitates were washed twice in 1% Nonidet P-40 lysis buffer, once in 10m M Tris, pH 7.4, 0.5 m LiCl and once in kinase buffer (10 m M Tris, pH 7.4, 10 m M MgCl2, and 10 m M MnCl2) as described previously (14). Reactions were performed at room temperature for 5 min in 25 m M of kinase buffer containing 10 μCi of [γ-32P]ATP (4500 Ci/ml, ICN Pharmaceuticals, Orsay, France); 5 m M of cold ATP, and 1 m M of KCl of band 3 (a kind gift of R. Wange, National Institutes of Health, Bethesda, MD) as an exogenous substrate. The reactions were stopped by adding an equal volume of 2x Laemmli sample buffer containing 200 m M dithiothreitol, 10 m M EDTA and boiling for 5 min. Samples were resolved by SDS-polyacrylamide gel electrophoresis; gels were treated with KOH for 1 h at 55 °C and dried, and phosphorylated proteins were visualized by autoradiography. Quantitation of 125I-labeled ZAP-70 and 3P-labeled ZAP-70 and band 3 was performed as described above. Gels were stained with Coomassie Blue to ascertain that equal amounts of band 3 were added to the reactions.

Determination of the Intracellular Free Calcium Concentration—Measurements of free cytosolic calcium concentration were performed using a method described previously (29). Three 106 cells were incubated for 1 h at 106/ml in RPMI without serum containing 20 μM of the
calcium-sensitive fluorescent dye fluo 3-acetoxymethyl ester (Molecular Probes, Eugene, OR). After washing with RPMI containing 1% fetal calf serum, cells were resuspended in the same medium at $1.5 \times 10^6$ cell/ml and stimulated with anti-TCR mAb (101.5.2) at 1:500 dilution of ascites or thapsigargin (1 $\mu$m). Mean fluorescence intensity ($F$) was recorded every 30 s in a FACSscan cytometer (Beckton & Dickinson Co., Mountain View, CA) and represents the average response of 2000 viable cells. Each recording was calibrated by determining the maximal uptake of calcium ($F_{max}$) in the presence of 2 $\mu$g/ml calcium ionophore A23187 as well as the level of autofluorescence ($F_{min}$), after quenching fluo 3-acetoxymethyl ester fluorescence with 1 mM Mn$^{2+}$. Intracellular calcium concentration, [Ca$^{2+}$]_i, was calculated using the equation (30): 

$$[\text{Ca}^{2+}]_i = \frac{K_d (F - F_{min})}{(F_{max} - F)}$$

where $K_d$ is the dissociation constant of fluo 3-acetoxymethyl ester set to 400 nM.

**RESULTS**

Overexpression of a Y492F/Y493F ZAP-70 Mutant in Jurkat Cells Inhibits TCR-induced NFAT Activation—A transient transfection assay was used to assess whether TCR-mediated activation of Jurkat cells was affected by overexpressing ZAP-70 in which both Tyr-492 and Tyr-493 were mutated to F (ZAP-70FF mutant). As a read-out, we measured the activation of NFAT, a nuclear factor enhancing IL-2 gene transcription (31). We used an NFAT-luciferase reporter construct (NFAT-luc) containing a trimer of an IL-2-derived cis-acting element that binds the NFAT transcription complex (31). A constitutive SV40 promoter-β-galactosidase reporter plasmid was used to normalize for transfection efficiency. Jurkat cells transfected with the empty vector used to express ZAP-70, together with NFAT-luc, displayed an approximately 100-fold induction of luciferase activity (set to 100%, Fig. 1A), when stimulated with an optimal dose of anti-TCR mAb. In contrast, the vector containing the ZAP-70FF mutant cDNA markedly reduced TCR-stimulated luciferase activity in a dose-dependent fashion, whereas no inhibition was observed with wild-type ZAP-70 cDNA (ZAP-70WT) (Fig. 1A). A similar dose-dependent inhibition was also found when expressing a kinase-defective ZAP-70 mutant (ZAP-70KD) in which Asp-461, a highly conserved residue in the kinase domain of PTKs essential for catalytic activity, was substituted by N (32). The expression of ZAP-70 constructs, all VSV-G-tagged at their COOH termini (see “Materials and Methods”), was confirmed by Western blotting (data not shown). Moreover, no apparent inhibition was noticed on constitutive β-galactosidase activity in any one of the transfections (data not shown), thus excluding a generalized suppressive effect on transcription by the expressed mutants. Fig. 1B shows that the inhibitory effect of ZAP-70 mutants on NFAT activity was essentially overcome by stimulating transfected cells with the combination of PMA and calcium ionophore. Both of these pharmacological agents are required for inducing an active NFAT transcriptional complex, bypassing proximal TCR-induced signals (33, 34). Altogether, these experiments

![Fig. 1. Effect of wild-type ZAP-70 and mutants on NFAT activation. Jurkat cells were cotransfected with NFAT-luciferase and pSV-β-galactosidase reporter plasmids (10 and 30 µg, respectively) together with pSRapuro expression vector either empty (5 µg) or containing the cDNA of ZAP-70 wild-type (WT) or ZAP-70 mutants (FF or KD) at the indicated doses. The DNA content of each transfection was made even with empty expression vector. After 24 h incubation, cells were stimulated for an additional 8 h with anti-TCR mAb precoated to plastic wells (A) or with PMA (50 ng/ml) plus calcium ionophore (A23187) (2 µg/ml) (B). Luciferase activity was determined and normalized to β-galactosidase activity to correct for transfection efficiency. Basal luciferase activity in unstimulated cells transfected with empty vector or ZAP-70 WT construct (C). Data in A and B indicate in percentage the induced luciferase activity measured in cells transfected with ZAP-70WT or mutants divided by the activity measured with empty expression vector (approximately 30,000 AU in A and 100,000 AU in B) and represent the average of two to five independent experiments. C, constitutive luciferase activity in cells transfected with the indicated doses of ZAP-70WT construct represents in percentage the luciferase activity of unstimulated cells divided by the activity after anti-TCR stimulation. The data are the average of three independent experiments. Bars, S.E.
performed by scanning the blots in a PhosphorImager and by analysis ofJurkat transfectants. Quantitation of the bands corresponding to the exogenousexogenous/endoogenous ratio of FFS and KD mutant in the WT15–8 and KD12 transfectants were about 1.9- and 2.6-fold higher than endogenous ZAP-70, respectively.

Jurkat Cells Stably Overexpressing ZAP-70FF Mutant Are Defective for TCR-induced NFAT Activation and Intracellular Calcium Increase — We tested the ability of the transfectants expressing ZAP-70FF to activate NFAT upon TCR triggering compared to J05.2 control cells. Fig. 3A shows that FF18-22 was severely impaired (80–90% reduction) in its ability to activate NFAT after TCR stimulation, whereas the response of FF10 was only reduced by 45% compared to J0 5.2 cells. This is consistent with the levels of ZAP-70FF mutant expressed in the two cell lines. The experiment presented in Fig. 3B indicates that transfection of ZAP-70WT in FF18-22 cells reversed NFAT activation inhibition whereas the empty vector had no restoring effect. This result supports the conclusion that the strongly reduced NFAT activity in FF18-22 cells was directly caused by ZAP-70FF interference with the normal function of ZAP-70. Because the levels of inhibition observed in FF10 cells were rather weak, further experiments were focused on transfectants overexpressing ZAP-70FF.

NFAT activation requires both calcium/calcineurin- and Ras-regulated pathways (34). We, therefore, initially investigated whether TCR-induced increase of free cytosolic calcium concentration ([Ca^{2+}]_i) was inhibited as a consequence of ZAP-70FF overexpression. A typical experiment is presented in Fig. 4A. J0 5.2 cells demonstrated a transient and marked [Ca^{2+}]_i increase, later reaching a plateau at a lower level. By contrast, FF18-22 cells responded by a weak, yet sustained, increase in [Ca^{2+}]_i, reaching a maximum which was only 13% of that of control cells. Similar reductions of [Ca^{2+}]_i increase were seen with another clone, FF18-21, which expressed levels of ZAP-70FF comparable to those detected in FF18-22 cells (data not shown). As a control, cells were stimulated with thapsigargin, a drug able to induce calcium release from internal stores and provoke extracellular calcium influx (35, 36). Fig. 4B shows that thapsigargin (1 μM) induced [Ca^{2+}]_i increase in FF18-22 at even higher levels than in J0 5.2 cells.

We, therefore, attempted to reverse the inhibition by adding either calcium ionophore or PMA to TCR-stimulated ZAP-70FF-expressing cells to test the possibility that only one of the two pathways leading to NFAT activation (e.g. calcium/calcineurin or Ras) was blocked. Neither calcium ionophore (Fig. 3A) nor PMA (data not shown) treatment was able to restore the response when combined with anti-TCR stimulation, compared with similarly treated J0 5.2 cells. Comprehensively, these data suggested that ZAP-70FF mutant inhibited both calcium/calcineurin and PMA (e.g. most likely protein kinase C/Ras) regulated pathways needed to form an active NFAT transcriptional complex.

The ZAP-70FF Mutant Binds to the TCR ζ Subunit and Can Be Tyrosine-phosphorylated — To investigate whether more proximal TCR-mediated signaling events were inhibited, we first asked if the ZAP-70FF mutant was still able to bind to the TCR ζ subunit and to be phosphorylated on tyrosine after TCR stimulation. To this end, anti-ζ immunoprecipitates from untreated and TCR-stimulated FF18-22 and control J0 5.2 cell transfectants were analyzed by anti-phosphotyrosine immunoblotting (Fig. 5). J0 5.2 cells displayed a weak basal tyrosine phosphorylation of ζ that was substantially increased after stimulation with an anti-TCR mAb and led to the recruitment of externalizing signal molecules, indicating that ZAP-70FF was able to recruit to the TCR ζ subunit and to be phosphorylated on tyrosine after TCR stimulation.
of ZAP-70 (ZAP-70end). Remarkably, in unstimulated FF18-22, the ζ chain was strongly tyrosine-phosphorylated and was associated with ZAP-70FF, itself phosphorylated on tyrosine. Upon TCR activation, phosphorylated ζ and ZAP-70FF increased, and a 69-kDa species migrating as endogenous ZAP-70 also appeared. The elevated basal and TCR-induced phosphorylation of ζ in FF18-22 cells was most likely due to the overexpression of ZAP-70FF. Indeed, in FF10 cells that expressed approximately 5-fold less mutant basal as well as induced expression of ZAP-70FF, it was predominantly binding to ζ compared to the endogenous wild-type ZAP-70, providing, therefore, an explanation for the inhibitory effects on downstream signaling events.

ZAP-70FF could still be phosphorylated in vivo on tyrosine residue(s). Thus, we measured the in vitro autophosphorylation capacity of ZAP-70FF and its kinase activity on the exogenous substrate band 3 and compared them with the activity of ZAP-70WT and ZAP-70KD. Anti-tag immunoprecipitates from the transfectants FF18-22, WT15.8, and KD12 were split and subjected to either an in vitro kinase assay (Fig. 6) or to a quantitative immunoblotting with anti-ZAP-70 antibody and 125I-labeled protein A to normalize for the same amounts of protein (data not shown). As expected, the ZAP-70KD mutant did not have significant kinase activity. The in vitro autophosphorylation of ZAP-70FF was found to be increased with respect to ZAP-70WT (after normalization for the levels of precipitated ZAP-70-tagged proteins). Moreover, the ZAP-70FF displayed a 3-fold increased basal kinase specific activity on band 3 compared with ZAP-70WT. Similar results were found with ZAP-70FF immunoprecipitated from FF10 cells (data not shown).

This result may at least in part explain why ζ-associated ZAP-70FF was highly phosphorylated in vivo (Fig. 5), although it lacked two major sites of phosphorylation.

**ZAP-70FF Mutant Is Able to Interact with p56lck**—We have previously provided evidence that p56lck binds directly, via its SH2 domain, to tyrosine-phosphorylated, ζ-associated ZAP-70 shortly after TCR triggering (22, 23). To know if this physical association could still occur between p56lck and the ZAP-70FF mutant, lysates of FF18-22 or J05.2 cells, unstimulated or stimulated by TCR cross-linking, were immunoprecipitated by anti-p56lck antibody, and the associated ZAP-70 was detected by an anti-phosphotyrosine immunoblot (Fig. 7A, left panel). In J0 5.2 cells, tyrosine-phosphorylated ZAP-70 coprecipitated with p56lck after activation, and smaller amounts were seen in nonactivated cells. The results presented above in Fig. 5 demonstrated that in nonactivated FF18-22 cells, overexpressed ZAP-70FF mutant was already tyrosine-phosphorylated to a sizable level. Consistent with this, the ZAP-70FF mutant coprecipitated with p56lck from unstimulated FF18-22 cells, and the level of associated phosphorylated ZAP-70FF increased after TCR stimulation. Similar amounts of p56lck were expressed by J0 5.2 and FF18-22 cells, as demonstrated by anti-p56lck Western blots on total lysates of stimulated cells (Fig. 7A, right panel). The position of p56lck and p60lck are indicated, the latter being mostly visible after TCR stimulation (37). In agreement with the above results, recombinant p56lckSH2 domain bound ZAP-70FF before activation, and the amounts increased after activation as revealed by Western blot with anti-tag antisera (Fig. 7B). These data indicate that Tyr-492 and Tyr-493 are not required for p56lck binding to ZAP-70.

**Overexpression of ZAP-70FF Results in Reduced Phosphorylation of the Grb2-associated pp36-38 Phosphoprotein and Electrophoretic Mobility Shift of ERKs—Grb2, a well characterized
adapter protein playing a central role in the activation of the Ras pathway (38), associates with a number of tyrosine-phosphorylated proteins after TCR stimulation (39–43). These phosphoproteins, including pp36-38, may have a role in connecting the TCR-activated PTKs to several signaling pathways such as those controlled by Ras and phospholipase C-\(\gamma\). We, therefore, examined whether in cells overexpressing the ZAP-70FF mutant, the association of phosphoproteins to Grb2 was altered. Cell lysates from FF18-22, FF18-21, and control J05.2 transfectants were subjected to immunoprecipitation with an anti-Grb2 antibody, and associated proteins were revealed by anti-phosphotyrosine immunoblotting. Fig. 8A shows that in J05.2 cells, pp36-38 was coprecipitated with Grb2 after TCR activation consistent with previous reports (39,41). In contrast, both clones overexpressing the ZAP-70FF mutant (FF18-22 and FF18-21), a marked inhibition of tyrosine-phosphorylated pp36-38 associated with Grb2 was observed. A strong decrease of an unidentified 60-kDa phosphoprotein coprecipitating with Grb2 was also seen in these cell lines. Anti-Grb2 immunoblots on the immunoprecipitates showed similar amounts of Grb2 protein (Fig. 8A, lower panel).

We next examined whether a more distal event in the Ras pathway, the activation of ERKs (44, 45), was also affected by the overexpressed ZAP-70FF mutant. To test this hypothesis, we investigated whether ERK-1 (44 kDa) and ERK-2 (42 kDa) electrophoretic mobility shift, an alteration associated to their activation, was inhibited after TCR stimulation in FF18-22 and FF18-21 cells. Fig. 8C shows that the mobility shift of both ERKs was considerably reduced in these cell lines but was clearly detectable in J0 5.2 control cells. The visibility of the mobility change of the 42-kDa molecular species representing ERK-2 is partially hindered by the unshifted 44-kDa ERK-1.

These data provide evidence that the dominant-negative ZAP-70 mutated in the regulatory loop of the kinase domain affects both the SH2-mediated association of pp36-38 with Grb2 and on the activation of ERKs.

**DISCUSSION**

In this report, we demonstrated that in Jurkat T cells, overexpression of ZAP-70 mutated on both Tyr-492 and Tyr-493 inhibited TCR-mediated signaling pathways controlling trans-
the phosphorylated form of ERK-1 and ERK-2 are indicated on polyacrylamide SDS-gels (acrylamide:bis-acrylamide ratio, 14:9:1) and analyzed by immunoblotting with anti-tag antiserum. Molecular weight markers are indicated in thousands.

Inhibition of pp36-38 association to Grb2 and ERKs

Electrophoretic mobility shift in cells overexpressing ZAP-70FF. A, upper panel: the indicated cells (10^7) were left unstimulated (-) or stimulated for 2 min with anti-TCR mAb (+). Nonidet P-40 cell lysates were subjected to immunoprecipitation with anti-Grb2 antibody, and immunocomplexes were separated on 10% polyacrylamide SDS-gel and analyzed by anti-phosphotyrosine mAb 4G10 immunoblotting. Position of molecular weight markers and immunoprecipitating antibodies (Ig) are indicated on the left. A, lower panel: the same blot was stripped and reprobed with the anti-Grb2 antibody. B, equal amount of proteins (determined by Bradford assay) from total cell lysates from unstimulated (-) or anti-TCR-stimulated cells (+) were separated on a 12.5% polyacrylamide SDS-gels (acylamide:bis-acylamide ratio, 14:9:1) and then immunoblotted using anti-ERK antibody. Position of unphosphorylated and phosphorylated forms of ERK-1 and ERK-2 are indicated on the right and on the left, respectively.

Fig. 7. ZAP-70FF mutant can associate with p56<sub>Lck</sub>. J0 5.2 and FF18-22 transfectants (8 × 10^6 cells) were left unstimulated (-) or stimulated with anti-TCR mAb for 2 min (+). A, left panel: after solubilization in 1% Nonidet P-40-containing buffer, p56<sub>Lck</sub> was immunoprecipitated, and proteins were separated on 8% polyacrylamide SDS-gel and analyzed by immunoblotting using anti-phosphotyrosine mAb 4G10. A, right panel: half of the total cell lysates prepared from stimulated cells (+) was loaded on the same gel and analyzed by anti-p56<sub>Lck</sub> immunoblotting. B, Nonidet P-40 lysates from J0 5.2 and FF18-22 cells (10^7) unstimulated (-) or stimulated (+) as above were incubated with maltose-binding protein-p56<sub>Lck</sub>-SH2 covalently coupled to Sepharose beads. Bound proteins were separated on a 7% polyacrylamide SDS-gel, transferred to nitrocellulose, and analyzed by immunoblotting with anti-tag antiserum. Molecular weight markers are indicated in thousands.

Fig. 8. Inhibition of pp36-38 association to Grb2 and ERKs

Formation of an active NFAT requires both calcium-regulated calcineurin and Ras-regulated mitogen-activated protein kinase (ERK) pathways (34). Our data indicated that ZAP-70FF overexpression negatively affected both pathways because it reduced TCR-mediated intracellular calcium concentration increase and ERK protein mobility shift. Upon TCR stimulation, pp36-38, probably the Lnk protein whose gene has been recently cloned (48), binds to Grb2 and phospholipase Cγ1 and has been proposed to play a role in connecting TCR-regulated PTKs to the Ras pathway and phospholipase Cγ1 activation (39, 41, 49). Reduced levels of tyrosine-phosphorylated pp36-38 bound to Grb2 were observed in Jurkat cell transfectants overexpressing ZAP-70FF compared with control cells. In agreement with our data, overexpression of the ZAP-70 SH2 domains alone (SH2 N+C), which inhibited TCR-induced NFAT activation, was also shown to provoke a similar effect on PMA-8 (50). Our preliminary results indicate that lower amounts of tyrosine-phosphorylated pp36-38 were associated with phospholipase Cγ1 as a consequence of ZAP-70FF overexpression. Thus, the inhibition of both calcium increase and ERK activation by the ZAP-70 mutant might be the conse-

mally controlled by ZAP-70. Binding to ITAM and subsequent phosphorylation/activation of ZAP-70 appear to be obligatory events for TCR-mediated NFAT activation (5), and ZAP-70FF might primarily interfere at this level. Using stable transfec-

1. ZAP-70FF mutant can associate with p56<sub>Lck</sub>

2. J0 5.2 and FF18-22 transfectants (8 × 10^6 cells) were left unstimulated (−) or stimulated with anti-TCR mAb for 2 min (+). A, left panel: after solubilization in 1% Nonidet P-40-containing buffer, p56<sub>Lck</sub> was immunoprecipitated, and proteins were separated on 8% polyacrylamide SDS-gel and analyzed by immunoblotting using anti-phosphotyrosine mAb 4G10. A, right panel: half of the total cell lysates prepared from stimulated cells (+) was loaded on the same gel and analyzed by anti-p56<sub>Lck</sub> immunoblotting. B, Nonidet P-40 lysates from J0 5.2 and FF18-22 cells (10^7) unstimulated (−) or stimulated (+) as above were incubated with maltose-binding protein-p56<sub>Lck</sub>-SH2 covalently coupled to Sepharose beads. Bound proteins were separated on a 7% polyacrylamide SDS-gel, transferred to nitrocellulose, and analyzed by immunoblotting with anti-tag antiserum. Molecular weight markers are indicated in thousands.

3. Inhibition of pp36-38 association to Grb2 and ERKs

4. Electrophoretic mobility shift in cells overexpressing ZAP-70FF. A, upper panel: the indicated cells (10^7) were left unstimulated (−) or stimulated for 2 min with anti-TCR mAb (+). Nonidet P-40 cell lysates were subjected to immunoprecipitation with anti-Grb2 antibody, and immunocomplexes were separated on 10% polyacrylamide SDS-gel and analyzed by anti-phosphotyrosine mAb 4G10 immunoblotting. Position of molecular weight markers and immunoprecipitating antibodies (Ig) are indicated on the left. A, lower panel: the same blot was stripped and reprobed with the anti-Grb2 antibody. B, equal amount of proteins (determined by Bradford assay) from total cell lysates from unstimulated (−) or anti-TCR-stimulated cells (+) were separated on a 12.5% polyacrylamide SDS-gels (acylamide:bis-acylamide ratio, 14:9:1) and then immunoblotted using anti-ERK antibody. Position of unphosphorylated and phosphorylated forms of ERK-1 and ERK-2 are indicated on the right and on the left, respectively.

5. Formation of an active NFAT requires both calcium-regulated calcineurin and Ras-regulated mitogen-activated protein kinase (ERK) pathways (34). Our data indicated that ZAP-70FF overexpression negatively affected both pathways because it reduced TCR-mediated intracellular calcium concentration increase and ERK protein mobility shift. Upon TCR stimulation, pp36-38, probably the Lnk protein whose gene has been recently cloned (48), binds to Grb2 and phospholipase Cγ1 and has been proposed to play a role in connecting TCR-regulated PTKs to the Ras pathway and phospholipase Cγ1 activation (39, 41, 49). Reduced levels of tyrosine-phosphorylated pp36-38 bound to Grb2 were observed in Jurkat cell transfectants overexpressing ZAP-70FF compared with control cells. In agreement with our data, overexpression of the ZAP-70 SH2 domains alone (SH2 N+C), which inhibited TCR-induced NFAT activation, was also shown to provoke a similar effect on PMA-8 (50). Our preliminary results indicate that lower amounts of tyrosine-phosphorylated pp36-38 were associated with phospholipase Cγ1 as a consequence of ZAP-70FF overexpression. Thus, the inhibition of both calcium increase and ERK activation by the ZAP-70 mutant might be the conse-

1. ZAP-70 Regulation of T-cell Activation

2. Formation of an active NFAT requires both calcium-regulated calcineurin and Ras-regulated mitogen-activated protein kinase (ERK) pathways (34). Our data indicated that ZAP-70FF overexpression negatively affected both pathways because it reduced TCR-mediated intracellular calcium concentration increase and ERK protein mobility shift. Upon TCR stimulation, pp36-38, probably the Lnk protein whose gene has been recently cloned (48), binds to Grb2 and phospholipase Cγ1 and has been proposed to play a role in connecting TCR-regulated PTKs to the Ras pathway and phospholipase Cγ1 activation (39, 41, 49). Reduced levels of tyrosine-phosphorylated pp36-38 bound to Grb2 were observed in Jurkat cell transfectants overexpressing ZAP-70FF compared with control cells. In agreement with our data, overexpression of the ZAP-70 SH2 domains alone (SH2 N+C), which inhibited TCR-induced NFAT activation, was also shown to provoke a similar effect on PMA-8 (50). Our preliminary results indicate that lower amounts of tyrosine-phosphorylated pp36-38 were associated with phospholipase Cγ1 as a consequence of ZAP-70FF overexpression. Thus, the inhibition of both calcium increase and ERK activation by the ZAP-70 mutant might be the conse-

ZAP-70 Regulation of T-cell Activation

Recognition of diminished phosphorylation of pp36-38. However, recent data suggest that ERK activation may not necessarily be related to phosphorylation of pp36-38 (49). In any event, the present as well as previous results (50) suggest that ZAP-70 may contribute to phosphorylation of pp36-38.

Several lines of evidence have suggested that p56\(^{ck}\) may have a regulatory role on ZAP-70 by phosphorylating Tyr-492 and Tyr-493 (14, 15). Other studies have implied that the interaction of these two PTKs is required for effective signaling in T cells (51). We and others have reported that p56\(^{ck}\) associates via its SH2 domain to tyrosine-phosphorylated ZAP-70 (22, 23, 52). Although the functional consequences of this physical interaction are not yet known, important effects in TCR signaling may be hypothesized, such as p56\(^{ck}\)-mediated ZAP-70 phosphorylation on regulatory tyrosines and/or p56\(^{ck}\) colocalization with the activated TCR, an event that could facilitate phosphorylation of additional substrates. The association of these two PTKs is most reminiscent of the SH2-mediated interaction of Src kinases with focal adhesion kinase, implicated in integrin-mediated signal transduction (53) or with platelet-derived growth factor and colony-stimulating factor-1 receptor tyrosine kinases (54, 55). In all three cases, the relevant tyrosine-containing motif has been mapped outside of the kinase domain. TCR \(\zeta\)-associated ZAP-70FF was phosphorylated on tyrosines other than Tyr-492 and Tyr-493 by this mechanism that is still unclear (discussed below), and this resulted in its efficient \textit{in vitro} binding to the p56\(^{ck}\)/SH2 domain and association with p56\(^{ck}\) \textit{in vivo}. These data formally rule out that these two tyrosines of ZAP-70 mediate the association with p56\(^{ck}\). In the case of focal adhesion kinase, \textit{in vivo} \textit{and} \textit{in vitro} studies have shown that Tyr-397, responsible for the SH2-mediated Src kinase binding, is the major autophosphorylation site (53, 56). Moreover, phosphorylation of Tyr-576 and Tyr-577, in the putative activation loop of focal adhesion kinase domain, as well as two other tyrosines, is essentially mediated by Src PTKs (56). Indeed, mutation of Tyr-397 dramatically reduced tyrosine phosphorylation of focal adhesion kinase and its \textit{in vitro} kinase activity (56). It is tempting to speculate that an analogous scheme applies to ZAP-70. Once recruited to the ITAMs, ZAP-70, which possesses a basal kinase activity (Refs. 14–16 and the present study) may undergo autophosphorylation (24) to generate the binding site for p56\(^{ck}\) which, in turn, would phosphorylate ZAP-70 on Tyr-492 and/or Tyr-493 (and perhaps on other tyrosines), resulting in up-regulation of ZAP-70 kinase activity and efficient phosphorylation of its substrates. In keeping with this model, p56\(^{ck}\)-carrying a point mutation that abolished binding of its SH2 domain to phosphotyrosine, was shown to be unable to reconstitute TCR-induced NFAT activation in a p56\(^{ck}\)-null Jurkat cell line (52). Our results suggest that p56\(^{ck}\) recruited by ZAP-70 may not be directly implicated in the activation of the calcineurin and Ras pathways. However, because our observations were restricted to TCR-mediated NFAT activation, we cannot exclude that in normal T cells, p56\(^{ck}\) controls key events of those pathways mediated through, for instance, the CD4/CD8 co-receptors (57).

It has been reported recently that overexpression of ZAP-70 SH2(N + C) domains alone in unstimulated Jurkat cells resulted in increased basal levels of tyrosine phosphorylation of \(\zeta\) (50) and that a kinase domain-deleted Syk mutant provoked the same effect on the FcRI \(\beta\) and \(\gamma\) chains in unstimulated RBL cells (58). This receptor clustering-independent ITAM phosphorylation was also observed by overexpressing ZAP-70FF. As discussed previously (58), this effect may be best explained by assuming that an ongoing ITAM phosphorylation is carried out under basal conditions by Src kinases, but accumulation of phosphorylated ITAMs is prevented by the action of a phosphatase that dephosphorylates them faster than they are phosphorylated. Thus, an excess of ZAP-70 mutant or wild type may lead to the protection of ITAM tyrosine residues by the SH2 domains and accumulation of phosphorylated ITAMs. Under normal circumstances, TCR engagement may create the conditions for accelerating ITAM phosphorylation or reducing their dephosphorylation (e.g., by sequestration from phosphatases). It is interesting that, as reported here, wild-type ZAP-70 transiently overexpressed in Jurkat cells was able to induce NFAT activation in the absence of TCR cross-linking. This suggests that clustering onto the ITAMs and subsequent activation of ZAP-70 (probably by p56\(^{ck}\)) is, by itself, not only necessary but also sufficient to ignite the signaling cascade leading to calcium/calcineurin and Ras pathway activation.

The dominant-negative effect on NFAT activation by ZAP-70FF was of similar magnitude to that observed with the ZAP-70 kinase-defective mutant, yet the former bound to TCR \(\zeta\) was found to be effectively phosphorylated on tyrosine. Which was the PTK responsible for phosphorylating ZAP-70FF? Others have reported that a kinase-deleted mutant of Syk associated to \(\beta\)\(\gamma\) ITAM of the FcRI receptor in RBL cells could be tyrosine-phosphorylated when overexpressed (58), and we have obtained similar data with the ZAP-70 kinase-defective mutant transiently expressed in Jurkat cells (data not shown). Thus, endogenous ZAP-70 may in part contribute to phosphorylate the expressed mutants and, by the mechanism proposed above, another PTK, such as p56\(^{ck}\), may further mediate this event. However, in the case of the ZAP-70FF mutant, there was an additional complication because its \textit{in vitro} kinase activity was found to be augmented. Similar results were obtained by analyzing Y492F or Y492FY493F ZAP-70 mutants expressed in Cos cells (14), and it was speculated that these tyrosines have distinct roles in the activation of the kinase. Our data suggest that ZAP-70 mutated on both Tyr-492 and Tyr-493 was able to \(\text{trans}\)-phosphorylate itself when bound to the ITAM but was unable to phosphorylate cellular substrates, possibly reflecting a fine mechanism of kinase activity regulation by Tyr-492 and Tyr-493. Perhaps phosphorylation of Tyr-492 (or the phenylalanine substitution) may unlock the catalytic site, thus facilitating the autophosphorylation step, whereas phosphorylation of Tyr-493 may be essential for stabilizing the kinase activation loop (21) important for full accessibility to more distal and/or less available cellular substrates.

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