Tyrosine 319, a Newly Identified Phosphorylation Site of ZAP-70, Plays a Critical Role in T Cell Antigen Receptor Signaling*

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Following T cell antigen receptor (TCR) engagement, the protein tyrosine kinase (PTK) ZAP-70 is rapidly phosphorylated on several tyrosine residues, presumably by two mechanisms: an autophosphorylation and a trans-phosphorylation by the Src-family PTK Lck. These events have been implicated in both positive and negative regulation of ZAP-70 activity and in coupling this PTK to downstream signaling pathways in T cells. We show here that Tyr319 and Tyr419 in the interdomain B of ZAP-70 are autophosphorylated in vitro and become phosphorylated in vivo upon TCR triggering. Moreover, by mutational analysis, we demonstrate that phosphorylation of Tyr419 is required for the positive regulation of ZAP-70 function. Indeed, overexpression in Jurkat cells and in a murine T cell hybridoma of a ZAP-70 mutant in which Tyr419 was replaced by phenylalanine (ZAP-70-Y319F) dramatically impaired anti-TCR-induced activation of the nuclear factor of activated T cells and interleukin-2 production, respectively. Surprisingly, an analogous mutation of Tyr319 had little or no effect. The inhibitory effect of ZAP-70-Y319F correlated with a substantial loss of its activation-induced tyrosine phosphorylation and up-regulation of catalytic activity, as well as with a decreased in vivo capacity to phosphorylate known ZAP-70 substrates, such as SLP-76 and LAT.

Collectively, our data reveal the pivotal role of Tyr319 phosphorylation in the positive regulation of ZAP-70 and in TCR-mediated signaling.

The signaling ability of the TCR1 depends upon its coordinated interaction with protein tyrosine kinases (PTKs) belonging to the Src and Syk families (1). Several members of these PTK families are expressed in T cells, including Lck, Fyn, Syk, and ZAP-70. Genetic evidence has indicated that among the Syk-PTKs, ZAP-70 plays a major role in both T lymphocyte development and functional activation. Analysis of patients suffering from a severe immunodeficiency due to the lack of this PTK showed that only CD4+ T cells develop, although they are unresponsive to TCR stimulation (2–4). Moreover, development of both CD4+ and CD8+ single positive thymocytes is impaired in ZAP-70-null mice (5) but not affected by Syk gene disruption (6, 7). According to current models of the early activation steps following TCR triggering, the Src-PTK Lck phosphorylates the tyrosine residues in the ITAMs of the receptor invariant chains (CD3 and ζ) (8, 9) and allows ZAP-70 recruitment, via its tandem SH2 domains, to the ITAMs (8, 10). Thereafter, ZAP-70 becomes tyrosine-phosphorylated as a result of both autophosphorylation and trans-phosphorylation by Lck (11, 12). Tyrosine phosphorylation of ZAP-70 correlates with its increased kinase activity (13) and is thought to generate docking sites for several SH2 or PTB domain-containing enzymes or adapters, including Lck (14), Abl, Ras-GTPase-activating protein (11), Vav (15), Cbl (16), She (17), and SH2-containing phosphatase-1 (18). These proteins may be involved in regulation of ZAP-70 activity and/or coupling to downstream signaling pathways.

So far, tyrosine residues of ZAP-70 that have been shown to be phosphorylated in vivo following TCR engagement include Tyr292, Tyr492, and Tyr493 (19). Mutational analysis of Tyr292 indicated that this residue negatively regulates ZAP-70 function without affecting its catalytic activity (20, 21). This negative effect could be mediated by Cbl binding to phosphorylated Tyr292 (22, 23). Phosphorylation of Tyr492 appears also to have a negative regulatory role, as its mutation to phenylalanine results in increased ZAP-70 catalytic activity (20, 21, 24). In contrast, up-regulation of ZAP-70 catalytic activity appears to depend on the phosphorylation of Tyr493 by Lck, as indicated by co-expression experiments in heterologous cell systems (12, 24). Phosphorylation of this residue is essential for connecting ZAP-70 to downstream signaling pathways (12), presumably because it enables this PTK to phosphorylate its substrates (25).

Nonetheless, several pieces of evidence have suggested the existence of other phosphorylation sites in ZAP-70. We have previously shown that when Tyr492 and Tyr493 are simultaneously mutated to phenylalanine, ZAP-70 is still phosphorylated after TCR triggering and can still interact with Lck via an SH2-mediated interaction (25). Moreover, although not proven to be phosphorylated, Tyr315 is thought to be the binding site for the SH2 domain of Vav (15) and has been shown to play a critical role in antigen receptor signaling (26). Finally, recent reports have suggested a regulatory role in TCR signaling for other tyrosine residues in ZAP-70 (27, 28), raising the possibility that other phosphorylation sites exist.
In an attempt to gain additional clues on the regulatory role of ZAP-70 tyrosine phosphorylation, we focused on conserved residues found in the linker region between the C-terminal SH2 domain and the catalytic domain of this PTK (called interdomain B; Ref. 10), namely Tyr315 and Tyr319. We demonstrate here that both tyrosines are phosphorylated in vitro and in vivo and that Tyr319 plays a critical role in the regulation of ZAP-70. Overexpression of ZAP-70 bearing a Y319F mutation (ZAP-70-Y319F) impaired TCR-induced events, such as activation of NFAT and IL-2 production. Mutation of Tyr319 substantially reduced the activation-dependent tyrosine phosphorylation of ZAP-70 and the up-regulation of its catalytic activity. Moreover, we show that ZAP-70-Y319F impaired TCR-mediated phosphorylation of known ZAP-70 substrates, such as SLP-76 and LAT. In contrast to a previous report (26), we found that a ZAP-70-Y315F had only minimal inhibitory effects on TCR signaling. Thus, our results indicate that phosphorylation of Tyr319 is a critical event for up-regulation of ZAP-70 activity and highlight the pivotal role of this residue in TCR signaling.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—The human leukemia Jurkat T cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 ml/ml penicillin, and 100 l/ml streptomycin (Life Technologies, Cergy Pontoise, France) (complete medium). Murine T cell hybridoma TAL.81 (T8.1), expressing human CD4 and TCR specific for a tetanus toxin peptide (tt830–843; QYIKANSFKGITE) (29) restricted for HLA-DR1*1102 was maintained in complete Dulbecco’s modified Eagle’s medium supplemented with 400 mM methotrexate, 1 mg/ml G418 and 50 l/ml 3-bromocaprotoehanol. Mouse L625.7 fibroblasts expressing HLA-DR1*1102 (30) were kindly provided by Dr. R. W. Karr (Monsanto Chemical Co., St. Louis, MO) and grown in complete minimum Eagle’s medium containing 250 mg/ml G418. Mouse monoclonal antibodies (mAbs) were as follows: anti-human TCR Vb8.101.5.2 (IgM, provided by E. L. Reinherz, Dana-Farber Cancer Institute, Boston, MA), anti-CD3 VIT3 (IgM, provided by W. Knapp, University of Vienna, Austria), anti-phosphotyrosine 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY), and anti-vesicular stomatitis virus-protein G epitope tag 5G5D (IgGb; hybridoma kindly provided by Dr. T. E. Kreis, University of Geneva, Switzerland) (31). Rabbit polyclonal antibodies were as follows: affinity-purified anti-Lck antibody 2102 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ZAP-70 antisera 4.06 (25) and ZAP-4 (gift of S. C. Ley) (32), and anti-vesicular stomatitis virus-protein G epitope tag antiserum (provided by M. Arpin, Institut Curie, Paris, France) (33).

Constructions and Plasmids—The GST-(SH2)ZAP-70 fusion protein (containing residues 255 through 619 of human ZAP-70) was obtained by polymerase chain reaction using oligonucleotide-directed mutagenesis and confirmed by nucleotide sequencing. This construct was expressed in COS-1 cells and purified by glutathione affinity chromatography. The ZAP-70 WT construct bearing a C-terminal vesicular stomatitis virus-protein G epitope tag was described previously (25). ZAP-70-Y315F and -Y319F mutants were both derived from this construct by polymerase chain reaction: the same 5’ primer (base pairs 713–734) was used, encompassing the Mutl unique site. The 3’ primers, encoding either the Y315F or the Y319F mutation, included base pairs 1147–1184 or 1157–1184, respectively, and both contained a SacI site. The Mutl-SacI-digested polymerase chain reaction products were ligated with both a SacI-NsiI fragment (base pairs 1179–1736) and a 3.8-kilobase pair ZAP-70 WT pBS fragment restricted with Mutl and NsiI. ZAP-70 constructs were finally subcloned into the EcoRl-XbaI sites of the pSRo-puro expression vector (a gift of R. P. Sekaly, Institut de Recherches Cliniques, Montreal, Quebec, Canada) (25). Mutations were verified by nucleotide sequencing.

Cell Transfection and NFAT Activity Assays—Jurkat cells (107) were transiently transfected by electroporation (25) with the indicated amounts of pSRo-puro vector empty or containing ZAP-70 WT or ZAP-70 mutant cDNAs together with the NFAT-luciferase (10 lsg) and pSV-β-galactosidase (30 lsg) reporter plasmids (25). 24 h after transfection, cells were left unstimulated or stimulated at 37 °C for 8 h with 101.5 anti-TCR mAb precoated to wells at 1:1000 dilution of ascites or with phorbol 12-myristate 13-acetate (50 ng/ml) and the calcium ionophore A23187 (2 lM lsg) (Sigma). β-Galactosidase and luciferase assays were performed by using the specific assay systems (Promega). Luciferase activities, determined in duplicate samples, were normalized to the β-galactosidase values to correct for transfection efficiency. Stable T8.1 transfectants were obtained by electroporation under the same conditions and were then selected in complete Dulbecco-modified Eagle’s medium containing methotrexate (400 mM), G418 (1 mg/ml), and puromycin (1 lsg/ml).

In Vitro and in Vivo Phosphorylation Analysis—The GST-(SH2)ZAP-70 fusion protein was autophosphorylated for 30 min at room temperature in 1 mM Tris buffer, pH 7.5, 7.5 mM NaCl, 25 mM Hepes, 10 mM MnCl2, 0.05% Nonidet P-40, containing 10 lC of [γ-32P]ATP and, after SDS-PAGE, transferred onto nitrocellulose membranes. 32P-Labeled bands were excised and incubated with 0.5 lC CNBr (Fluka) in 70% formic acid for 1.5 h at room temperature (33). Cleavage products were separated on Tris-tricine gels (16.5% total acrylamide concentration, 3% cross-linker) as described (34). For further tryptic digestion, gel-separated 32P-peptides were transferred onto nitrocellulose membranes and digested in situ by trypsin (Worthington) in 50 mM NH4HCO3, overnight at 37 °C. Peptides were then identified by performic acid and separated by thin layer electrophoresis in pH 1.9 buffer for 15 min at 1000 V, followed by thin layer chromatography for 10 h in phosphochromatography buffer (35). Phosphorylated peptides were visualized by autoradiography.

For automated Edman degradation, gel-separated 32P-peptides were transferred onto a polyvinylidene difluoride membrane, excised, and eluted in 40% trifluoroacetic acid, 0.1% acetonitrile at 70 °C. They were covalently coupled onto a Sequelon™-AA filter (Millipore) and sequenced in an Applied Biosystems 494 sequencer (36). ATZ amino acid derivatives were collected and monitored by liquid scintillation counting.

Metabolic labeling was performed by incubating Jurkat cells (3.3 × 106/ml) for 4 h in phosphate-free medium containing 0.3 mM 32P (ICN). Cells were then stimulated by anti-CD3 antibody (VIT3) at 1:200 dilution of ascites for 3 min or perevanadate for 5 min.

Immunoprecipitation and Immunoblotting—Jurkat cell transfecants, stimulated with anti-CD3 mAb (1:200 dilution of ascites) for 3 min at 37 °C or perevanadate for 5 min, were lysed in 1% Nonidet P-40-containing buffer and immunoprecipitated with the relevant antibody (25). Immunoprecipitation, immunoblotting, and detection of proteins by enhanced chemiluminescence (Amersham Pharmacia Bio- tech) were performed as described previously (14). [32P]T Protein A detection followed by PhosphorImager scanning of anti-ZAP-70 immunoblots were used to quantify the relative expression of exogenous versus endogenous ZAP-70 proteins in stable transfecants as described (25). Metabolic labeling of in vitro kinase activity—ZAP-70 catalytic activity was assessed in vitro by incubating anti-tag immunoprecipitates for 5 min at room temperature in 25 mM MES, pH 6.5, 10 mM MnCl2, 5 lM ATP, containing 10 lC of [γ-32P]ATP and 0.3 lC of the cytoplasmic fragment of the erythrocyte band 3 protein (cF3b) as a substrate (24, 37). Antigen Stimulation and IL-2 Production Measurement—T cell hybridomas (1 × 106 cells) were incubated overnight at 37 °C with L625.7 fibroblasts (1 × 105) prepuited or not with increasing concentration of tt830–843 (0.05–5 lM) as described previously (29). IL-2 released in the supernatants was then measured by using the DuoSet ELISA kit (Genzyme, Cambridge, MA) according to the manufacturer’s instructions. Analysis of protein phosphorylation in total cell lysates was performed by anti-phosphotyrosine immunoblotting as described previously (38).

RESULTS

Residues Tyr315 and Tyr319 in ZAP-70 Are Phosphorylated in Vitro and in Vivo—In order to establish whether Tyr315 and Tyr319 are phosphoacceptor sites, we first made use of a GST-ZAP-70 fusion protein lacking the SH2 domains (GST-(SH2)ZAP-70, containing residues 255–619 of human ZAP-70). Although trypsin digestion has been previously employed to identify phosphorylated tyrosines in ZAP-70 (19), inspection of the amino acid sequence surrounding Tyr315 and Tyr319 (Fig. 1) indicated the presence of multiple peptide bonds known to be

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cleaved with low efficiency or not cleaved at all by trypsin (e.g. Arg306-Pro307, Lys304-Pro305, and Lys325-Lys329; see Ref. 35).

Thus, in order to avoid complexity of peptide maps due to partial digestion products, we initially chose to perform phosphopeptide mapping after protein cleavage by CNBr. This approach had also the advantage of allowing the isolation of relatively large-sized peptides, analyzable by one-dimensional gel electrophoresis.

According to the human ZAP-70 sequence (39), CNBr cleavage at Met310 and Met359 would produce a peptide containing Tyr315 and Tyr319 and having a M, of about 6000. Indeed, when GST-(ΔSH2)ZAP-70 was autophosphorylated in vitro with [γ-32P]ATP and cleaved with CNBr, five phosphorylated peptides could be detected (Fig. 2A), which included a major product (peptide I) migrating at a M, of about 6000. Edman degradation of peptide I (which, as expected, contained only phosphotyrosines; data not shown) showed two 32P-labeled amino acids at cycles 5 and 9, as predicted for Tyr315 and Tyr319 (Fig. 2B). Because GST-(ΔSH2)ZAP-70 contains only these two tyrosines separated by four residues, we concluded that Tyr315 and Tyr319 are autophosphorylated under these conditions. Although the amount of radioactivity recovered at cycle 5 was usually higher compared with cycle 9, suggesting a possible preferential phosphorylation at Tyr315, this is likely not to be the case, as recovery of radioactivity at cycle 9 (Tyr319) suffers from a low yield due to poor cleavage at P318, as we verified by sequencing a synthetic peptide encompassing Tyr315 and Tyr319 (not shown).

Peptides I and II were further digested by trypsin and subjected to two-dimensional peptide mapping. As shown in Fig. 2C, these peptides gave an identical phosphopeptide pattern, indicating that peptide II was a partial cleavage product including peptide I. As no trypsin cleavage site exists between Tyr315 and Tyr319, the multiplicity of spots is due to partial digestion products (as predictable from peptide I sequence, see above). Calculation of the electrophoretic and chromatographic mobilities for the expected tryptic peptides (35) indicated that Tyr315/Tyr319 doubly phosphorylated peptides would migrate more toward the anode (Fig. 2C, right) and have a decreased mobility in the chromatographic dimension compared with their mono-phosphorylated counterparts. This prediction was confirmed by Edman degradation, as the three peptides indicated by arrowheads (Fig. 2, C and D) contained phosphorylated amino acids at both cycles 5 and 9. Moreover, these peptides selectively disappeared in tryptic peptide maps of peptide I from GST-(ΔSH2)ZAP-70-Y319F or -Y315F mutants.3 Thus, these data show that peptides indicated by arrowheads (Fig. 2, C and D) are actually isomers phosphorylated on both Tyr315 and Tyr319, whereas the additional phosphopeptides observed in tryptic digest of peptide I (in the upper part of the plate, Fig. 2, C and D) are likely to represent peptides phosphorylated at either residue.

As peptide III was not visible in all digestions, and peptides IV (M, ~23,000) and V (M, ~26,000–28,000) migrated with an M, ≥ ½ of the entire GST-(ΔSH2)ZAP-70 (M, ~67,000) (Fig. 2A), these peptides are likely to be partial cleavage products also containing Tyr(P)315 and/or Tyr(P)319. Phosphorylation of ZAP-70 at residue Tyr292, previously identified as an autophosphorylation site (Ref. 19), was not evident with this construct.

In vivo phosphorylation of Tyr315 and Tyr319 upon cell activation was demonstrated by comparing CNBr and trypsin cleavage products of in vitro autophosphorylated GST-(ΔSH2)ZAP-70 with those derived from metabolically 32P-labeled ZAP-70 immunoprecipitated from anti-CD3-treated Jurkat cells. CNBr cleavage of in vivo phosphorylated ZAP-70 generated two peptides co-migrating with peptides I and II (Fig. 2A). Analysis of tryptic maps of peptide I from in vivo and in vitro phosphorylated ZAP-70 and their mixture revealed that both samples shared three peptides migrating as Tyr315/Tyr319 doubly phosphorylated forms (Fig. 2E). Singly phosphorylated peptides migrating in the upper part of the plates (see above) were only barely detectable in the in vivo labeled peptide I. Moreover, an extra phosphopeptide appeared in this sample that was not observed in the in vitro labeled protein.

Identification of this additional phosphopeptide was precluded by the very low radioactivity recovered after in vivo labeling. Similar tryptic peptide patterns were obtained with ZAP-70 from pervanadate-activated Jurkat (not shown). Preliminary experiments using peptide-specific antibodies and ZAP-70 mutants suggest that peptides from in vivo labeled ZAP-70 (Fig. A, filled and open arrowheads) contain phosphorylated Tyr292 and Tyr319, respectively.3 Collectively, these results demonstrate that both Tyr315 and Tyr319 are phosphorylated in vivo following TCR engagement, and the in vitro data suggest that this event may be the consequence of ZAP-70 auto-phosphorylation.

Dominant Negative Effect of ZAP-70-Y319F on TCR-stimulated NFAT Activity—To investigate the possible role of the phosphorylation on Tyr315 and/or Tyr319 in the signaling ability of ZAP-70, a tagged ZAP-70-Y315F or ZAP-70-Y319F mutant was first transiently overexpressed in Jurkat T cells. As a readout for cellular activation, TCR-induced stimulation of NFAT activity was measured. Transfection of ZAP-70-Y319F-encoding plasmid resulted in a dose-dependent inhibition of the TCR-induced NFAT activity compared with the empty vector (Fig. 3A). We have previously shown that a similar inhibition was exerted by a kinase-defective mutant of ZAP-70 (ZAP-70KD) or by a ZAP-70 mutant carrying a double mutation Y492F/Y493F, whereas transfection of ZAP-70WT had no effect in this system (25). On the other hand, only a modest inhibition of NFAT activity was observed when transfecting a vector encoding ZAP-70-Y315F (Fig. 3A). This mild effect on TCR-induced signaling, which was also observed in another T cell model system (see below and Ref. 38), contrasts with a previous report that showed that the same mutation strongly impaired the ability of ZAP-70 to reconstitute antigen receptor signaling in a Syk-deficient chicken B cell line (26). These discrepancies are possibly due to the different experimental model used.

Mutation of Tyr319 Results in Decreased Activation-induced ZAP-70 Tyrosine Phosphorylation and Kinase Activity Up-regulation—The molecular basis of the signaling defect due to the Y319F mutation was analyzed in Jurkat cells stably overexpressing ZAP-70-Y319F. The presence of the C-terminal epitope tag increased the electrophoretic mobility of the transfected construct compared with endogenous ZAP-70 and allowed us to measure the relative expression levels of the exog-

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enous versus endogenous ZAP-70 in each cell line by anti-ZAP-70 immunoblotting, as described previously (25). Two representative independent transfectants, named 1.40 and 1.60, expressing ZAP-70-Y319F at levels 3–4-fold over the endogenous ZAP-70 were used in most experiments, as well as two cell lines expressing the ZAP-70-Y315F mutant (2.21/2 and 2.21/14). Jurkat cells transfected with an empty vector (J05.2) or a representative cell line (15.8) stably expressing a 2-fold excess of tagged ZAP-70 (ZAP-70WT) (Ref. 25 and data not shown) were utilized as controls. The relative expression levels of the exogenous ZAP-70 constructs (collectively designated ZAP-70tag) in these transfectants are shown in Fig. 3C. All of these cell lines expressed comparable levels of TCR/CD3 and Lck (data not shown).

Fig. 3B shows that the dominant negative effect of ZAP-70-Y319F, but not that of ZAP-70-Y315F, could be confirmed in these stable transfectants. Again, TCR-induced NFAT activation was strongly reduced (by ~80%) in both cell lines expressing ZAP-70-Y319F, whereas only a limited inhibition was noted in cells expressing ZAP-70-Y315F (37 and 12% in 2.21/2 and 2.21/14 cell lines, respectively), despite higher (2–3-fold) expression levels of this mutant (Fig. 3C) compared with ZAP-70-Y319F. Collectively, data shown in Fig. 3, A and B, demonstrate that the Y319F mutation has a dramatic effect on TCR-induced signaling, whereas the Y315F mutation has only a modest inhibitory effect (see also below).

Based on the results described above, we focused on biochemical analysis on ZAP-70-Y319F. In order to evaluate the impact of the Y319F mutation on ZAP-70 phosphorylation level in vivo, ZAP-70-Y319F and ZAP-70WT were immunoprecipitated using an anti-tag antiserum and analyzed by SDS-PAGE and anti-phosphotyrosine immunoblotting. These experiments...
revealed a strong reduction of ZAP-70-Y319F tyrosine phosphorylation compared with ZAP-70WT (Fig. 4A, upper panel) after CD3 cross-linking, despite similar amounts of immunoprecipitated protein (Fig. 4A, lower panel). These experiments also showed that ZAP-70-Y319F, like ZAP-70WT, co-precipitated with the tyrosine-phosphorylated ζ-chain (Fig. 4A, upper panel), thus indicating that the mutation did not affect the ability of ZAP-70 to bind the ITAMs.

Considering that at least five tyrosine residues of ZAP-70 are known to be phosphorylated upon TCR triggering (Ref. 19 and this work), the absence of Tyr319 cannot in itself explain the considerably decreased phosphorylation of ZAP-70-Y319F and suggests that other phosphorylation sites can be indirectly affected. Thus, we asked whether the catalytic activity of ZAP-70 was impaired by the mutation. To this aim, ZAP-70WT and ZAP-70-Y319F were immunoprecipitated by an anti-tag antiserum from Jurkat cells unstimulated or stimulated with the tyrosine phosphatase inhibitor pervanadate, and their ability to phosphorylate an exogenous substrate in vitro was assessed. Pervanadate was routinely used in these experiments as it reproducibly gave a higher increase in ZAP-70 kinase activity over the basal level compared with anti-CD3 stimulation. As the in vivo phosphorylation pattern of ZAP-70 was superimposable after pervanadate or anti-CD3 antibody stimulation of Jurkat cells (data not shown; see also Ref. 12) and because the induction of ZAP-70 tyrosine kinase activity correlates with its tyrosine phosphorylation (12), these two stimulatory agents can be considered equivalent from a qualitative point of view. Moreover, because the expression levels of the ZAP-70tag proteins were slightly dissimilar in the different cell lines, 32P incorporation in the substrate was normalized in each sample for the relative amount of immunoprecipitated protein.
Phosphorylation of ZAP-70 at Tyr^{319} Regulates TCR Signaling

FIG. 3. Dominant negative effect of the ZAP-70-Y319F on TCR-induced NFAT activity. A, Jurkat cells were co-transfected by electroporation with 0.2 μg (black bars), 1 μg (open bars), or 5 μg (hatched bars) of pSGag-puro vector, either empty (vector) or containing Y315F or Y319F mutant cDNAs, together with the NFAT-luc and pSV-β-galactosidase reporter plasmids. Transfected cells were stimulated for 8 h with either anti-TCR mAb or phorbol 12-myristate 13-acetate + calcium ionophore A23187. Luciferase activities are corrected for transfection efficiency and expressed as a percentage of the maximal luciferase activity measured after stimulation with phorbol 12-myristate 13-acetate (PMA) + A23187. Comparable expression of both mutants was confirmed by anti-tag immunoblot (not shown). Data are mean + S.E. from 2–4 independent experiments. B, Jurkat cells stably expressing ZAP-70-Y319F (1.40 and 1.60), ZAP-70-Y315F (2.21/2 and 2.21/4), or the empty pSGag-puro vector (J05.2) were co-transfected with NFAT-luc and pSV-β-galactosidase plasmids and left unstimulated (open bars) or stimulated with anti-TCR mAb for 8 h (black bars). Luciferase activities (mean + S.E. from 2–6 independent experiments for each clone) were normalized and expressed as described above. C, PhosphorImager scanning showing the relative expression levels of exogenous ZAP-70 proteins (WT or mutants) in stably transfected Jurkat cells. Equal amount of proteins (as assessed by Bradford assay) from the indicated cell line were subjected to SDS-PAGE and immunoblotting with the anti-tag antiserum. Detection was performed by 125I-labeled protein A, followed by PhosphorImager scanning of the membrane.

protein measured by anti-tag immunoblot. As shown in Fig. 4B, these experiments revealed that the ability of unstimulated ZAP-70-Y319F to phosphorylate the cytosolic fragment of c-fos was comparable to that of ZAP-70WT. However, the increase in the kinase activity of ZAP-70-Y319F after pervanadate treatment of both 1.40 and 1.60 cell lines was markedly reduced compared with ZAP-70WT. Thus, ZAP-70-Y319F appeared to be defective in the activation-dependent up-regulation of its catalytic activity. A possible explanation for this phenotype would be that mutation of Tyr^{319} induced a structural change in ZAP-70 that impaired the increase in its kinase activity following TCR triggering. Alternatively, as this residue is phosphorylated in vivo, it could serve as a docking site for other molecules required for up-regulating ZAP-70 catalytic activity (see under “Discussion”).

Dominant Negative Effect of ZAP-70-Y319F on Antigen-induced IL-2 Production—We then wanted to confirm the dominant negative effect of ZAP-70-Y319F in a different T cell model and to evaluate the effect of this mutant on antigen-induced signaling. Thus, ZAP-70-Y319F was stably overexpressed in the murine T cell hybridoma T8.1, expressing a human TCR specific for the tetanus toxin peptide t830–843 (29). ZAP-70-Y315F and ZAP-70KD were also overexpressed in these cells as a control. As shown in Fig. 5A, the expression of these ZAP-70 constructs in a number of representative clones was comparable and at levels over 10-fold higher than the endogenous ZAP-70, as measured by PhosphorImager scanning of anti-ZAP-70 immunoblot. Hybridomas were stimulated using as APCs the human class II MHC-expressing murine fibroblasts L625.7 (29) pre pulsed with increasing amounts of antigenic peptide. Overexpression of ZAP-70-Y319F markedly decreased IL-2 production in response to antigen (Fig. 5B). Indeed, this mutant induced a shift of the dose-response curve of about 1 order of magnitude compared with untransfected T8.1 cells. A similar effect was found in cells overexpressing comparable levels of ZAP-70KD, whereas no effect of ZAP-70-Y315F overexpression on IL-2 production was detectable (Fig. 5B). Moreover, no effect either was seen in T8.1 cells overexpressing similar levels of ZAP-70 wild-type (data not shown). Collectively, these results indicate that the inhibitory effect of ZAP-70-Y319F on TCR-induced signaling cannot be overcome by using more physiological stimulatory conditions (i.e., antigen presentation by an APC able to deliver appropriate co-stimulatory signals (38)) and underline the critical function of Tyr^{319} of ZAP-70 in TCR-mediated T cell activation.

Effects of ZAP-70-Y319F Overexpression on Tyrosine Phosphorylation of ZAP-70 Substrates—Because we have shown that ZAP-70-Y319F was defective in the activation-dependent up-regulation of its catalytic activity, it was expected that phosphorylation of ZAP-70 substrates would be impaired in cells overexpressing this mutant. We took advantage of the higher expression levels of ZAP-70-Y319F obtained in the T8.1 hybridoma, compared with Jurkat transfectants, to analyze the effects of this mutant on the pattern of tyrosine phosphorylation induced by antigen stimulation. Moreover, we were also able to compare it to ZAP-70KD-expressing cells. As shown in Fig. 5C, anti-phosphotyrosine immunoblotting on total cell lysates revealed an increased tyrosine phosphorylation of several proteins of 120–130, 95, 76, 66, and 36–38 kDa in antigen-stimulated T8.1 cells compared with unstimulated cells. In particular, two phosphoproteins of about 76 and 36–38 kDa appeared to be the major substrates upon antigen stimulation. The 76-kDa phosphoprotein, migrating as a doublet, was previously identified as being SLP-76 (41, 42), whereas the 36–38-kDa-migrating phosphoprotein band is likely to represent LAT (43, 44). Both of these proteins are thought to play important roles in connecting TCR-activated PTKs to downstream signaling pathways and have been recently shown to be phosphorylated by ZAP-70 (44–46). Antigen-induced tyrosine phos-
phorylation of both SLP-76 and LAT appeared to be markedly reduced in cells overexpressing ZAP-70-Y319F. Similarly to our previous observation about IL-2 production (see Fig. 5B), this effect was comparable to that exerted by the kinase-defective mutant ZAP-70KD (Fig. 5C). Thus, the inhibition of tyrosine phosphorylation of known ZAP-70 substrates in ZAP-70-Y319F-expressing hybridomas can be accounted for by the lack of activation-induced increase in kinase activity of this mutant and may explain the impairment of antigen-induced IL-2 production.

DISCUSSION

In this work, we demonstrated that both Tyr315 and Tyr319, located in the interdomain B of ZAP-70, are phosphorylated in vivo upon TCR signaling. Moreover, we provide multiple pieces of evidence that Tyr319, but not Tyr315, is a critical positive regulatory site for ZAP-70 function and TCR-mediated signaling.

Other groups had failed in identifying Tyr315 and Tyr319 as phosphorylation sites (19). This discrepancy could be accounted for, at least in part, by the different protein cleavage (i.e. CNBr instead of trypsin digestion) and phosphopeptide analysis methods that we employed. Indeed, although chemical digestion of ZAP-70 by CNBr was not complete (see Fig. 2A), it could have facilitated the recovery and thus the identification of phosphopeptides containing Tyr315 and Tyr319, as trypsin digestion was predicted to generate multiple partial digestion products containing those residues. Comparison of in vivo and in vitro labeled peptide I shows that this peptide is almost exclusively phosphorylated at both Tyr315 and Tyr319 in vivo, whereas the monophosphorylated isoform of this peptide can be readily detected after in vitro labeling (Fig. 2F). This difference suggests that phosphorylation of both tyrosines is most efficient in vivo, possibly due to the proximity and/or correct orientation of ZAP-70 molecules bound to the ITAMs of engaged TCR/CD3 complexes, compared with the phosphorylation of molecules in solution obtained during in vitro labeling experiments. However, it cannot be ruled out that this difference is accounted for by the particular GST-(ΔSH2)ZAP-70 construct used for in vitro labeling. Also, we suspect that the direct fusion of GST protein to the interdomain B might have precluded in vivo phosphorylation of Tyr329, perhaps as a consequence of steric hindrance. Our experiments also show that in vivo labeled peptide I contained an extra spot that could not be identified. This spot, containing only phosphotyrosine residues, likely derives from a CNBr-cleaved peptide co-migrating with peptide I and including a tyrosine that cannot be auto-phosphorylated by ZAP-70 in vitro.

Previous works have demonstrated that the corresponding residues of the homologous PTK Syk (Tyr342 and Tyr352) are autophosphorylation sites in vitro (47). Our data showing that GST-(ΔSH2)ZAP-70 autophosphorylates in vivo on both Tyr315 and Tyr319 are in agreement with that report and suggest that in vivo phosphorylation of Tyr315 and Tyr319 could similarly be achieved by autophosphorylation of ZAP-70, although contribution of other PTKs (e.g. Lck) cannot be ruled out. Indeed, as shown by Neumeister et al. (11), trans-phosphorylation between adjacent ZAP-70 molecules on multiple tyrosine residues occurs following their binding to phosphorylated ITAMs on the TCR-ζ chain. This mechanism, which would not require phosphorylation of ZAP-70 by Src-PTKs (see below), generates binding sites for multiple substrates, effectors, or other regulatory molecules on ZAP-70 and could lead to phosphorylation of Tyr315 and Tyr319.

Several pieces of evidence underscore the functional importance of the interdomain B of Syk-PTKs: in particular, this region contains several phosphorylatable tyrosine residues in both ZAP-70 (Ref. 19 and this work) and Syk (47). For example, Tyr329, Tyr315, and Tyr319 in ZAP-70 are phosphorylated upon TCR triggering. Tyr329 is thought to be a negative regulatory site, as its mutation to phenylalanine increases TCR-mediated signaling (20, 21). On the other hand, we show now that an analogous mutation of Tyr319 results in a strong impairment of
TCR-dependent T cell activation (Figs. 3 and 5), thus indicating a positive regulatory role for this residue. The role of phosphorylation at Tyr\(^{315}\) remains so far unclear. It has been proposed that this residue is the binding site for the SH2-domain of Vav (15). Moreover, Wu et al. (26) have shown that the Y315F mutation profoundly altered the ability of ZAP-70 to reconsti-

**Fig. 5.** Overexpression of ZAP-70-Y319F inhibits antigen-induced IL-2 production and tyrosine phosphorylation of ZAP-70 substrates. A. PhosphorImager scan showing the expression levels of endogenous wild-type ZAP-70 (ZAP-70endo) versus exogenous ZAP-70 mutants (ZAP-70tag) in T8.1 hybridomas stably expressing ZAP-70KD, -Y315F, and -Y319F. Equal amounts of proteins (as assessed by Bradford assay) from the T8.1 hybridoma or the indicated transfectant were analyzed by SDS-PAGE and immunoblot with the 4.06 anti-ZAP-70 antiserum. The transfected constructs (ZAP-70tag) have a decreased electrophoretic mobility compared with the endogenous form (ZAP-70endo) due to the presence of the C-terminal epitope tag. Detection and analysis were performed as outlined in Fig. 3C. B, T8.1 T cell hybridoma or transfectants stably expressing the indicated mutant (KD, Y315F, or Y319F) were stimulated by APC prepulsed with increasing amount of tt830–843 peptide antigen. IL-2 released in the supernatant was measured by enzyme-linked immunosorbent assay. Each point represents the average of triplicate samples. C, T8.1 T cell hybridoma or transfectants stably expressing ZAP-70-Y319F (clone 11; cf. Fig. 3B) or ZAP-70KD (clone 3.3) were stimulated by APC prepulsed with 10 \(\mu\)g of tt830–843 peptide antigen. Cells were then lysed, and the phosphorylation of cellular proteins was assessed by SDS-PAGE and anti-phosphotyrosine immunoblotting. Similar results were also obtained with two other independent transfectants expressing ZAP-70-Y319F and another two expressing ZAP-70KD.
tute antigen receptor signaling in a Syk-negative chicken B cell line, in contrast with the modest effect of ZAP-70-Y319F on TCR-induced NFAT activation in Jurkat cells (Fig. 3) and on antigen-stimulated IL-2 production in the T8.1 hybridoma (Fig. 5). We have also previously shown that ZAP-70-Y315F overexpression in the T8.1 hybridoma did not have a noticeable effect on TCR-induced tyrosine phosphorylation of Vav and SLP-76 (38). These discrepancies may be ascribed to the different cellular systems used. Nonetheless, our data on the functional role of Tyr319 further underscore the view of interdomain B as a critical regulatory region for ZAP-70 function.

It should be noted, however, that data from Zhao and Weiss (20) indicated that expression of a ZAP-70 mutant in which residues 265–331 (spanning about 80% of the interdomain B) were deleted did not significantly affect TCR signaling, in contrast with the evidence on the functional importance of Tyr319 and Tyr319 cited above. Although further investigations are required to clarify these discrepancies, the simultaneous elimination of negative and positive regulatory mechanisms depending on Tyr292 and Tyr319, respectively, could account for the apparent neutral effect of the interdomain B deletion.

Our data show that TCR-induced tyrosine phosphorylation of ZAP-70-Y319F is dramatically reduced compared with the wild-type molecule (Fig. 4A). This finding is not explained by an alteration of the intrinsic kinase activity of the mutant, as its ability to phosphorylate cH3 in vitro is comparable to that of the wild-type molecule when both are immunoprecipitated from unstimulated cells (Fig. 4B). Moreover, this mutation did not alter the ability of ZAP-70 to bind to phosphorylated ITAMs (Fig. 4A), a defect previously observed for a naturally occurring deletion in the interdomain B of Syk (48). These results argue against a structural alteration of the PTK induced by the mutation of Tyr319. On the other hand, ZAP-70-Y319F appears to be defective in the activation-induced up-regulation of its kinase activity, a finding that correlates with the lack of in vivo phosphorylation of known ZAP-70 substrates, i.e. SLP-76 and LAT (see Fig. 5C). As these proteins have been shown to couple ZAP-70 to the activation of downstream signaling pathways leading to NFAT activation (44–46), our data indicate that the inability of ZAP-70-Y319F to phosphorylate SLP-76 and LAT in vivo is responsible for the inhibition of TCR-stimulated cellular activation.

However, the molecular mechanism underlying the inhibitory effect of the Y319F mutation remains to be fully ascertained. The simultaneous mutation of Syk residues Tyr348 and Tyr352 (homologous to ZAP-70 residues Tyr315 and Tyr319, respectively) has been shown to affect the SH2-mediated binding of PLCγ1 to Syk and the tyrosine phosphorylation of the former by Syk (49). Although we cannot rule out the possibility that Tyr319 is similarly involved in the binding of PLCγ1 to ZAP-70, it would be difficult to explain how the mutation of Tyr319 and the consequent lack of PLCγ1 binding could dramatically impair activation-induced tyrosine phosphorylation and catalytic activity of ZAP-70 (see Fig. 4), as well as phosphorylation of known substrates of this PTK (see Fig. 5). In this context, it is worth noting that the strong inhibitory effect of ZAP-70-Y319F contrasts with those seen associated with the mutation of Tyr292 (20, 21) and, more recently, with Tyr509/Tyr508 (28), both resulting in a gain-of-function phenotype, or with the mutation of Tyr315 and Tyr474 (this work and Ref. 27), which have a mild inhibitory effect in T cell signaling. Rather, the phenotype observed for ZAP-70-Y319F is reminiscent of that caused by mutation of tyrosines present in the activation loop of ZAP-70, Y493F or Y492F/Y493F. Indeed, we and others have previously shown that these mutations result in impaired calcium mobilization and NFAT activation after antigen receptor stimulation (12, 25) and in decreased pp36–38 phosphorylation and extracellular signal-regulated kinase activation (25). These effects could be explained if Tyr319 and its neighboring residues were the docking site for other molecules directly involved in the regulation of ZAP-70 catalytic activity. A possible candidate is the Src-PTK Lck, as this enzyme is responsible for the phosphorylation of Tyr493 (12), an event that has been shown to up-regulate ZAP-70 catalytic activity and to be required for this PTK to phosphorylate its substrates (25). Several biochemical and functional pieces of evidence recently obtained in our laboratory support this hypothesis: we have found that Tyr319, but neither Tyr315 nor other in vitro phosphorylated tyrosines of ZAP-70 (25), specifically binds the SH2 domain of Lck. Consistent with these data, replacement by mutagenesis of the natural sequence Y319S of ZAP-70 by an optimal binding motif for the SH2 domain of Src-PTKs (sequence YEEI; Ref. 50) results in strong gain-of-function mutant inducing augmentation of NFAT transcriptional activity in Jurkat cells at levels >10-fold higher compared with the wild-type molecule and a more efficient ZAP-70 phosphorylation.5 Binding of Lck to ZAP-70 to up-regulate its catalytic activity bears an analogy with the model proposed for the FAK PTK, the activation of which depends on an SH2-mediated association with Src and/or Fyn PTKs (36, 51). Thus, the SH2-mediated binding of Lck to ZAP-70 would ensure, by a relatively stable interaction, persistent phosphorylation/activation of the latter and downstream signaling while TCR is engaged with the antigen.

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