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Phosphorylation of Tyr319 in ZAP-70 is required for T-cell antigen receptor-dependent phospholipase C-γ1 and Ras activation

Brandi L.Williams[1], Brenda J.Irvin[1], Shari L.Sutor[2], Claudia C.S.Chini[2], Elaine Yacyshyn[3], Juliane Bubeck Wardenburg[4], Mark Dalton[4], Andrew C.Chan[4] and Robert T.Abraham[1,2,5,6]

[1]Department of Immunology, [2]Division of Oncology Research, [3]Division of Rheumatology, Department of Medicine, Mayo Clinic, Rochester, MN 55905, [4]Division of Rheumatology, Departments of Medicine and Pathology, and Howard Hughes Medical Institute, Washington University School of Medicine, St Louis, MO 63110, USA

[5]Present address: Room C333B, LSRC building, Department of Pharmacology and Cancer Biology, Box 3813, Duke University Medical Center, Durham, NC 27710, USA

[6]Corresponding author
e-mail: abrah008.mc@duke.edu

B.L.Williams and B.J.Irvin contributed equally to this work

Accumulating evidence indicates that the interdomain B regions of ZAP-70 and Syk play pivotal roles in the coupling of T-cell antigen receptor (TCR) stimulation to the activation of downstream signaling pathways. The interdomain B region of ZAP-70 contains at least three candidate sites of tyrosine phosphorylation. In this report, we identify Tyr319 as a functionally important phosphorylation site in the ZAP-70 interdomain B region. TCR crosslinkage triggered a rapid increase in the phosphorylation of Tyr319 in Jurkat T cells. Although mutation of Tyr319 to Phe had no effect on the tyrosine kinase activity of ZAP-70, the resulting ZAP(Y319→F) mutant failed to reconstitute TCR-dependent Ca^{2+} mobilization, Ras activation, CD69 expression and NFAT-dependent transcription in ZAP-70-deficient Jurkat cells. These defects were correlated with reduced tyrosine phosphorylation of phospholipase C (PLC)-γ1 and the LAT adapter protein in the ZAP(Y319→F)-expressing cells. On the other hand, ZAP(Y319→F)-expressing cells displayed normal increases in SLP-76 phosphorylation and ERK activation during TCR stimulation. Phosphorylation of Tyr319 promoted the association of ZAP-70 with the SH2 domains of two key signaling molecules, Lck and PLC-γ1. These studies suggest that Tyr319 phosphorylation is required for the assembly of a ZAP-70-containing signaling complex that leads to the activation of the PLC-γ1- and Ras-dependent signaling cascades in antigen-stimulated T cells.

Keywords: NFAT/signal transduction/T-cell antigen receptor/ZAP-70

Introduction

T-cell antigen receptor (TCR) ligation initiates a series of intracellular signaling events that, depending on the maturational stage of the T cell and the setting in which receptor stimulation occurs, culminate in T-cell activation, anergy or apoptosis. The initiation of signal output from the TCR requires the sequential activation of two families of protein tyrosine kinases (PTKs) (Iwashima *et al*., 1994; Chan and Shaw, 1996; Qian and Weiss, 1997). The Src family PTKs, Lck and Fyn, phosphorylate the immunoreceptor-based tyrosine activation motifs (ITAMs) found in the cytoplasmic domains of the TCR-ζ and -CD3 subunits. A critical function of the phosphorylated ITAMs is to bind the tandem Src homology 2 (SH2) domains of Syk family PTKs, including ZAP-70. The ITAM-bound ZAP-70 undergoes full catalytic activation upon phosphorylation of the Tyr493 residue, which is located in the activation loop of the catalytic domain (Chan *et al*., 1995). Once ZAP-70 is activated, Lck and ZAP-70 presumably act in concert to phosphorylate specific downstream substrates, which in turn orchestrate the cytoplasmic signaling cascades leading to T-cell activation.

The central roles of Lck and ZAP-70 in TCR function have been amply documented by phenotypic analyses of T cells derived from humans with a severe combined immunodeficiency syndrome, from gene-targeted mice, and by studies of Jurkat T cell-derived somatic mutants that fail to express either of the PTKs (Molina *et al*., 1992; Arpaia *et al*., 1994; Chan *et al*., 1994; Elder *et al*., 1994; Negishi *et al*., 1995; Gelfand *et al*., 1995; Hashimoto *et al*., 1996). In particular, comparative studies of Lck- versus ZAP-70-deficient cell lines lend strong support to the sequential activation model outlined above (Straus and Weiss, 1992; van Oers *et al*., 1996; Williams *et al*., 1998). The major challenges at this point are to understand the mechanisms whereby specific cytoplasmic substrates are recruited to the TCR-associated PTKs, and to define the contribution of individual PTKs to the phosphorylation of each substrate. A series of adapter proteins, including LAT, SLP-76 and Cbl, play important roles in the recruitment of phospholipase C (PLC)-γ1, phosphoinositide 3-kinase, and activators of the Ras and Rho families of GTPases to the aggregated receptor complex (Wange and Samelson, 1996; Peterson *et al*., 1998). These translocation events promote downstream signal propagation by colocalizing protein substrates with the TCR-linked PTKs, and by drawing certain signaling enzymes, such as PLC-γ1, into close proximity with their lipid substrates in the plasma membrane.

An additional mechanism of substrate recruitment to the aggregated TCR–PTK complex is considerably more direct. During TCR stimulation, ZAP-70 undergoes the auto- or *trans*-phosphorylation of this PTK on several tyrosine residues (Watts *et al*., 1994; van Oers and Weiss, 1995). In certain cases (e.g. Tyr493), these phosphorylation events exert *cis*-modulatory effects on the activity of the PTK domain, while the phosphorylation of other sites

generates target sequences for specific SH2 domain-containing cytoplasmic proteins. The binding of heterologous proteins to activated ZAP-70 would facilitate the phosphorylation of these proteins by drawing them into the vicinity of the ZAP-70 PTK domain. In addition, phosphorylated ZAP-70 may act as a scaffold for the presentation of substrates to the co-localized Src kinases, and for the formation of multi-protein signaling complexes that are tethered directly to the TCR (Neumeister *et al*., 1995). A potential 'hot spot' for such intermolecular interactions is located in the interdomain B region of ZAP-70, which links the tandem SH2 domains to the C-terminal PTK domain. In human ZAP-70, the interdomain B region spans amino acid residues 254–338, and contains several known or suspected sites of tyrosine phosphorylation (van Oers and Weiss, 1995). A similar interdomain B region is found in the ZAP-70-related PTK, Syk, and although this region in Syk exhibits only 30% overall sequence identity to the ZAP-70 interdomain B region, many of the known or suspected sites of tyrosine phosphorylation are conserved (Furlong *et al*., 1997).

Three tyrosine residues (Tyr292, Tyr315 and Tyr319) in the interdomain B region of ZAP-70 have attracted particular attention as sites that might regulate ZAP-70 activity and/or binding to heterologous proteins. Substitution of Tyr292 with Phe generates an activated ZAP-70 mutant [ZAP(Y292→F)] that, when expressed in Jurkat T-leukemic cells, drives a NFAT-mediated transcriptional response in the absence of TCR ligands (Kong *et al*., 1996; Zhao and Weiss, 1996). These results suggest that phosphorylation of Tyr292 exerts a negative regulatory effect on the signaling functions of ZAP-70 in T lymphocytes. Recent studies have shown that the Cbl proto-oncogene product binds to the phosphorylated Tyr292 site in ZAP-70, and to the orthologous phospho-Tyr323 residue in human Syk (Lupher *et al*., 1997). Genetic data suggest that Cbl is a negative regulator of PTK-dependent signaling pathways in both *Caenorhabditis elegans* and mammalian cells (Ota and Samelson, 1997; Murphy *et al*., 1998). These results support the notion that Cbl binding to phospho-Tyr292 in activated ZAP-70 dampens the signal-transducing functions of this PTK during TCR engagement.

In contrast to Tyr292, a second interdomain B site, Tyr315, may play a positive role in ZAP-70 signaling by serving as an inducible binding site for the SH2 domain of Vav, a Rho-specific guanine nucleotide exchange factor (Katzav *et al*., 1994; Wu *et al*., 1997). The homologous residue (Tyr348) in Syk has also been identified as a phosphorylation-dependent Vav binding site (Deckert *et al*., 1996). Introduction of a ZAP(Y315→F) mutant into wild-type Jurkat cells inhibits several TCR-dependent responses, including the tyrosine phosphorylation of Vav and the transcription of an NFAT-dependent reporter gene. Furthermore, while ectopic expression of wild-type ZAP-70 reconstitutes B-cell antigen receptor (BCR) signaling functions in $Syk^{-/-}$ chicken B-cells, the ZAP(Y315→F) mutant is almost completely defective in this model system.

The third candidate phosphorylation site in the interdomain B region of ZAP-70 is Tyr319. Although the functional importance of this site in ZAP-70 is unknown, a previous report presented indirect evidence that the orthologous Tyr352 residue in Syk is involved in the association of PLC-γ1 with this PTK (Law *et al*., 1996). Unfortunately, the analysis was limited to a Syk(Y348,352→F) double mutant; hence, the authors were unable to assign the PLC-γ1-binding activity to either one or both of the interdomain B tyrosine residues in Syk. The conservation of sequence surrounding the corresponding tyrosines in ZAP-70 (Tyr315 and Tyr319) implies that this sub-region of interdomain B also participates in the recruitment of PLC-γ1 and/or other critical signaling molecules during TCR stimulation.

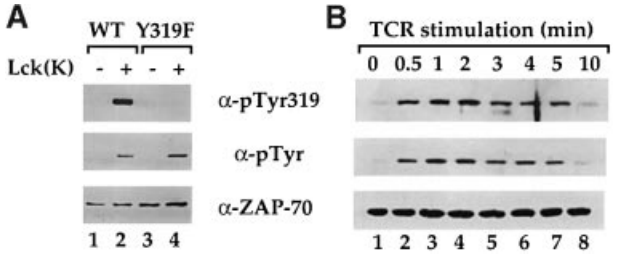

Fig. 1. Identification of Tyr319 as an inducible phosphorylation site in ZAP-70. (**A**) Phosphorylation of recombinant wild-type ZAP-70 and ZAP(Y319→F) in insect cells. Sf9 insect cells were infected with baculovirus encoding GST-tagged wild-type ZAP-70 (WT) or the ZAP(Y319→F) substitution mutant (Y319F) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of the Lck catalytic domain. At 36 h post-infection, the cells were harvested, lysed, and the GST-tagged ZAP-70 fusion proteins were isolated with glutathione–Sepharose. After separation by SDS–PAGE, the immunoprecipitated proteins were immunoblotted sequentially with phospho-Tyr319-specific antibodies (α-pTyr319), anti-phosphotyrosine antibodies (α-pTyr), and the α-ZAP-70 mAb, 2F3.2. (**B**) Tyr319 is phosphorylated in response to TCR crosslinkage. Jurkat cells were stimulated with anti-TCR mAb (C305) for the indicated times, and ZAP-70 was immunoprecipitated from cellular extracts with anti-ZAP-70 antiserum (1225). The immunoprecipitated proteins were immunoblotted sequentially with α-pTyr319, α-pTyr and α-ZAP-70 as described in (A).

The objective of the present study was to define the role of phosphorylation at Tyr319 in the signaling functions of ZAP-70 in T cells. Through the use of phospho-Tyr319-specific antibodies, we demonstrate that this site is rapidly phosphorylated in response to antibody-mediated TCR crosslinkage in Jurkat cells. The functional importance of Tyr319 phosphorylation was documented by the failure of a ZAP(Y319→F) mutant to reconstitute TCR-dependent PLC-γ1 and Ras activation in a ZAP-70-deficient Jurkat T-cell line. Biochemical studies identified the C-terminal SH2 domain of PLC-γ1 and the SH2 domain of Lck as candidate ligands for the phosphorylated Tyr319 site in ZAP-70. These results indicate that phosphorylation of Tyr319 in ZAP-70 plays a critical role in the coupling TCR ligation to the PLC-γ1- and Ras-dependent signaling cascades in T lymphocytes.

Results

Activation-dependent phosphorylation of the interdomain B Tyr319 residue in ZAP-70

The interdomain B region of ZAP-70 contains two closely spaced tyrosine residues ($Y^{315}ESPY^{319}SDP$), both of which are followed by proline at the +3 position. Recent studies have provided indirect evidence suggesting that both sites may be phosphorylated following stimulation of multi-chain antigen receptors on T and B cells (Law *et al*.,

1996; Wu *et al*., 1997). To determine whether the Tyr319 site undergoes TCR-inducible phosphorylation in T cells, we generated polyclonal antibodies that specifically recognized a ZAP-70-derived peptide containing phospho-Tyr319 (see Materials and methods). The phospho-Tyr319-specific antibody reacted with baculovirus-expressed ZAP-70 only when this PTK was co-expressed with the Lck catalytic domain (Figure 1A, upper panel), a setting in which ZAP-70 becomes catalytically active (Chan *et al*., 1995). In contrast, a ZAP-70 mutant containing a Tyr319→Phe substitution [ZAP(Y319→F)] was not recognized by the antibody after expression in insect cells, in the absence or presence of Lck. Nonetheless, Lck co-expression caused a significant increase in the overall phosphorylation of the ZAP(Y319→F) mutant on tyrosine residues, as indicated by immunoblotting with anti-phosphotyrosine antibodies (Figure 1A, middle panel).

The effect of TCR stimulation on the phosphorylation of the Tyr319 in ZAP-70 was subsequently examined in Jurkat-derived P116 cells that were stably transfected with epitope-tagged, wild-type ZAP-70. The results demonstrate that ZAP-70 phosphorylation on Tyr319 is rapid and time-dependent, with the maximal response observed after 1–2 min of TCR stimulation. Thus, the kinetics of phosphorylation at the Y319 site correlate with the changes in overall protein tyrosine phosphorylation stimulated by TCR crosslinkage in Jurkat cells (Williams *et al*., 1998).

Failure of the ZAP(Y319→F) mutant to restore TCR-dependent NFAT activation in P116 cells

The functional importance of Tyr319 phosphorylation was initially determined in NFAT-dependent transactivation assays. In these experiments, P116 cells, which are deficient in both ZAP-70 and Syk (Williams *et al*., 1998), were transiently transfected with a pNFAT-Luc reporter plasmid, together with wild-type ZAP-70, ZAP(Y315→F) or ZAP(Y319→F). As reported previously (Williams *et al*., 1998), anti-CD3 antibody stimulation fails to trigger NFAT activation in the absence of co-transfected ZAP-70 (Figure 2A). Unexpectedly, the ZAP(Y315→F) mutant, which lacks the major binding site for Vav (Wu *et al*., 1997), also reconstituted TCR-dependent NFAT activation in P116 cells. In contrast, P116 cells transfected with the ZAP(Y319→F) mutant or with a ZAP(Y315,319→F) double mutant were non-responsive to anti-CD3 antibody stimulation in these reporter assays. Identical results were obtained in a panel of P116-derived subclones that stably expressed either the wild-type or mutant ZAP-70 constructs, at protein levels approximating those found in the parental Jurkat cell line (Figure 2B; data not shown).

To determine whether the ZAP(Y319→F) mutant was a dominant inhibitor of the signaling functions of wild-type ZAP-70, the parental Jurkat E6 cells were cotransfected with pNFAT-Luc plus various amounts of

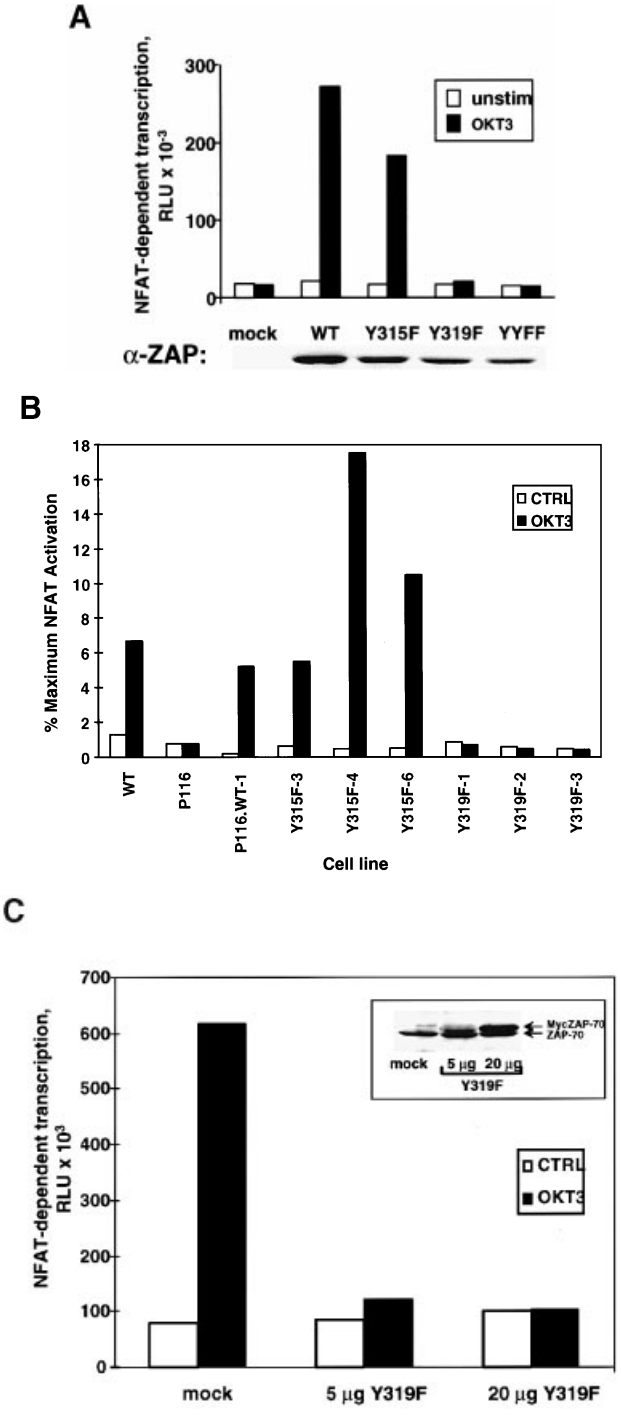

Fig. 2. Effect of mutations at Tyr315 and Tyr319 in the interdomain B region on ZAP-70-dependent NFAT activation. (**A**) Transient transfection assays. P116 cells were transfected with 10 μg plasmid DNA encoding no protein (mock), wild-type ZAP-70 (WT), ZAP-Y315F (Y315F), ZAP-Y319F (Y319F) or ZAP-YYFF (YYFF). The cells were co-transfected with 10 μg pNFAT-Luc reporter plasmid. The transfected cells were cultured for 12 h, and were stimulated for an additional 6 h with medium only (open bars), or with 1 μg per ml anti-CD3 antibody (OKT3; filled bars). Cellular extracts were prepared, and duplicate aliquots of each sample were assayed for luciferase activity and for expression of the ZAP-70 proteins. (**B**) NFAT activation in stably-transfected P116 cells. P116 cells were transfected with wild-type ZAP-70 (P116.WT-1), ZAP(Y319→F) (Y319F) or ZAP(Y315→F) (Y315F). The indicated subclones were selected for the expression of ZAP-70 polypeptides at levels approximating those in the parental Jurkat cell line (results not shown). The cell lines were transiently transfected with the pNFAT-Luc reporter plasmid. Cells were stimulated and luciferase activities were determined as described in (A). (**C**) Dominant-suppressive effect of ZAP-70 mutants in Jurkat T cells. Jurkat cells were cotransfected with pNFAT-Luc and the indicated amounts of plasmid DNA encoding no protein (mock) or ZAP(Y319→F). Cells were stimulated and luciferase activity determined as in (A). The inset shows an anti-ZAP-70 immunoblot of cell extracts from the same cell populations. The arrows on the right depict the locations of the endogenous ZAP-70 and Myc-tagged ZAP(Y319→F).

ZAP(Y319→F) expression plasmid (Figure 2C). Electroporation of Jurkat cells with 5 μg of ZAP(Y319→F) plasmid DNA effectively abolished the increase in NFAT-dependent transcription normally provoked by TCR ligation.

Catalytic activity of ZAP(Y319→F) mutant

A possible explanation for the functional defects displayed by the ZAP(Y319→F) mutant is that the replacement of Tyr319 with Phe inhibits either the intrinsic or inducible PTK activity of ZAP-70. To address this issue, we examined the *in vitro* autokinase activities of wild-type and mutant ZAP-70 proteins isolated from stably transfected P116 cells. The cells were left unstimulated or were stimulated with OKT3 antibody for 2 min prior to cell harvest. The immune complex kinase assays were done in the presence of [γ-^{32}P]ATP, and incorporation of radioactive phosphate into ZAP-70 was determined as a measure of autokinase activity. The results in Figure 3A show that TCR crosslinkage increases the autophosphorylation of wild-type ZAP-70. The mutant ZAP-70 immunoprecipitated from the ZAP(Y319→F)-expressing P116 cells showed higher levels of basal and inducible autophosphorylation that were explained by the relative expression level of the mutant protein in this experiment (Figure 3A; data not shown). Although the autokinase activity of the ZAP(Y315→F) mutant was also increased in response to TCR ligation, both the basal and stimulated kinase activities were consistently lower than those observed in samples containing equivalent levels of wild-type ZAP-70 (also see Figure 3B). These findings indicate that the loss of function associated with replacement of Tyr319 with Phe is not due to suppression of the TCR-inducible autophosphorylating activity of ZAP-70. Moreover, in spite of the reduction in autokinase activity induced by the Tyr315→Phe substitution, the ZAP(Y315→F) mutant retains substantial signaling activity when expressed in P116 cells.

The relative kinase activities of the wild-type and mutant ZAP-70 proteins toward an exogenous substrate paralleled those observed in the autophosphorylation assays. In these experiments, P116 cells were transiently transfected with Myc epitope-tagged versions of wild type ZAP-70 or the indicated ZAP-70 mutants, and the transfected cells were either left unstimulated or were stimulated with pervanadate, a pharmacological stimulus for ZAP-70 activation (Secrist *et al*., 1993), prior to cell lysis. The recombinant ZAP-70 polypeptides were immunoprecipitated with anti-ZAP-70 antibodies, and immune complex kinase assays were done in the presence of [γ-^{32}P]ATP and the glutathione *S*-transferase–cytoplasmic domain of band 3 (GST–cdb3) substrate. Stimulation of the transfected P116 cells with pervanadate increased the *in vitro* kinase activities of both the wild-type and mutated ZAP-70 proteins (Figure 3B). The specific activity of the ZAP(Y319→F) mutant towards the exogenous substrate was similar to that of wild-type ZAP-70. In contrast, the PTK activity of ZAP(Y315→F) isolated from unstimulated or pervanadate-stimulated cells was reproducibly lower than those obtained with the corresponding wild-type ZAP-70 and ZAP(Y319→F) immunoprecipitates.

Finally, we examined the effect of anti-CD3 antibody

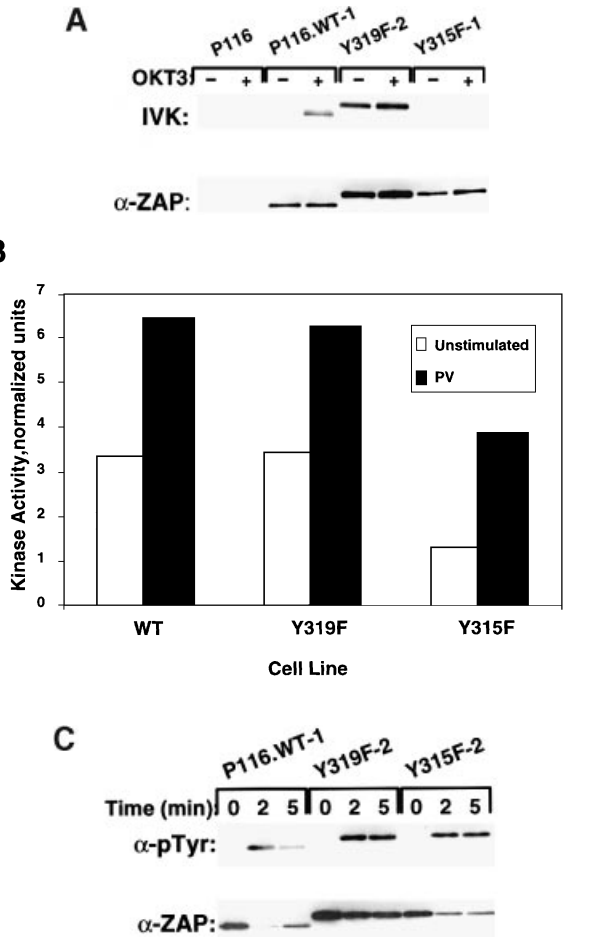

Fig. 3. Catalytic activities of ZAP-70 interdomain B mutants. (**A**) TCR-inducible autokinase activity of ZAP-70 polypeptides. P116 cells or stable transfectants expressing the indicated ZAP-70 polypeptides were left unstimulated or were stimulated for 2 min with OKT3 antibodies. The cells were lysed, and extracts were immunoprecipitated with ZAP-70-specific polyclonal antibodies. Immune complex kinase assays were done in the presence of [γ-^{32}P]ATP, and the reaction products were separated by SDS–PAGE. Radiolabeled ZAP-70 was detected by autoradiography (IVK, upper panel) and total ZAP-70 protein in each sample was determined by immunoblotting with anti-ZAP-70 antibodies (lower panel). (**B**) Catalytic activities of ZAP-70 polypeptides toward an exogenous substrate. P116 were transiently transfected with wild-type (WT) or the indicated ZAP-70 interdomain B region mutants. The cells were either left unstimulated or were stimulated for 5 min with pervanadate, and the ZAP-70 polypeptides were immunoprecipitated for immune complex kinase assays as described in (A). Assays were performed with GST–cdb3 as the substrate. Incorporation of radioactivity into GST–cdb3 was quantitated and normalized to the amount of ZAP-70 protein in each reaction, based on the level of Myc tag-specific immunoreactivity, which was quantitated by luminescence detection with a Molecular Dynamics Storm 840 system. (**C**) Tyrosine phosphorylation of ZAP-70 polypeptides in intact cells. P116 subclones that stably expressed the indicated wild-type and mutated ZAP-70 proteins were stimulated for various times with anti-CD3 (OKT3) antibodies as described in (A). The cells were lysed, and cellular extracts were immunoprecipitated with anti-ZAP-70 antibodies. The immunoprecipitated proteins were separated by SDS–PAGE, and immunoblotted with anti-phosphotyrosine (α-pTyr) mAb. The membrane was then stripped and re-probed with anti-ZAP-70 antibody.

stimulation on the tyrosine phosphorylation of ZAP-70 in intact P116 cells. In these experiments, the wild-type and mutated ZAP-70 proteins were immunoprecipitated from stably transfected P116 subclones at various times after

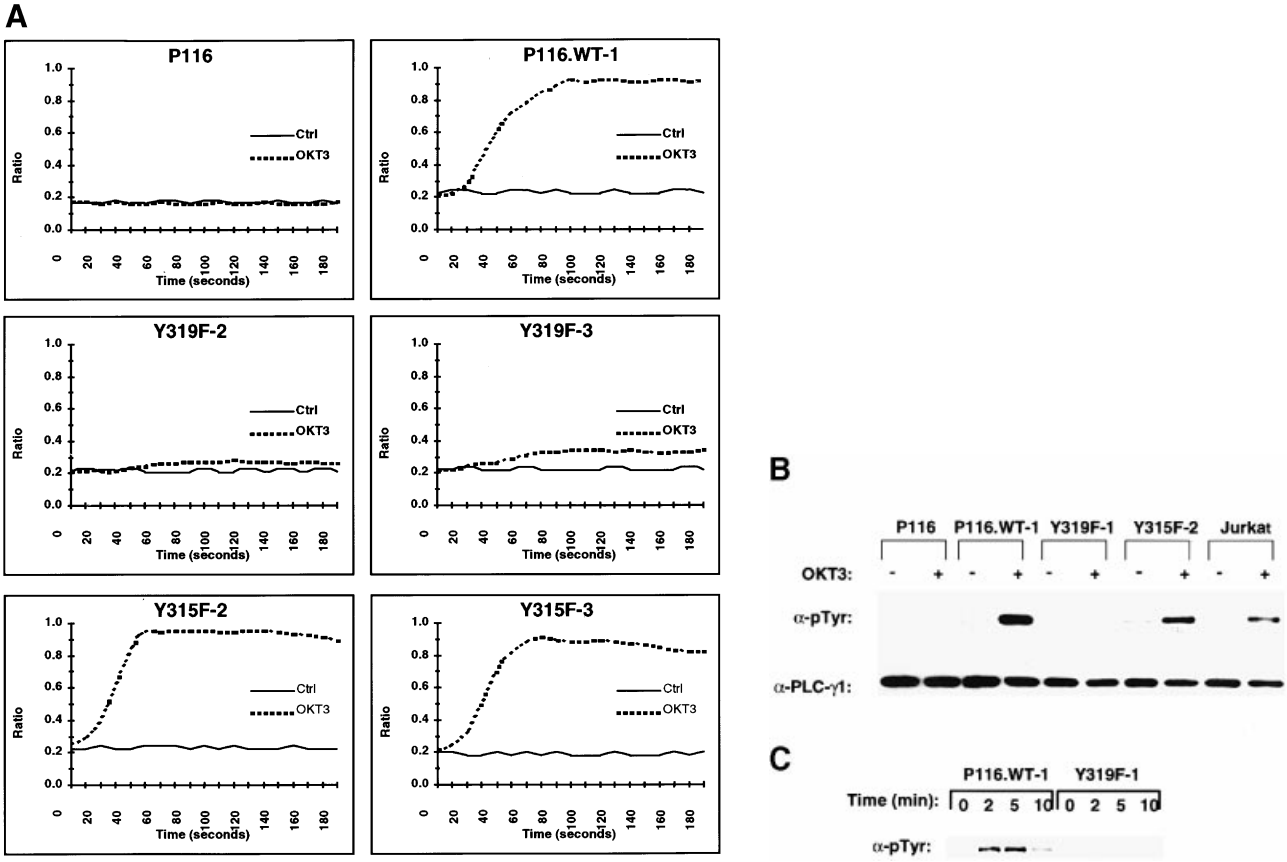

Fig. 4. Activation of the PLC-γ1-dependent signaling pathway in P116 cell transfectants. (**A**) TCR-induced calcium mobilization in P116-derived subclones. Non-transfected or stably transfected P116 cells were loaded with the Ca^{2+}-indicator dye, indo-1. The cells were stimulated with 1 μg/ml anti-CD3 antibody, and the Indo-1 fluorescence emission ratio was monitored as a function of time after addition of the stimulus. (**B**) Tyrosine phosphorylation of PLC-γ1. The indicated cell lines were left unstimulated or were stimulated for 2 min with OKT3 antibody. Anti-PLC-γ1 immunoprecipitates were prepared from cellular extracts and separated by SDS–PAGE. Membranes were immunoblotted with anti-phosphotyrosine mAb, and then stripped and re-probed with anti-PLC-γ1 anti-serum. (**C**) Time course of TCR-induced PLC-γ1 tyrosine phosphorylation. Stably transfected P116.WT-1 or Y319F-1 cells were stimulated for the indicated times with OKT3 antibody. PLC-γ1 was immunoprecipitated from cellular extracts, and immunoblotted with anti-phosphotyrosine antibodies.

stimulation, and alterations in ZAP-70 tyrosine phosphorylation were determined by anti-phosphotyrosine immunoblotting (Figure 3C). The results demonstrate that TCR stimulation provokes comparable increases in the tyrosine phosphorylation of the wild-type ZAP-70, ZAP(Y319→F) and ZAP(Y315→F) proteins.

Effect of ZAP(Y319→F) on Ca^{2+} mobilization and PLC-γ1 activation

The induction of NFAT-dependent transcription in T cells is dependent on an increase in intracellular free Ca^{2+} triggered by TCR engagement (Crabtree and Clipstone, 1994). The profound defect in NFAT activation observed in ZAP(Y319→F)-expressing P116 cells suggested that this ZAP-70 mutant might fail to restore the coupling between TCR ligation and intracellular Ca^{2+} mobilization in these cells. To address this possibility, stably transfected P116 subclones were loaded with the Ca^{2+} indicator dye, indo-1, and changes in intracellular Ca^{2+} concentration were monitored by flow cytometry (Figure 4A). As reported previously (Williams *et al.*, 1998), anti-CD3 antibody stimulation induces virtually no increase in intracellular free Ca^{2+} in ZAP-70-deficient P116 cells. This defect is fully reversed by stable expression of wild-type ZAP-70 in these cells. In contrast, the ZAP(Y319→F) mutant fails to reconstitute the TCR-dependent Ca^{2+} mobilization response in P116 cells. Once again, the ZAP(Y315→F) mutant restored the normal increase in intracellular Ca^{2+} in the activated cells, indicating that this response is not dependent on the phosphorylation of Tyr315 in ZAP-70.

The Ca^{2+} mobilization defect displayed by ZAP(Y319→F)-expressing P116 cells suggests that the mutant ZAP-70 may be incapable of mediating TCR-dependent phosphorylation and activation of PLC-γ1. The importance of ZAP-70 in this process was documented by the failure of TCR stimulation to induce a detectable increase in the tyrosine phosphorylation of PLC-γ1 in P116 cells (Williams *et al.*, 1998; Figure 4B). This PLC-γ1 phosphorylation defect was corrected by reconstitution of P116 cells with either wild-type ZAP-70 or the ZAP(Y315→F) mutant. However, the TCR-dependent tyrosine phosphorylation of PLC-γ1 was dramatically reduced in ZAP(Y319→F)-expressing P116 cells (Figure 4B), even after prolonged periods of receptor engagement (Figure 4C).

Phosphorylation of SLP-76 and LAT

Recent studies have implicated two adapter proteins, SLP-76 and LAT, as positive upstream regulators of the tyrosine

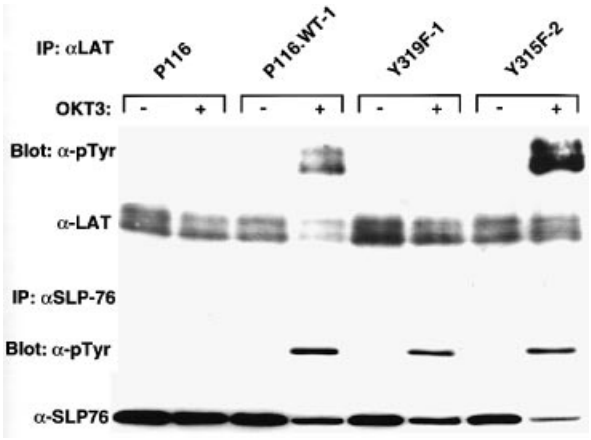

Fig. 5. Effect of interdomain B mutations in ZAP-70 on the TCR-dependent phosphorylation of LAT and SLP-76. P116 cells that stably expressed the indicated wild-type (WT) or mutated ZAP-70 polypeptides were stimulated for 2 min with crosslinked OKT3 antibodies, and either LAT (top panel) or SLP-76 (bottom panel) was precipitated from detergent extracts with the appropriate polyclonal antibodies. Tyrosine phosphorylation of each protein was examined by anti-phosphotyrosine (α-pTyr) immunoblotting. The immunoblots were stripped and reprobed with LAT- or SLP-76-specific antibodies to detect total protein loaded in each lane. The apparent reduction in total LAT protein in samples from activated Jurkat cells reflects an anomalous decrease in the immunoreactivity of the α-LAT antibody with phosphorylated LAT (unpublished observations).

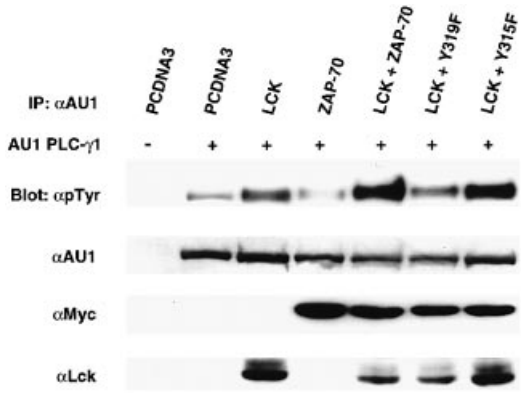

Fig. 6. Co-expression studies in COS-1 cells. COS-1 cells were transfected by electroporation with equivalent total amounts of plasmid DNA encoding the indicated proteins. In the upper two panels, cellular extracts were immunoprecipitated with anti-AU1 antibodies (αAU1), and the phosphorylation of AU1-PLC-γ1 was examined by immunoblotting with anti-phosphotyrosine antibodies (top panel). The immunoblot was stripped and reprobed with αAU1 to verify equivalent sample loading. In the bottom two panels, whole-cell extracts were immunoblotted directly with αMyc (to detect expression of Myc-tagged ZAP-70 polypeptides), followed by anti-Lck antibodies.

phosphorylation of PLC-γ1 in activated T cells (Yablonski *et al*., 1998; Zhang *et al*., 1998). The profound defect in TCR-dependent PLC-γ1 phosphorylation in ZAP (Y319→F)-expressing P116 cells prompted an examination of the tyrosine phosphorylation of SLP-76 and LAT in these cells (Figure 5). Non-transfected P116 cells displayed dramatic reductions in the tyrosine phosphorylation of SLP-76 and LAT following anti-CD3 antibody stimulation, which is consistent with earlier evidence that both proteins are substrates for ZAP-70 in T cells (Wardenburg *et al*., 1996; Zhang *et al*., 1998). Stable expression of either wild-type ZAP-70 or ZAP(Y315→F) in P116 cells reversed the defects in TCR-dependent SLP-76 and LAT tyrosine phosphorylation. In contrast, introduction of the ZAP(Y319→F) mutant differentially affected the phosphorylation of the two adapter proteins. Whereas SLP-76 tyrosine phosphorylation was restored to an almost wild-type level, the tyrosine phosphorylation of LAT remained profoundly depressed in ZAP(Y319→F)-expressing P116 cells. Thus, the failure of ZAP(Y319→F) to restore the coupling between TCR ligation and PLC activation may be explained, at least in part, by the inability of this mutant to support the tyrosine phosphorylation of the LAT adapter protein.

Role of Tyr319 in the interaction of ZAP-70 with PLC-γ1

The results described above suggested that the Tyr319 phosphorylation site plays an important role in ZAP-70-mediated PLC-γ1 phosphorylation. To further substantiate this model, co-expression studies were performed in the primate kidney cell line, COS-1. The cells were transfected with an AU1-tagged PLC-γ1 expression plasmid, together with various combinations of plasmids encoding Lck and wild-type or mutant ZAP-70. After 24 h, the cells were lysed in detergent-containing buffer, and the tyrosine phosphorylation of PLC-γ1 was examined by immunoblotting (Figure 6). PLC-γ1 was tyrosine phosphorylated at a low level when overexpressed in COS-1 cells, and as reported previously (Law *et al*., 1996), this basal level of phosphorylation was increased by co-expression of Lck. In the presence of ZAP-70 only, PLC-γ1 phosphorylation remained at the basal level, which is consistent with the strong dependence of ZAP-70 catalytic activity on phosphorylation of this PTK by a Src family kinase (Kolanus *et al*., 1993; Latour *et al*., 1996). As predicted, the tyrosine phosphorylation of PLC-γ1 was increased markedly by co-expression of both Lck and wild-type ZAP-70 in COS-1 cells. The ZAP(Y315→F) mutant supported a similar increase in PLC-γ1 phosphorylation when co-expressed with Lck. On the other hand, expression of the ZAP(Y319→F) mutant failed to increase the phosphorylation of PLC-γ1 above the level observed with Lck alone. Thus, in spite of the fact that ZAP(Y319→F) displays essentially normal catalytic activity *in vitro* (Figure 4), this interdomain B mutant is unable to mediate the phosphorylation of PLC-γ1 in COS-1 cells.

Role of Tyr319 in ZAP-70 binding to SH2 domains of PLC-γ1 and Lck

The sequence adjacent to the Tyr319 residue in ZAP-70 ($\underline{Y}^{319}$SD$\underline{P}$) suggested that phosphorylation of this site might create a target motif for the C-terminal SH2 domain of PLC-γ1 (Songyang *et al*., 1993; Law *et al*., 1996; Furlong *et al*., 1997). Alternatively, the phosphorylated Tyr319 site may be required for an interaction between activated ZAP-70 and SH2 domain of Lck, which may explain the lack of cooperation between the ZAP(Y319→F) mutant and Lck in the PLC-γ1 phosphorylation studies described above. To determine whether phosphorylation of Tyr319 was required for the association of ZAP-70 with PLC-γ1 or Lck, we performed precipitation assays with immobilized GST fusion proteins containing the N- or C-terminal SH2 domains [SH2(N) and SH2(C) domains, respectively] of PLC-γ1, or the SH2 domain of Lck. Detergent extracts were prepared from unstimulated

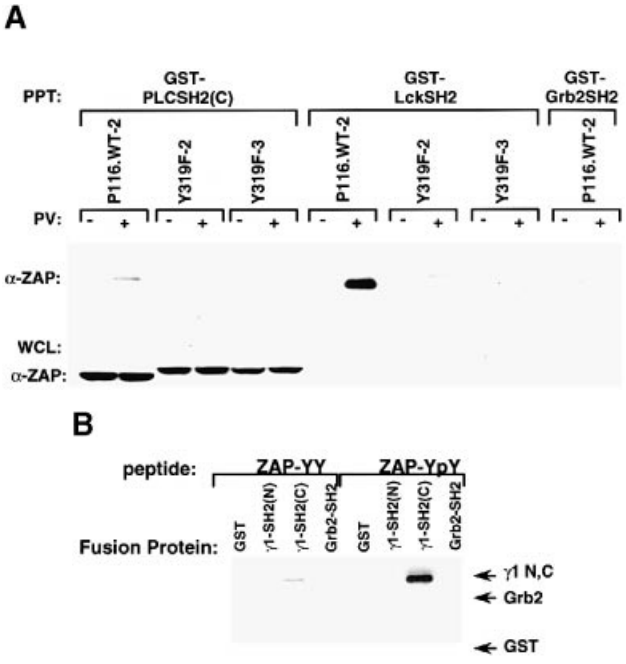

Fig. 7. Binding of SH2 domains of PLC-γ1and Lck to wild-type and mutated ZAP-70 proteins. (**A**) Precipitations with GST fusion proteins. P116 subclones that stably expressed the indicated wild-type (P116.WT-2) or mutated ZAP-70 polypeptides were stimulated for 5 min with 100 μM pervanadate, and cellular extracts were precipitated with GST fusion proteins containing the SH2(C) domain of PLC-γ1, or the SH2 domains of Lck and Grb2. The precipitated proteins were separated by SDS–PAGE, and immunoblotted with anti-ZAP-70 antibodies. The bottom panel shows an anti-ZAP-70 immunoblot of the cellular extracts used in the upper panel. (**B**) Phosphopeptide-binding assays. Biotinylated peptides derived from the interdomain B region of ZAP-70 were prepared with tyrosines (YY) at residues 315 and 319 (ZAP-YY) or with phosphotyrosine (YpY) at residue 319. The peptides were bound to streptavidin–agarose beads, and mixed with the indicated GST fusion proteins. After washing the beads, the bound fusion proteins were eluted, separated by SDS–PAGE, and immunoblotted with rabbit polyclonal α-GST antibodies.

or pervanadate-stimulated P116 cells that stably expressed either wild-type ZAP-70 (P116.WT-2) or ZAP(Y319→F). The GST–PLCSH2(C) fusion protein precipitated wild-type ZAP-70 from pervanadate-stimulated P116.WT-2 cells, but not from unstimulated cells (Figure 7A). In contrast, this fusion protein precipitated little or no detectable ZAP-70 from two P116-derived subclones that stably expressed comparable levels of the mutant ZAP(Y319→F) protein. Qualitatively similar results were obtained with the GST–LckSH2 fusion protein as the precipitating agent. These results indicate that Tyr319 in plays an important role in the binding of activated ZAP-70 to both the SH2(C) domain of PLC-γ1 and the single SH2 domain of Lck. The specificity of these Tyr319-dependent interactions is indicated by the failure of GST fusion proteins containing the Grb2 SH2 domain or the SH2(N) domain of PLC-γ1 (data not shown) to precipitate wild-type ZAP-70 from pervanadate-stimulated P116.WT-2 cells (Figure 7A). In control experiments, the integrity of the SH2 domains of the GST–PLCSH2(N) and GST–Grb2SH2 fusion proteins was verified by the finding that both fusion proteins will precipitate phosphorylated LAT from pervanadate-stimulated P116.WT-2 cells (data not shown).

The selective binding of the PLC-γ1 SH2(C) domain to the phosphorylated Tyr319 site in ZAP-70 was confirmed with phosphopeptides derived from the ZAP-70 interdomain B region (Figure 7B). Biotinylated peptides spanning residues 308–327 of ZAP-70 were synthesized with either Tyr (ZAP-YY) or phosphotyrosine (ZAP-YpY) at the position corresponding to Tyr319 in ZAP-70. The peptides were mixed with fusion protein containing the PLC-γ1 SH2(N) or SH2(C) domain, or the Grb2 SH2 domain. The immobilized ZAP-YpY peptide effectively precipitated the GST–PLCSH2(C) fusion protein, but not GST alone, GST–PLCSH2(N) or GST–Grb2SH2. A very low level of the GST–PLCSH2(C) protein was detected in the ZAP-YY peptide precipitates. This background level of association likely results from the supra-physiological concentrations of the peptide and fusion protein reactants used in these assays. These peptide-binding results substantiate the hypothesis that phosphorylation of Tyr319 in ZAP-70 promotes the direct interaction of this PTK with the SH2(C) domain of PLC-γ1.

ERK activation by ZAP-70 interdomain B region mutants

TCR engagement triggers rapid increases in the activities of the MAP kinase family members, ERK1 and ERK2 (Cantrell, 1996). In the absence of ZAP-70, antibody-mediated TCR crosslinkage causes only a very low level of ERK2 activation, as determined by both the phosphorylation of the ERK substrate, MBP (Figure 8A), and by immunoblotting of the activated forms of the protein kinase with phospho-ERK-specific antibodies (Figure 8B). The ERK activation defect in P116 cells is corrected by the expression of wild-type ZAP-70. Interestingly, expression of either of the ZAP-70 interdomain B region mutants, ZAP(Y319→F) or ZAP(Y315→F), also reversed the ERK activation defect in P116 cells. Thus, although the ZAP(Y319→F) mutant is severely impaired in terms of coupling the TCR to the PLC-γ1 signaling pathway, this ZAP-70 mutant is competent to transduce the signals required for the activation of ERKs during TCR stimulation.

Defective activation of Ras in ZAP(Y319→F)-expressing P116 cells

In many receptor systems, the accumulation of the active, GTP-bound form of Ras is an obligate upstream event leading to the activation of ERKs (Davis, 1993). The restoration of TCR-mediated ERK activation by expression of the ZAP(Y319→F) mutant therefore suggested that the mutated PTK would also reverse the Ras activation defect in P116 cells. An indirect measure of TCR-dependent Ras activation in T cells is the induction of CD69 expression on the cell surface (D'Ambrosio *et al*., 1994). The ZAP-70-deficient P116 cell line displays a severe defect in surface CD69 expression following anti-CD3 antibody stimulation (Figure 9A). This abnormality is corrected by stable expression of wild-type ZAP-70, indicating that ZAP-70 is required for effective coupling of the TCR to Ras. Unexpectedly, however, P116 subclones that stably expressed ZAP(Y319→F) showed a significant impairment of the TCR-mediated increase in surface CD69 expression. Thus, in spite of the apparently normal MAP kinase activation response, the Ras-mediated increase in

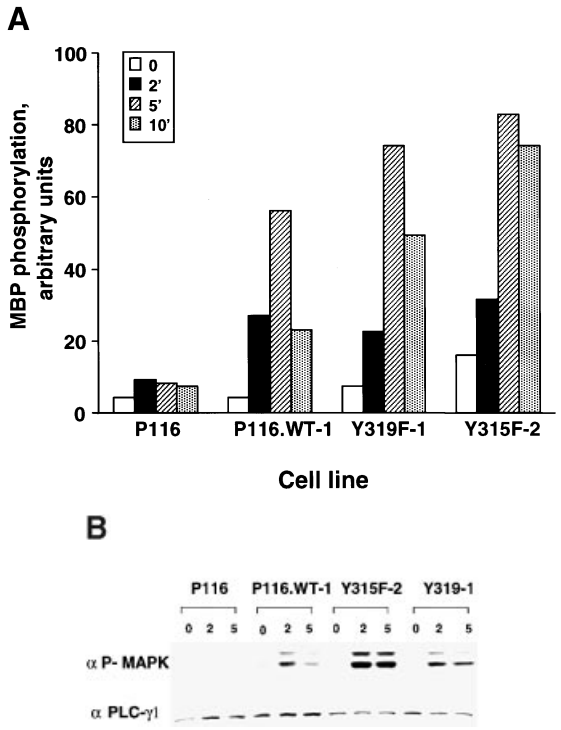

Fig. 8. TCR-dependent ERK activation in P116 cell transfectants. (**A**) Non-transfected or stably transfected P116 cells were stimulated for the indicated times with OKT3 antibodies. The cells were lysed, and ERK2 was immunoprecipitated from the cleared extracts. ERK2 activity was measured in an immune complex kinase assay with [γ-^{32}P]ATP and myelin basic protein (MBP) as the substrate. Incorporation of radioactivity into MBP was quantitated with a PhosphorImaging system. (**B**) The indicated P116 cell lines were stimulated with OKT3 antibodies, and ERK activity was determined by immunoblotting with phospho-ERK-specific antibodies (upper panel). The same samples were immunoblotted with PLC-γ1-specific antibodies to verify equal loading of cellular proteins in each lane (lower panel).

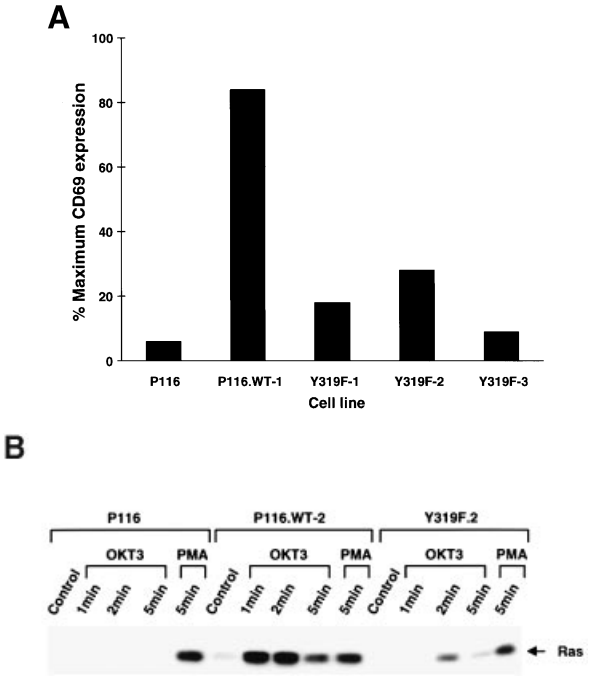

Fig. 9. Defective activation of Ras in ZAP(Y319→F)-expressing P116 cells. (**A**) Stably transfected P116 cells were stimulated for 16 hours with anti-CD3 antibodies or with the phorbol ester, PMA. The cells were assayed for cell surface expression of CD69 by staining with anti-CD69 antibody, followed by flow cytometry. The percentage of CD69-positive cells was normalized to the maximal response obtained with the pharmacologic stimulus, PMA. (**B**) TCR-mediated Ras activation. Stable P116 transfectants expressing wild-type ZAP-70 (P116.WT-2) or ZAP(Y319→F) were stimulated for various times with OKT3 antibodies, or with PMA. The cells were lysed, and cellular extracts were precipitated with GST–Raf fusion protein containing the binding region for GTP-bound Ras. The precipitated proteins were eluted, separated by SDS–PAGE, and the bound Ras protein was determined by immunoblotting with a Ras-specific antibody. The results shown are representative of those obtained in four independent trials.

CD69 expression is severely compromised in ZAP(Y319→F)-expressing P116 cells.

These seemingly discrepant results prompted us to examine more directly the level of TCR-inducible Ras activation in the stable ZAP(Y319→F) transfectants. TCR-dependent alterations in the levels of GTP-bound Ras were monitored by precipitating cellular extracts with an immobilized fusion protein containing the Ras interaction domain of Raf-1 (Brtva *et al*., 1995). This fusion protein selectively captures the GTP-bound form of Ras, the level of which is determined by anti-Ras immunoblotting of the precipitates. The results confirmed that reintroduction of ZAP-70 was necessary for the TCR-mediated accumulation of GTP-bound Ras in anti-CD3 antibody-stimulated P116 cells (Figure 9B). In contrast, P116 cells transfected with ZAP(Y319→F) consistently displayed a significant defect in TCR-dependent Ras activation. Similar results were obtained with a second, independently derived ZAP(Y319→F)-expressing subclone (data not shown). Immunoblot analyses of whole-cell lysates with the same anti-Ras antibody indicated no significant differences in total Ras protein expression among the P116 and ZAP-70-transfected subclones used in these experiments (data not shown). Thus, loss of Tyr319 phosphorylation site in ZAP-70 interferes with the coupling mechanism between TCR stimulation Ras activation in Jurkat T cells.

Discussion

Accumulating evidence indicates that the interdomain B regions of ZAP-70 and Syk play multiple roles in the regulation of the catalytic and functional activities of these PTKs in lymphoid cells. The regulatory functions of the interdomain B region are attributable, at least in part, to the phosphorylation of specific tyrosine residues, several of which are conserved in Syk and ZAP-70. Three candidate interdomain B phosphorylation sites in ZAP-70 are Tyr292, Tyr315 and Tyr319. In this report, we demonstrate that TCR stimulation triggers a rapid increase in the phosphorylation of ZAP-70 at Tyr319 in Jurkat T cells. Substitution of Tyr319 with a non-phosphorylatable phenylalanine residue does not significantly alter the PTK activity of ZAP-70; however, the resulting ZAP(Y319→F) mutant is incapable of reconstituting TCR signaling functions in the ZAP-70-deficient P116 cell line. Our results support the hypothesis that Tyr319 serves as a TCR-regulated binding site for an SH2 domain-containing protein(s) whose association with ZAP-70 is required for initiation of the PLC-γ1- and Ras-dependent signaling cascades in activated T cells.

The possibility that the Tyr315 and Tyr319 residues play active roles in ZAP-70-mediated signaling in T cells

was first raised by the finding that the orthologous sites in human Syk (Tyr348 and Tyr352, respectively) were required for the phosphorylation of PLC-γ1 by this PTK (Law *et al*., 1996). These studies further demonstrated that the SH2(C) domain of PLC-γ1 binds directly to activated Syk, but not to a Syk(Y348,352→F) double mutant. Whether one or both interdomain B tyrosine residues were required for the interaction with PLC-γ1 was not established. Similarly, we found that expression of the corresponding homologous ZAP(Tyr315,319→F) double mutant also fails to reverse the PLC-γ1 phosphorylation and activation defects displayed by the ZAP-70-deficient P116 cell line (results not shown). A comparison of the ZAP(Y319→F) and ZAP(Y315→F) single mutants revealed that the ability of this PTK to restore TCR–PLC-γ1 coupling in P116 cells was exclusively dependent on the integrity of the Tyr319 site. The most straightforward explanation for these results is that auto- or *trans*-phosphorylation of Tyr319 drives the interaction between ZAP-70 and an SH2 domain-containing protein required for the tyrosine phosphorylation and activation of PLC-γ1 by ZAP-70. As discussed below, mutually non-exclusive candidates for this pivotal phospho-Tyr319 interactor are Lck and PLC-γ1 itself.

The functional capabilities of the ZAP(Y315→F) single mutant in P116 cells were unexpected, based on earlier evidence that Tyr315 is an important positive regulatory site in ZAP-70 (Wu *et al*., 1997). The phosphorylated Tyr315 residue binds to the SH2 domain of Vav, a guanine nucleotide exchange factor for Rho family GTPases (Crespo *et al*., 1997). We have confirmed that overexpression of ZAP(Y315→F) in the parental (ZAP-70 positive) Jurkat cell line partially inhibits the increase in NFAT activity provoked by TCR crosslinkage (B.L.Williams and R.T.Abraham, unpublished observations). Similarly, while introduction of wild-type ZAP-70 fully restored BCR-mediated Ca^{2+} mobilization and NFAT activation in $Syk^{-/-}$ B cells, the ZAP(Y315→F) mutant displayed severe defects in this model system (Wu *et al*., 1997). Nonetheless, we observed that expression of ZAP(Y315→F) in ZAP-70-deficient P116 cells fully restores several TCR-dependent signaling events, including PLC-γ1 tyrosine phosphorylation, intracellular Ca^{2+} mobilization and NFAT activation. Similar results were obtained by Acuto and colleagues, who reported that overexpression of the ZAP(Y315→F) mutant failed to interfere with either Vav tyrosine phosphorylation or IL-2 production in activated T-hybridoma cells (Michel *et al*., 1998). The explanation for the conflicting results obtained with the ZAP(Y315→F) mutant in different B- and T-cell lines remains unclear. On the one hand, the dysfunctional state of ZAP(Y315→F) in $Syk^{-/-}$ B cells may be attributable to fundamental differences in the regulation of substrate phosphorylation by BCR-associated PTKs, such that Tyr315 in ZAP-70 is crucial for the activation of PLC-γ1 and other downstream signaling proteins in B cells. On the other hand, the ZAP(Y315→F) mutant may function as a partial antagonist of TCR signaling in settings where the mutant PTK is greatly overexpressed relative to the endogenous, wild-type ZAP-70.

Expression of the ZAP(Y319→F) mutant in P116 cells restored many of the protein tyrosine phosphorylation events normally induced by TCR ligation in Jurkat cells. However, examination of whole-cell anti-phosphotyrosine immunoblots revealed that a band which co-migrated with PLC-γ1 was notably absent from ZAP(Y319→F)-transfected P116 cell extracts (B.L.Williams and R.T.Abraham, unpublished observations). Subsequent studies confirmed that the TCR-mediated tyrosine phosphorylation of PLC-γ1 was profoundly reduced in ZAP(Y319→F)-expressing P116 cells. The failure of TCR stimulation to induce PLC-γ1 phosphorylation provides a rational explanation for both the Ca^{2+} mobilization and NFAT-activation defects displayed by these cells. The present results suggest that the Tyr319→Phe mutation may disrupt a binding site that mediates the recruitment of PLC-γ1 to activated ZAP-70, which then phosphorylates and activates the bound phospholipase. However, other, less direct, models for the role of the Tyr319 site in PLC-γ1 activation are equally possible.

A particularly relevant candidate for the hypothetical phospho-Tyr319-dependent intermediate between ZAP-70 and PLC-γ1 is the adapter protein, LAT. TCR-dependent tyrosine phosphorylation of LAT was sharply reduced in ZAP(Y319→F)-expressing P116 cells. Previous studies have shown that TCR stimulation triggers the formation of a complex containing phosphorylated LAT and PLC-γ1 (Zhang *et al*., 1998). The functional importance of this interaction was underscored by recent observations that TCR ligation fails to induce the tyrosine phosphorylation of PLC-γ1 in LAT-deficient Jurkat cells (Finco *et al*., 1998; W.Zhang, B.J.Irvin, R.F.Trible, R.T.Abraham and L.E.Samelson, submitted). Interestingly, a similar phenotypic defect was recently reported in a Jurkat-derived somatic mutant that lacks a distinct adapter protein, SLP-76 (Yablonski *et al*., 1998). Thus, expression of both LAT and SLP-76 appears essential for the coupling of TCR stimulation to PLC-γ1 phosphorylation. However, we found that TCR-mediated SLP-76 phosphorylation is unimpaired in ZAP(Y319→F)-expressing P116 cells, which suggests that SLP-76 is providing its normal stimulatory input into PLC-γ1 in these cells. Our results focus attention on the contribution of the LAT phosphorylation defect to the disruption of TCR-mediated PLC-γ1 activation in the ZAP(Y319→F)-expressing cells. The interaction between PLC-γ1 and LAT may be direct, as we have recently confirmed a report (Stoica *et al*., 1998) that the predominant ligand for the SH2(N) domain of PLCγ1 is tyrosine-phosphorylated LAT (B.J.Irvin and R.T.Abraham, unpublished observation).

The findings presented in this report support a working model that is compatible with many of the findings presented in this report (Figure 10). First, TCR stimulation causes the sequential activation of Lck and ZAP-70. We propose that Lck then initiates LAT phosphorylation on a subset of the tyrosine residues modified during TCR stimulation. This scenario is supported by our observation that TCR crosslinkage induces a very low level of LAT tyrosine phosphorylation in ZAP-70-deficient P116 cells (B.J.Irvin and R.T.Abraham, unpublished results). The partially phosphorylated LAT protein engages the SH2(N) domain of PLC-γ1, while the SH2(C) domain of this phospholipase binds to the phosphorylated Tyr319 motif in ZAP-70. PLC-γ1 therefore acts as bridge between LAT and ZAP-70, an interaction that not only promotes the phosphorylation of PLC-γ1 by ZAP-70, but also draws

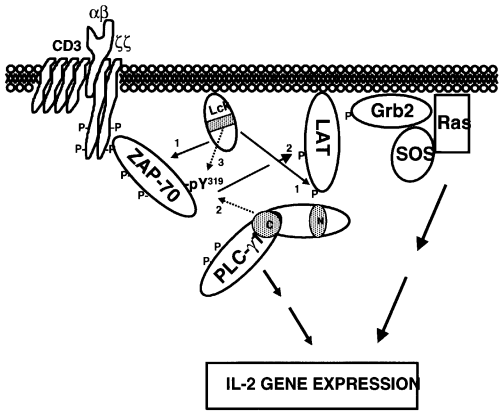

Fig. 10. A 'bridge' model TCR-mediated PLC-γ1 and Ras activation. TCR ligation triggers Lck-dependent activation of ZAP-70 and low-level phosphorylation of LAT (step 1). PLC-γ1 binds to the phosphorylated Tyr319 site in ZAP-70 via its SH2(C) domain (SH2 domains are denoted by stippling), and to partially phosphorylated LAT via its SH2(N) domain (step 2). The PLC-γ1 'bridge' facilitates full phosphorylation of both LAT and PLC-γ1 by ZAP-70, leading to phosphoinositide hydrolysis and Ras activation. Finally, the PLC-γ1 SH2(C) domain is displaced from phospho-Tyr319 by other SH2 domain-containing ligands, including Lck (step 3).

LAT into the vicinity of the active PTK, thereby driving full phosphorylation of LAT. We speculate that the relative selectivity of the SH2(N) domain of PLC-γ1 for phosphorylated LAT allows the fully phosphorylated PLC-γ1 to be effectively 'handed off' to LAT, leading in turn to phosphoinositide hydrolysis.

This PLC-γ1 'bridge' model, though speculative, predicts that a reduction in PLC-γ1 expression levels would negatively affect the phosphorylation of LAT in response to TCR crosslinkage. Indeed, our preliminary studies indicate that LAT tyrosine phosphorylation is reduced in a PLC-γ1-deficient Jurkat subclone (B.J.Irvin and R.T.Abraham, unpublished results). On the other hand, we emphasize that another phospho-Tyr319-dependent ligand(s), including the Lck SH2 domain, may be crucial for downstream signaling from ZAP-70. Phosphorylation of Tyr319 site may drive the previously reported interaction between ZAP-70 and CD4-bound Lck (Thome *et al*., 1995). A plausible scenario is that PLC-γ1 and Lck compete for binding to activated ZAP-70, with both interactions contributing to the T-cell-activation response. If the interaction of Lck with the ZAP-70 interdomain B region proves to be physiologically relevant, then it must be required for only a subset of the Lck-dependent signaling events in T cells since many of these events are preserved in ZAP(Y319→F)-expressing P116 cells. Obviously, further studies in genetically tractable model systems, including ZAP-70- and LAT-deficient Jurkat cells, will be required to fully understand the interplay between Lck, ZAP-70, LAT and PLC-γ1 during the early stages of TCR signaling.

The Tyr319→Phe substitution in the interdomain B region of ZAP-70 also led a marked defect in TCR-dependent Ras activation in P116 cells. On the other hand, TCR crosslinkage triggered the normal increase in ERK activity in ZAP(Y319→F)-expressing P116 cells. Our studies and others (Griffith *et al*., 1998) with P116 cells have shown that activation of ERKs in response to TCR stimulation is largely, but not entirely, dependent on ZAP-70. In many growth factor receptor systems, ERK activity is regulated by a linear signaling cascade that proceeds from Ras to Raf-1 to MEK1, which serves as the proximate activator of ERK1 and ERK2 (Davis, 1993). Thus, we were surprised to find that TCR-dependent ERK activation was unimpeded in the ZAP(Y319→F)-transfected P116 cells, in spite of the significant decrease in GTP-bound Ras in these cells. One explanation for these findings is that the reduced levels of activated Ras induced by TCR stimulation in ZAP(Y319→F)-expressing cells are nonetheless sufficient to fully activate the signaling pathway leading to ERK activation. Interestingly, the same cells show a severe defect in TCR-dependent CD69 expression, suggesting that this response is more tightly linked to the level of GTP-bound Ras than is the activation of ERKs. Activation of Ras appears to be both necessary and sufficient for the induction of CD69 expression during TCR ligation (D'Ambrosio *et al*., 1994). Again, the relatively tight linkage of CD69 expression to the level of activated Ras may simply reflect the threshold level of Ras activation needed to provide a signal of sufficient strength and duration to drive the transcription and translation of the CD69 gene.

A more mechanistic explanation for the apparent quantitative difference in Ras activation thresholds for ERKs and CD69 expression is that induction of each response depends on qualitatively different sets of downstream Ras effectors. For example, while activation of Raf-1 may suffice to fully activate ERKs, higher levels of GTP-bound Ras might be required to activate Raf-1 and other effectors, e.g. Rho family GTPases, which, in turn, collaborate to induce CD69 gene expression. A more speculative alternative is that a substantial proportion of the increase in ERK activity induced by the TCR occurs through an as yet undefined Ras-independent pathway, while CD69 induction is wholly Ras dependent. Clearly, the molecular mechanisms whereby the TCR couples to the activation of Ras and ERK remain important areas for future investigations.

In summary, we have identified the Tyr319 residue in the interdomain B region of ZAP-70 as a critical phosphorylation site for the activation of PLC-γ1 and Ras by TCR ligands. The complex roles of the interdomain B region in the regulation of ZAP-70 function are gradually being unraveled, primarily through mutational approaches similar to those employed in this study. The interplay between the negative modulator (possibly Cbl) that binds to the Tyr292 site and the positive interactors at Tyr315 and Tyr319 is critical to our understanding of the signaling functions of ZAP-70 in T cells. In normal T cells, the simultaneous engagement of positive and negative modulators by activated ZAP-70 may contribute to the generation of graded activation responses to different classes of TCR ligands. Qualitative or quantitative differences in the balance of signals delivered through the interdomain B region of ZAP-70 may help determine the outcome—activation, death or anergy—of antigenic stimulation in immature and mature T lymphocytes.

Materials and methods

Cells

Wild-type Jurkat (clone E6) and Jurkat-derived P116 cell lines (Williams *et al*., 1998) were maintained in standard growth medium [RPMI 1640

supplemented with 10% fetal calf serum (FCS), 10 mM HEPES pH 7.4, 2 mM L-glutamine and 50 μM β-mercaptoethanol] at cell densities $<5\times10^5$ cells/ml. P116 subclones stably transfected with wild-type or mutated ZAP-70 expression plasmids were generated as described (Williams *et al*., 1998), and were maintained in standard growth medium supplemented with 1 mg/ml of G418 (Life Technologies, Inc., Gaithersburg, MD). COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) buffered to pH 7.4 with 10 mM HEPES and supplemented with 50 μM β-mercaptoethanol, 1% non-essential amino acids (Life Technologies, Inc.) and 10% FCS.

Antibodies and peptides

The human CD3-ε-specific mAb, OKT3 (Kung *et al*., 1979), and the 9E10 mAb, which recognizes an epitope tag sequence derived from the Myc protein (Evan *et al*., 1985), were purified from murine ascites fluids by chromatography over protein G–agarose affinity columns. PLCγ1-specific polyclonal anti-serum was prepared as described previously (Ting *et al*., 1992, 1995). Antisera directed against Lck and ZAP-70 were generously supplied by Dr David McKean (Mayo Clinic, Rochester, MN) and Dr Larry Samelson (National Institutes of Health), respectively. Monoclonal anti-phosphotyrosine antibody 4G10 and anti-ZAP-70 antibody 2F3.2 (UBI, Lake Placid, NY), mAb AU1 and 9E10 (Babco, Richmond, CA), monoclonal pan-Ras Ab-3 (Calbiochem, La Jolla, CA), fluorescein isothiocyanate (FITC)-conjugated monoclonal CD69 antibody (Becton Dickinson, San Jose, CA), polyclonal anti-ERK2 antibody (Santa Cruz Biotechnology), goat-anti-mouse immunoglobulin G (IgG) (Cappel, Aurora, OH), and rabbit anti-mouse IgG (Pierce, Rockford, IL) were purchased from the indicated commercial suppliers. Polyclonal antibodies specifc for phosphorylated, activated forms of the ERKs were obtained from New England Biolabs (Beverly, MA).

The antiserum specific for phospho-Tyr319 in ZAP-70 was developed by immunization of rabbits with a keyhole limpet hemocyanin-conjugated phosphopeptide (ESPpYSDPEEC) prepared by Quality Control Biochemicals (Hopkinton, MA). The antiserum was initially depleted of peptide-reactive antibodies by absorption with the corresponding non-phosphorylated peptide coupled to beads. The phospho-Tyr319-specific antibodies were then affinity-purified by chromatography over beads conjugated to the phospho-Tyr319-containing peptide used for immunization. The specificity of the phospho-Tyr319-directed antibodies was confirmed by immunoblot analyses of gluthathione *S*-transferase (GST)-tagged wild-type ZAP-70 and ZAP(Y319→F) fusion proteins purified from baculovirus-infected Sf9 insect cells (Chan *et al*., 1995).

Biotinylated peptides derived from the ZAP-70 interdomain B region, MPMDTSVY^{315}ESPY^{319}SDPEELKD, were synthesized in the Mayo Clinic Protein Sequencing and Peptide Synthesis Facility. The peptides were prepared with either tyrosine or phosphotyrosine at the Y315 or Y319 positions.

Plasmids and fusion proteins

The NFAT-luciferase (pNFAT-Luc) and IL-2-luciferase (pIL2-Luc) reporter constructs were provided by Dr David McKean (Mayo Clinic, Rochester, MN). A pBJ1-based expression plasmid encoding Myc epitope-tagged Lck was kindly provided by Dr Carrie Micelli (Lewis *et al*., 1997). The Myc-tagged ZAP-70 (mZAP) cDNA was cloned into the pcDNA3 expression vector as described previously (Williams *et al*., 1998). Mutations of Tyr315 and and/or Tyr319 to phenylalanine were introduced into the pcDNA3-mZAP plasmid by site-directed mutagenesis using the Transformer kit (Clontech, Palo Alto, CA) and the following primers (mutagenic codons are underlined): GACACGAGCGTG<u>TTT</u>GAGAGCCCCTAC (Y315F); TATGAGAGC CCC<u>TTC</u>AGCGACCCAGAG (Y319F); GACACGAGCGTG<u>TTT</u>GAGAGCCCC<u>TTC</u>AGCGACCCAGAG (Y315,319F double mutant). The resulting plasmids were designated pcDNA3-ZAP-Y315F, pcDNA3-ZAP-Y319F and pcDNA3-ZAP-YYFF, respectively.

The GST–PLCγ1SH2/SH3 fusion protein was prepared as described previously (Secrist *et al*., 1993). GST–Grb2SH2 was kindly provided by Dr Gary Koretzky (University of Iowa). To prepare the GST–γ1SH2(N) and GST–γ1SH2(C) fusion proteins, PCR primers were designed to overlap the appropriate regions of the bovine PLC-γ1 cDNA with 5′ extensions, and to add *Eco*RI or *Xho*I restriction sites at the respective 5′ and 3′ ends of the PCR product. The products of the PCR were digested with *Eco*RI and *Xho*I, and were ligated into the pGEX-KT GST expression vector to create pGEX-SH2(N) (amino acids 541–656 of PLC-γ1) and pGEX-SH2(C) (amino acids 657–772 of PLCγ1). *Escherichia coli* strain DH5α cells were transformed with these expression plasmids, and fusion protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside. After 3 h, the bacteria were lysed by sonication in phosphate-buffered saline (PBS), 10 mM EDTA pH 7.2, containing 1% (w/v) Triton X-100, 1 mM dithiothreitol (DTT), and a protease inhibitor cocktail (10 μg/ml leupeptin, 5 μg/ml aprotinin and 5 μg/ml pepstatin). GST fusion proteins were captured from the cleared lysates by glutathione–agarose affinity chromatography.

Transfections and luciferase assays

P116 cells were transfected with 30 μg of total plasmid DNA by electroporation with a BTX model T820 square-wave electroporator (San Diego, CA) as described previously (Williams *et al*., 1998). For stable expression of the ZAP(Y315→F) and ZAP(Y319→F) proteins, the transfected cells were selected in standard growth medium containing 2 mg/ml of G418, and the drug-resistant bulk populations were cloned by limiting dilution. Transient transfection and luciferase reporter assays were performed as described (Williams *et al*., 1998). For luciferase assays, the cells were transiently transfected with 10 μg of pNFAT-Luc or pIL2-Luc reporter plasmid DNA.

Cellular stimulation, immunoprecipitation and Western blot analysis

Jurkat-derived cell lines were stimulated with anti-CD3 antibody crosslinked with goat anti-mouse IgG or 100 μM pervanadate as described previously (Williams *et al*., 1998). In immunoprecipitation experiments, samples (1×10^7 cells) were terminated with 1 ml lysis buffer B (25 mM Tris–HCl, 100 mM NaCl, 5 mM EDTA, 50 mM β-glycerophosphate, pH 7.4, supplemented with 1% Brij-96, 1 mM sodium orthovanadate, and protease inhibitor cocktail) at the indicated times after stimulation. The detergent-soluble proteins were immunoprecipitated for 1 h at 4°C with 15 μl packed protein A–Sepharose beads and either 4 μl anti-PLC-γ1 or 2 μl anti-ZAP-70 antiserum. Immunoprecipitates were washed three times in lysis buffer B, and then boiled in 25 μl 2× SDS sample buffer. Whole-cell extracts for immunoblot analyses were prepared by lysis of 1×10^6 cells in 30 μl buffer A (Williams *et al*., 1998). The extracts were cleared by centrifugation, mixed with 10 μl 4× SDS sample buffer, and heated for 5 min at 100°C. The denatured proteins were resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and were transferred electrophoretically to an Immobilon P membrane (Millipore, Bedford, MA). Membranes were blocked in Tris-buffered saline (TBS; 10 mM Tris–HCl pH 8.0, 150 mM NaCl) containing 0.2% Tween 20 (TBST), and supplemented with 2% bovine serum albumin (BSA). Immunoblotting was carried out as described previously (Williams *et al*., 1998).

For anti-phospho-Tyr319 immunoblot analyses, Jurkat T cells were stimulated with the anti-TCR clonotype-specific antibody, C305. The stimulation conditions and methods for the preparation of cellular extracts were as described previously (Chan *et al*., 1995).

COS-1 cell expression studies

COS-1 cells were harvested from subconfluent cultures by detachment from culture dishes with PBS containing 5 mM EDTA. The cells were suspended in culture medium and were electroporated with 40 μg total plasmid DNA. Unless indicated otherwise, all inserts were cloned into the pcDNA3 (Invitrogen, Carlsbad, CA) expression plasmid. The amounts of the individual expression plasmids used in cotransfections were as follows: AU1-PLC-γ1, 10 μg; wild-type Myc-ZAP-70, 5 μg; Myc-ZAP (Y315→F), 10 μg; Myc-ZAP(Y319→F), 10 μg; Myc-ZAP-KD (catalytically inactive mutant), 25 μg; Myc-Lck, 5 μg. The electroporation conditions were similar to those described previously (Williams *et al*., 1998), except that the instrument settings were 330 V and 10 ms pulse duration. After 24 h in culture, the adherent cells were lysed in buffer C (40 mM HEPES, 60 mM NaCl, 50 mM β-glycerophosphate, pH 7.4, 1 mM $MgCl_2$, 5 mM iodoacetic acid, 1% Triton X-100, 1 mM sodium orthovanadate and protease inhibitor cocktail). Cellular extracts were cleared by centrifugation and total protein amounts were determined with the Bio-Rad Protein Assay Kit. Equivalent amounts of extract protein (300 μg) were mixed for 1 h at 4°C with 2 μl of AU1 ascites fluid, and immune complexes were immunoprecipitated with 15 μl packed protein A–Sepharose beads that had been pre-coated with 2 μg rabbit anti-mouse IgG. After 45 min, the beads were collected by centrifugation and the immunoprecipitates were washed three times in lysis buffer C. The bound proteins were solubilized with 50 μl of 2× SDS–PAGE sample buffer, and the samples were separated by SDS–PAGE through a 7.5% polyacrylamide gel. The immunoprecipitated proteins were analyzed by immunoblotting with the indicated antibodies.

ZAP-70 kinase assay

P116 cells were transiently transfected with Myc epitope-tagged versions of wild-type ZAP-70, ZAP(Y315→F), or ZAP(Y319→F) expression

plasmids. After 16 h, the transfected cell populations were divided into two equivalent samples, one of which was stimulated for 3 min with 100 μM pervanadate (Secrist *et al*., 1993) prior to cell lysis in 25 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, pH 7.4, containing 1 mM sodium orthovanadate, 1% Brij-96 and the protease inhibitor cocktail. The recombinant ZAP-70 polypeptides were immunoprecipitated from the cleared extracts with ZAP-70-specific polyclonal antibodies. The immunoprecipitates were washed and immune complex kinase reactions were done as described previously (Williams *et al*., 1998), with the exception that the substrate was a purified GST–cdb3 fusion protein (kindly provided by Dr A.Weiss, University of California at San Francisco, CA). The phosphorylation reaction products were separated by SDS–PAGE through a 10% gel, transferred to Immobilon P membranes, and incorporation of radioactive phosphate into GST–cdb3 was quantitated with a Molecular Dynamics Storm 840 PhosphorImager system equipped with ImageQuant software. The membranes were immunoblotted with the Myc-specific 9E10 mAb, and immunoreactive bands were detected with ECL Plus (Amersham, Piscataway, NJ). The level of Myc reactivity was quantitated by luminescence detection with the PhosphorImager system.

Ras and ERK kinase assays

For Erk assays, cells (5×10^6 cells per sample) were stimulated with OKT3 antibody crosslinked with goat anti-mouse IgG as described previously (Williams *et al*., 1998). After incubation for the indicated times at 37°C, the cells were lysed with 800 μl of buffer S (20 mM Tris–HCl, 40 mM sodium pyrophosphate, 50 mM NaF, 5 mM $MgCl_2$, 10 mM EGTA, pH 7.4, 1% Triton X-100 and 0.25% deoxycholate) supplemented with 20 nM microcystin, 1 mM sodium orthovanadate, 60 mM β-glycerophosphate and 40 mM *p*-nitro phenylphosphate (PNPP). Post-nuclear supernatants were incubated with 15 μl packed protein A–Sepharose beads and 2 μl anti-ERK2 antibody for 1 h at 4°C. The immunoprecipitates were washed twice in buffer S containing 0.1% SDS, and three times in kinase buffer (30 mM Tris–HCl, 0.1 mM EGTA, 10 mM magnesium acetate, 1 mM β-glycerophosphate, pH 7.4, supplemented with 1 mM DTT and 5 mM *n*-octyl β-D-glucopyranoside). ERK kinase reactions were initiated with 20 μl of kinase buffer containing 10 μM ATP, 5 μg MBP (obtained from UBI) and 10 μCi [γ-^{32}P]ATP (specific activity, 3500 Ci/mmol; ICN, Costa Mesa, CA). After 10 min at 30°C, the reactions were terminated with 8 μl 4× SDS sample buffer and heated at 100°C for 5 min. The reaction products were separated by SDS–PAGE through a 15% polyacrylamide gel. The proteins were transferred to Immobilon P and incorporation of radioactivity into MBP was quantitated with the PhosphorImager system.

Activation of ERKs was also monitored by immunoblotting of cellular extracts with phospho-ERK-specific antibodies. The cells (3×10^6 cells per sample) were stimulated as described above, and the reactions were terminated with 800 μl ice-cold PBS. The cells were pelleted by centrifugation, and were lysed in 100 μl of lysis buffer D (30 mM Tris–HCl, 100 mM β-glycerophosphate, 0.1 mM EGTA, 10 mM magnesium acetate, pH 7.4, containing 1 mM sodium orthovanadate, 20 nM microcystin, 40 mM PNPP and protease inhibitor cocktail. The cells were lysed by sonication, and the lysates were cleared of insoluble debris by centrifugation. The cleared extracts were assayed for total protein content, and equivalent amounts (50 μg) of extract protein were subjected to SDS–PAGE through a 10% gel. After electrophoretic transfer, the blotted proteins were probed simultaneously with phospho-ERK-specific antibodies, and with PLC-γ1-specific antibodies. The level of PLC-γ1 in each sample lane served as a control for equal protein loading. Immunoreactive proteins were visualized with protein A conjugated to horseradish peroxidase and the ECL reagent (Amersham) as described above.

To determine levels of GTP-bound Ras, cells ($1–2\times10^6$ cells per sample) were suspended in solution #2 and stimulated with crosslinked OKT3 antibody as described above. Reactions were terminated with cold PBS, and the cells were lysed in Ras lysis buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 10 mM $MgCl_2$, pH 7.5, containing 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 1 mM sodium orthovanadate and protease inhibitor cocktail). After 20 min on ice, the cell lysates were cleared by centrifugation, and the extracts were mixed with 15 μl glutathione (GSH)–agarose beads loaded with a freshly prepared GST fusion protein containing the Ras-binding domain (RBD) of Raf-1 (Brtva *et al*., 1995). The samples were rotated for 30 min at 4°C, and the precipitates were washed one time in Ras lysis buffer. The bound Ras protein was eluted by boiling the beads in sample buffer, and the eluted proteins were separated by SDS–PAGE through a 12.5% gel. The level of precipitated Ras was determined by immunoblotting with the pan-Ras-specific Ab-3.

Intracellular free Ca^{2+} measurements

Non-transfected or stably-transfected P116 cells were loaded with the Ca^{2+} indicator dye, indo-1/AM, and were stimulated with OKT3 as described previously (Williams *et al*., 1998). Stimulus-induced changes in intracellular Ca^{2+} concentration were determined by monitoring the fluorescence emission ratio of the Ca^{2+}-bound versus free form of indo-1 at 405 and 495 nm, respectively, on a FACStar Plus cell sorter (Becton Dickinson). The time-dependent changes in this ratio are directly proportional to stimulus-dependent alterations in the intracellular free Ca^{2+} concentration.

GST fusion protein binding assays

Cellular extracts from P116-derived subclones were prepared in lysis buffer B containing 1% Brij-96. The extracts were precleared for 30 min with 5 μg GST and 15 μl GSH–agarose beads. The cleared supernatants were transferred to tubes containing 15 μl GSH–agarose and 5 μg of the indicated fusion protein. Samples were rotated for 4 h at 4°C, and the beads were subsequently washed three times with 1 ml lysis buffer A. Proteins were eluted with 25 μl 2× sample buffer and resolved by SDS–PAGE. After electrophoretic transfer, the membrane-bound proteins were immunoblotted with the indicated antibodies.

Peptide binding assays

The indicated GST fusion proteins (2.5 μg) were mixed with biotinylated, ZAP-70-derived peptides (50 μM final concentration), and 15 μl of packed steptavidin–agarose beads. After 1 h at 4°C, the precipitates were washed three times with lysis buffer B and boiled in 25 μl 2× SDS sample buffer. Bound proteins were separated by SDS–PAGE and transferred to Immobilon P. The association of the GST fusion proteins with the ZAP-70 peptides was detected with an anti-GST rabbit antiserum as described above.

CD69 expression

P116-derived cell lines were harvested and resuspended in standard growth medium at a density of 3.3×10^5 cells/ml. The cells were stimulated for 16 h at 37°C with 50 ng of PMA per ml or 1 mg/ml of OKT3. The MEK inhibitor, PD098059 (30 μM; Biomol, Plymouth Meeting, PA), was added to the indicated samples at 30 min prior to stimulation with OKT3. The cells were harvested by centrifugation, resuspended in solution 2 (Hanks' balanced salt solution containing 5 mM glucose and buffered to pH 7.4 with 10 mM HEPES) and stained for 30 min on ice with 10 μl of FITC-conjugated anti-CD69 antibody. The cells were pelleted and resuspended in ice-cold solution 2 plus 0.05 % BSA prior to analysis for CD69 expression on a FACScan flow cytometer (Becton Dickinson).

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