T cell receptor signaling results both in T cell proliferation and apoptosis. A key enzyme at the intersection of these downstream pathways is phosphatidylinositol 3'-kinase (PI 3-kinase). In a previous report, we showed that the p85α subunit of the PI 3-kinase preferentially associated with the CD3ζ-membrane-proximal immunoreceptor tyrosine-based activation motif of the ζ chain (ζ-ITAM) (Exley, M., Varticovski, L., Peter, M., Sancho, J., and Terhorst, C. (1994) J. Biol. Chem. 269, 15140–15146). Here, we demonstrate that tyrosine phosphorylation of CD3ζ can recruit the PI 3-kinase enzyme in a T cell activation-dependent manner. In vivo studies with Jurkat cells stably transfected with a CD8-CD3ζ chimera (termed CD8ζ) shows that ligation of endogenous CD3ζ or CD8ζ by specific antibodies induces tyrosine phosphorylation of CD3ζ or CD8ζ, respectively. Increased tyrosine phosphorylation correlates with increased binding of p85α PI 3-kinase and recruitment of PI 3-kinase enzymatic activity to CD3ζ or CD8ζ proteins. Mutagenesis studies in COS-7 cells, transiently transfected with CD8ζ, p85α, and Fyn cDNAs in various combinations, show that both Tyr176 and Tyr181 within the CD3ζ-ITAM are required for efficient binding of p85α PI 3-kinase. Thus, replacement of Tyr176 by Phe (Y176F), or Tyr181 by Phe (Y181F) significantly reduces binding of p85α PI 3-kinase, whereas it does not affect binding of Fyn. Further in vitro experiments suggest that a direct binding of the tandem SH2 domains of p85α PI 3-kinase to the two phosphorylated tyrosines in a single CD3ζ-ITAM may occur. The data also support a model in which a single CD3ζ subunit can recruit distinct effector molecules by means of TCR-mediated differential ITAM phosphorylation.

The T cell antigen receptor (TCR-CD3) is a complex oligomer of TCR subunits α/β or γδ associated with the subunits of the CD3 (γ, δ, ε) and the ζ-ζ polypeptides (2, 3). The program of early responses to antigen-receptor activation proceeds as follows. Rapid induction of tyrosine phosphorylation precedes phosphatidylinositol-specific phospholipase C-γ1 activation, elevation of cytoplasmic free Ca2+, Ras-GTP accumulation, and activation of serine/threonine kinases (4). T cell activation culminates in cell division and induction of effector functions such as interleukin-2 secretion and cytolytic activity. Since none of the TCR-CD3 subunits have intrinsic tyrosine kinase activity, the earliest biochemical events are mediated by Lck and Fyn that associate with the CD4-CD8 and the TCR-CD3 complex, respectively (5, 6). TCR-CD3 ligation results in tyrosine phosphorylation of CD3ζ (7), CD3ε (8–10), and other intracellular substrates (11–14).

The individual cytoplasmic tails of the CD3 complex are necessary and sufficient for transduction of activating stimuli. The signal transduction function of the CD3 complex has been mapped to small sequence motifs, originally defined by Reth (15). This motif, termed the immunoreceptor tyrosine-based activation motif (ITAM), contains consensus sequence around two characteristic tyrosine residues spaced by 10 or 11 amino acids (YXX(L/I)XXX(L/I)). There are three ITAMs within the ζ chain, ζA, ζB, and ζC, and one ITAM in each CD3 chain, ε, δ, and γ. The presence of distinct motifs in individual cells offers the potential for regulation of activation pathways with different specificities and consequences (16). Motifs from different receptor subunits have partially distinct binding activity, consistent with their activation of distinct sets of effectors. The distinct selectivity of ITAMs for effector binding indicates that they may drive at least distinct biological responses (17).

The ITAMs contain structural information necessary for signal transduction, and they are rapidly tyrosine-phosphorylated on both tyrosines following receptor stimulation (7, 8, 18). The ability of phosphorylated CD3 cytoplasmic tails to transduce mitogenic signals is based on specific recruitment of signaling molecules. One candidate for signal transducing molecule is PI 3-kinase.

The abbreviations used are: TCR, T cell antigen receptor; PI, phosphatidylinositol; FCS, fetal calf serum; ITAM, immunoreceptor tyrosine-based activation motif; SH2, Src homology 2; SH3, Src homology 3; mAb, monoclonal antibody; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; WL, whole cell lysate; PC, preclearing; PVDF, polyvinylidene difluoride; PTK, protein-tyrosine kinase; WB, western blot.
3-kinase. This enzyme phosphorylates the D-3 position of the inositol ring of PI, PI-4-P, and PI-4,5-P2 (18). PI 3-kinase is a heterodimer consisting of a regulatory subunit (p85) and a catalytic subunit (p110) (20, 21). PI 3-kinase regulatory subunit p85 has an N-terminal Src homology 3 (SH3) region, two SH2 domains, and a domain with significant sequence homology to the product of the breakpoint cluster region gene (21–23). Although the downstream effectors of the phosphoinositide 3-phosphates generated by the enzyme are not well characterized, it has been shown that they activate protein kinase Cz, a non-calcium/diacylglycerol-regulated isoform of protein kinase C, and the RAC (related to the A and C kinases) serine threonine kinase (24). In addition, PI 3-kinase products have been implicated in regulation of multiple cellular events, such as vesicular trafficking, cytoskeletal rearrangements, and mitogenesis. T cell activation results in association of PI 3-kinase with the TCR and an increase in levels of 3'-phosphorylated polyphosphoinositides (1, 25).

In this study, we report association of p85a PI 3-kinase with the CD3-ε, which is dependent on phosphorylation of the two tyrosine residues of the CD3-ε-ITAM. The data support a model of TCR-mediated activation in which the extent of CD3-ε phosphorylation may serve to recruit distinct SH2 domain-containing signaling proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cells—Jurkat human T cells (ATCC) were used. Cells were grown in RPMI 1640 (Life Technologies, Inc.) with 10% FCS (BioWhittaker), penicillin, streptomycin, and 2 mM glutamine. Jurkat T cells were stably transfected by electroporation with the cDNA coding the CD3-ε chimera, and clones were selected by growth in media with neomycin. Primary sequences were verified by dideoxysequencing. The construct was used as a template to make mutants with Y170F and Y181F. Primary sequences were verified by dideoxysequencing.

**CD3-ε Polyclonal Antibody ZAP-4 (generously donated by Dr. Steven C. Ley, Transduction Laboratories); anti-Cbl and anti-Fyn polyclonal antibodies, RC20 (E120H, Transduction Laboratories); anti-p85 PI 3-kinase subunit antibody (Upstate Biotechnology, Inc.); anti-She pAb (Transduction Laboratories); anti-Cbl and anti-Fyn polyclonal antibodies (Santa Cruz, CA); anti-human Lck rabbit polyclonal antibody (against carboxyl terminus; Upstate Biotechnology, Inc.); anti-ZAP70 polyclonal antibody ZAP-4 (generously donated by Dr. Steven C. Ley, Medical Research Council, London); affinity-purified anti-CD3-ε anti-peptide polyclonal antibody (Dako); and anti-Ghr-2 monoclonal antibody (Transduction Laboratories) were used. Affinity-purified OKT3 mAb and OKT8 mAb were obtained from tissue culture supernatant of OKT3 and OKT8 hybridomas acquired from ATCC.

**cDNA Cloning**—For the construction of CD8-ε, 650- and 168-base pair cDNA fragments representing the human CD8α eotransmembrane and the mouse CD3-ε cytoplasmic domains, respectively, were amplified and then annealed at overlapping ends, filled in, and further amplified to produce the chimera. The chimera CD3-ε was generated by the polymerase chain reaction technique using the complete cDNA of human CD8α in pCD8α/pink2 (26) as template to amplify the fragment of base pairs 1–624 of human CD8α coding sequence (CD8α 5′-sense oligonucleotide, 5′-CCGGAAAGCTTGGCAGCCAGCAG-3′; CD8α 3′-antisense oligonucleotide, 5′-CTTGGCCTGGCCCTCCTTCTATTGTGAGTCCTAAGGGTGTGATA-3′). To amplify the fragment of base pairs 481–648 of murine CD3-ε cytoplasmic domain, the murine CD3-ε cDNA in pDL1 (27) was used as a template (3′-antisense oligonucleotide, 5′-CGGGATCCCTCGAGACTCCAGCAGATG-3′; CD3-ε 5′-sense oligonucleotide, 5′-GTTATCACGCTAGCATCTCCTCAGTC-3′). The products of both polymerase chain reactions were then annealed by another polymerase chain reaction. Polymerase chain reaction fragments were then digested with HindIII and BamHI and inserted in vector pCDNA3 (Invitrogen). This construct was used as a template to make mutants with Y170F and with Y181F. Primary sequences were verified by dideoxysequencing. Full-length cDNA encoding murine c-FynT were previously described (28); p58α cDNA was generously donated by Dr. C. Rudd (Dana-Farber Cancer Institute, Boston) (29).

**Peptide Synthesis Procedure**—The peptides were synthesized by the solid phase procedure using FMoc (N-(9-fluorenylmethoxycarbonyl) amino acids (30, 31) on a Ranin Symphony peptide synthesizer. The initial residue was linked to a p-alloxybenzyl alcohol resin providing a C-terminal carboxylic acid. The C-terminal carboxyl peptides were deprotected and cleaved from the resin by a mixture of trifluoroacetic acid/thiioanisole/ethanediol/thioanisol (9:0.5:0.3:0.2) to minimize side reactions. The individual peptides were precipitated from the trifluoroacetic acid cleavage mixture with diethyl ether and washed several times with cold ether to remove impurities generated by the cleavage reaction. Peptides were purified by high performance liquid chromatography.

**Preparation of Affinity Matrices**—The synthetic peptides were chemically coupled via N-terminal Cys with maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Pierce) to 1-Lys-Septaharose (Sigma) according to the manufacturer’s instructions and blocked with 1% bovine serum albumin. Coupling efficiency was monitored by determining the degree of binding of anti-peptide and anti-phosphotyrosine antibodies to the peptide affinity matrices as described (1).

**Cell Activation and Immunoprecipitations**—Jurkat cells were serum-starved by incubation for 4 h at 37 °C in RPMI 1640 with 0.1% FCS. Cells were washed with serum-free RPMI containing 20 mM HEPES, pH 7.4, and lysed with 1% Nonidet P-40 in 20 mM HEPES, pH 7.5, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and pepstatin (antipain, chymostatin, leupeptin, and pepstatin) at 1 μg/ml, 1 mM phenylmethylsulfonyl fluoride and 10 mM iodoacetamide. After two preclearings with Lys-Septaharose, lysates were incubated with CD3-ε peptide affinity matrices for 1 h at 4 °C. Beads were then washed twice in solution A (0.5% Nonidet P-40, 150 mM NaCl, 20 mM HEPES, pH 7.6, and 100 μM sodium orthovanadate), twice in solution B (0.1% Nonidet P-40, 150 mM NaCl, 20 mM HEPES, pH 7.6, and 100 μM sodium orthovanadate) and once in solution C (150 mM NaCl and 20 mM HEPES, pH 7.6).

Jurkat CD8-ε cells were serum starved by incubating for 4 h at 37 °C in RPMI 1640 with 0.1% FCS. Cells were washed and resuspended at 105 cells/100 μl in serum-free RPMI 1640 containing 20 mM HEPES, pH 7.4, and incubated at 37 °C for 10 min before activation. Jurkat cells were then incubated with anti-receptor mAb (5 μg/106 cells) at 37 °C for 5 min. In indicated experiments, secondary antibody was used to further cross-link the receptors, e.g. goat anti-mouse IgG (whole molecule) or the F(ab′)2 fragments (Cappel). The concentration of the secondary antibody used for activation was 20 μg/107 cells. In the latter case, cells were stimulated with the anti-receptor mAb for 2 min at 37 °C followed by addition of secondary antibody for an additional 3 min at 37 °C. Lysis was carried out as described above. Cells lysates were centrifuged at 14,000 × for 15 min at 4 °C. For immunoprecipitations with mAbs OKT3 and OKT8, 5 μg/107 cells of Ab were bound to 20 μl of protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) for 1 h at 4 °C after one preclearing with bovine serum albumin-Sepharose 1 h at 4 °C. Washes before loading were carried out as described previously. COS cells were lysed and subjected to immunoprecipitation with 5 μg/107 cells of mAb OKT8 and 20 μg of protein A-Sepharose CL-4B (Pharmacia) for 1 h at 4 °C after one preclearing with normal mouse serum (Sigma) and protein A-Sepharose CL-4B for 1 h at 4 °C.

**DNA Transfections**—COS-7 cells (10 × 104) were transfected with 2 μg of expression vectors, containing the appropriate cDNA insert, by the DEAE-Dextran method (32). Cells were harvested 72 h after transfection and lysed in a lysis buffer as described above. Aliquots of these lysates were subjected to immunoblotting with Abs to Fyn, p58α PI 3-kinase, and CD3-ε to confirm protein expression. In transfected cells, surface expression of the CD8-ε chimera was analyzed by flow cytometry with OKT8 antibody.

**Western Blotting**—Proteins were separated on SDS-PAGE and transferred to PVDF Immobilon membrane (Millipore Corp.). Filters were probed with specific antibodies as described previously (8). For detection, anti-mouse IgG (H + L) or anti-rabbit IgG (Fc) horseradish peroxidase conjugates (Promega) were used followed by chemiluminescence (ECL, Amersham Corp.). For anti-TyrP (Western blot), membranes were incubated with anti-phosphotyrosine directly coupled to horseradish peroxidase conjugated (Promega) followed by chemiluminescence (ECL system, Amersham Corp.). For stripping and reblotting, membranes were incubated for 10 min in stripping buffer (150 mM NaCl, 10 mM Tris- HCl, pH 2.3) at room temperature. Membranes were then washed for 3 × 10 min in TNA-Tween and reblotted.

**PI 3-Kinase Assay**—The PI 3-kinase assay was performed as described (33). Briefly, whole cell lysates were prepared as above and incubated with anti-phosphotyrosine antibody, with OKT3 or with...
RESULTS

In Jurkat and Jurkat CD8-e cells, stimulation of the TCR-CD3 or CD8-e receptors induces the association of p85a PI 3-kinase with the phosphorylated forms of CD3-e and CD8-e. We have previously shown that in vitro PI 3-kinase preferentially associates with the membrane-proximal ITAM of the ζ chain (ζA-ITAM). Maximal PI 3-kinase activity is recovered following TCR-CD3 activation and is dependent upon phosphorylation of both tyrosine residues of the ζA-ITAM (1). A chimera composed of the extracellular domain of CD8 and the cytoplasmic domain of CD3-e (termed CD8-e) was constructed to determine in vivo whether the intracellular domain of CD8-e chain was capable of associating with the p85 PI 3-kinase independently of the presence of the other TCR-CD3 subunits. To this end, the CD8-e chimera was stably transfected in Jurkat cells expressing the wild-type TCR-CD3 complex (hereafter termed Jurkat CD8-e cells or JCD8-e cells) and stimulated by the addition of the anti-CD8 mAb OKT8 alone or further crosslinked with a secondary antibody against mouse immunoglobulins (GmIg). In these cells, the overall pattern of OKT8-induced substrate tyrosine phosphorylation was similar, although somewhat weaker, than that induced after TCR stimulation with the anti-CD3 mAb OKT3 (data not shown). Regarding tyrosine phosphorylation of the CD8-e chimera, in unstimulated cells or cells stimulated with a goat anti-mouse IgG antibody alone, a basal level of tyrosine phosphorylation of CD8-e was observed (Fig. 1A, lanes 5 and 6), which was higher than the basal level of tyrosine phosphorylation of endogenous CD3-e (Fig. 1A, lanes 1 and 2). However, the level of CD8-e tyrosine phosphorylation induced upon stimulation of the chimera with anti-CD8 antibodies was similar to that observed in endogenous CD3-e upon stimulation with the anti-CD3 mAb OKT3 (Fig. 1A, compare lanes 7 and 8 with lanes 3 and 4). Despite that, more tyrosine-phosphorylated proteins seemed to co-immunoprecipitate with CD8-e than with CD8-e (Fig. 1A, compare lane 4 with lane 8). Therefore, evidence clearly demonstrates that both the CD8-e chimera and endogenous CD3-e result in tyrosine phosphorylation in response to receptor ligation.

To test in these cells whether an interaction between CD8-e and p85 PI 3-kinase may occur in vivo, we reblotted the filter with an anti-p85a PI 3-kinase antibody. As shown in Fig. 1B, p85 PI 3-kinase was weakly detected in OKT8 immunoprecipitates from unstimulated or GmIg-stimulated Jurkat CD8-e cells (Fig. 1B, lanes 5 and 6). This association was highly increased in OKT8 immunoprecipitates from OKT3- or OKT8 plus GmIg-stimulated cells (Fig. 1B, lanes 7 and 8). The basal level of association of p85 PI 3-kinase to CD8-e in nonstimulated and secondary antibody-stimulated cells (Fig. 1B, lanes 5 and 6) could be due to the higher basal level of tyrosine phosphorylation of CD8-e as compared with that of endogenous CD3-e (Fig. 1A, compare lanes 5 and 6 with lanes 1 and 2). Thus, p85a PI 3-kinase was not detected in OKT3 immunoprecipitates from unstimulated Jurkat CD8-e cells or from cells exposed to secondary antibody alone (Fig. 1B, lanes 1 and 2), whereas p85a was readily co-immunoprecipitated with endogenous CD3-e when Jurkat CD8-e cells were stimulated with OKT3 or OKT3 plus the secondary antibody p85a (Fig. 1B, lanes 3 and 4). Altogether, these results suggest a tyrosine phosphorylation-dependent mechanism for p85 PI 3-kinase binding to the intracellular domain of CD3-e, in which none of the other CD3 chains seems to play a role.

In Jurkat CD8-e Cells PI 3-Kinase Associates with CD3 and CD8-e in Response to Receptor Engagement—To test whether PI 3-kinase enzymatic activity was detected in association with the cytoplasmic tail of CD3-e, we performed kinase reactions on OKT3 or OKT8 immunoprecipitates from Jurkat CD8-e cells stimulated with anti-CD8 mAb OKT8 or with anti-CD3 mAb OKT3, respectively (Fig. 2). In agreement with the p85a immunoblot studies, an increase in PI 3-kinase activity associated with the chimera CD8-e in response to receptor engagement was clearly demonstrated (Fig. 2, first and third columns). This receptor-associated PI 3-kinase activity was further increased by extensive crosslinking of OKT8 or OKT3 with GmIg (Fig. 2, second and fourth columns). Since for PI 3-kinase enzymatic activity the association of both p85a and p110 subunits is required, these results suggest that in JCD8-e cells, CD8-e or TCR-CD3 stimulation leads to the binding of a p85a/p110 heterodimer to the tyrosine-phosphorylated cytoplasmic tail of CD3-e.

Co-transfection of Fyn and CD8-e Chimera Can Reconstitute CD8-e Tyrosine Phosphorylation in a Nonhematopoietic Cell Line—Taken together, the above results are consistent with a
mechanism for p85 PI3-kinase binding in which Lck (or Fyn) directly phosphorylates the CD8-e chimera and subsequently p85a binds direct or indirectly to the chimera. However, Jurkat T cells contain other hematopoietic-specific tyrosine kinases (such as ZAP-70, etc.) and other signaling molecules, which confound the drawing of definitive conclusions from the above experiments. A previously described reconstitution system was used (28) to determine whether Fyn was capable of directly phosphorylating the CD8-e chimera. In this system, COS-7 cells were transiently transfected with mouse Fyn, or mouse p85a PI 3-kinase, or CD8-e cDNAs alone or in various combinations. The CD8-e chimera was isolated by immunoprecipitation with the anti-CD8 mAb OKT8 and analyzed by SDS-PAGE and Western blotting with anti-phosphotyrosine mAb (Fig. 3A). Since COS-7 cells lack all known hematopoietic specific tyrosine kinases, only very weak CD8-e tyrosine phosphorylation occurred without exogenous kinase expression in COS-7 cells (Fig. 3A, lane 5). When the Fyn kinase was co-transfected with CD8-e in these cells, a marked increase in the level of CD8-e tyrosine phosphorylation occurred (Fig. 3A, lane 6). Moreover, co-expression of these two proteins resulted in the co-immunoprecipitation of a 59-kDa tyrosine phosphoprotein with CD8-e that was identified as Fyn (Fig. 3, panel A and panel B, lane 6). In contrast, in cells transfected with Fyn cDNA alone, the OKT8 antibody did not co-immunoprecipitate any 59-kDa band (Fig. 3B, lane 3), suggesting that the interaction of phosphorylated CD8-e with Fyn was specific. These results indicate that, without the ZAP-70 PTK, Fyn is able to directly tyrosine-phosphorylate the cytoplasmic tail of the CD8-e chimera, resulting in a tight association of this kinase with the CD8-e-ITAM.

In Transfected COS-7 Cells p85a PI 3-Kinase Binds Preferentially to Tyrosine-phosphorylated CD8-e Chimera—CD8-e chimera was also analyzed for association with transfected mouse p85a PI 3-kinase. While in COS-7 cells co-transfected with CD8-e and mouse p85a cDNAs, a low amount of p85a was co-immunoprecipitated with unphosphorylated CD8-e (Fig. 3C, lane 7), in cells co-transfected with CD8-e, p85a, and Fyn cDNAs, a significantly increased amount of p85a was co-immunoprecipitated with phosphorylated CD8-e (Fig. 3C, lane 8). In contrast, in cells transfected with p85a cDNA alone the p85a protein did not bind specifically to the OKT8 mAb used for immunoprecipitation (Fig. 3C, lane 4), suggesting that the CD8-e/p85a interactions were specific.

Fyn can efficiently bind to the CD8-e chimera (Fig. 3B, lane 6), and Src homology 3 domain of Fyn can mediate binding to PI 3-kinase in T cells (34). Therefore, the increased association of p85a to the CD8-e chimera observed in cells that were co-transfected with Fyn (Fig. 3C, lane 8) suggested that either Fyn was the linker between CD8-e and p85 PI 3-kinase or that tyrosine phosphorylation of the chimera could provide a high affinity binding site to the SH2 domains of p85 PI 3-kinase. To distinguish between these two possibilities, two mutated CD8-e chimeras were constructed. In one chimera, the first tyrosine of CD3-e-ITAM was replaced by phenylalanine (Y170F). In the second chimera the second tyrosine was replaced by phenylalanine (Y181F) (see "Experimental Procedures"). The data showed that in COS-7 cells co-transfected with Y170F or with Y181F and Fyn, the chimeras resulted in weak tyrosine phosphorylation as compared with wild-type CD8-e (Fig. 3C, compare lanes 10 and 14 with lane 6). Despite that, Fyn could still be detected associated with Y170F and Y181F chimeras (Fig. 3B, lanes 10 and 14). These results suggest that phosphorylation of only a single tyrosine residue within the CD3-e-ITAM is sufficient for high affinity binding of Fyn to the cytoplasmic tail of CD3-e.

In COS-7 cells transfected with Y170F or with Y181F and p85c cDNAs, the amount of p85a protein co-immunoprecipitated with the chimera was low (Fig. 3C, lanes 11 and 15).
Moreover, in cells transfected with Y170F or Y181F and with p85α and Fyn cDNAs, the amount of p85α protein co-immunoprecipitated with either Y170F or Y181F chimeras did not increase as compared with that in the double transfectants (Fig. 3C, compare lanes 12 and 16 with lanes 11 and 15, respectively). Therefore, these results suggest that Fyn is not the linker that couples p85 PI 3-kinase to the CD3-ε-ITAM. Moreover, the data suggest that there are two types of p85α/CD8 complexes depending upon the phosphorylation state of the two tyrosines within the CD3-ε-ITAM.

p85α Subunit of PI 3-Kinase Binds in Vitro to Tyrosine-phosphorylated CD3-ε-ITAM—To examine whether segments representing the CD3-ε-ITAM specifically bind p85α PI 3-kinase, we constructed chemically synthesized peptides representing individual ITAMs. The peptides were coupled via an N-terminal L-cysteine to L-lysine-Sepharose beads. The peptides used (see Table I) represented the unphosphorylated CD3-ε-ITAM (CT26), the phosphorylated CD3-ε-ITAM on both tyrosines (CT26P), and an unphosphorylated peptide representing the entire cytoplasmic tail of CD3-ε (CD3). Similar amounts of each synthetic peptide bound to lysine-Sepharose beads were used to precipitate proteins from Jurkat whole lysates. In these experiments, the efficiency of CD3-ε-ITAM binding was judged by the efficiency of the ITAM peptides to recruit the proteins from Jurkat T cell lysates. Fig. 4A shows an anti-phosphotyrosine Western blot of the proteins bound to the peptides. Multiple tyrosine-phosphorylated proteins bound both phosphorylated and unphosphorylated peptides, and only a phoshoprotein at 72-kDa bound exclusively to the phosphorylated CT26P peptide.

We next tested whether p85α PI 3-kinase interacted with CD3-ε-ITAM peptides by Western blot analysis with an anti-p85α pAb. A preferential binding of p85α PI 3-kinase subunit to doubly phosphorylated peptide (CT26P) was observed as compared with that of unphosphorylated CT26 or CD3 peptides (Fig. 4B, lane 5 versus lanes 4 and 3). These results suggested that association of the CD3-ε-ITAM with p85 PI 3-kinase is dependent on the phosphorylation of both tyrosine residues.

Requirements for p85α PI 3-Kinase Binding to CD3-ε-ITAM Peptides—That the in vitro binding of p85α PI 3-kinase to CD3-ε requires tyrosine phosphorylation of CD3-ε-ITAM was further confirmed by testing the ability of phosphotyrosine to block p85α PI 3-kinase with tyrosine-phosphorylated CT26P peptide. The addition of soluble phosphotyrosine to lysates from Jurkat cells just before mixing them with CT26P-Sepharose beads caused a dose-dependent inhibition of the association of p85α PI 3-kinase with CT26P (Fig. 5A, lanes 3–6).

We also tested the ability of other compounds to compete the association of p85 PI 3-kinase with CT26P peptide (Fig. 5B). The addition of 80 mM phosphotyrosine or 50 mM phenyl phosphate to the binding reaction completely blocked the association of p85 PI 3-kinase with the immobilized CT26P peptide.

Table I: Amino acid sequence of the peptides synthesized

| Peptide | Sequence |
|---------|----------|
| CD3     | CKRRAKAKYPVRGAGGQVRQKNKRPFPVPY |
| CT26    | CGDYEP1RKGRQDLYSGLNQRRI       |
| CT26P   | CGDYEP1RKGRQDLYSGLNQRASV      |

Thus, binding of p85α PI 3-kinase to phosphorylated CD3-ε-ITAM peptides, 20 × 10^6 Jurkat cells per point were lysed with 1% Nonidet P-40 lysis buffer. Cell lysates were precleared twice with lysine-Sepharose and incubated with the affinity matrices 1.5 μg CD3 (lane 3), 3 μg CT26 (lane 4), and 3 μg CT26P (lane 5). Proteins bound to beads were separated on 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with anti-phosphotyrosine RC20 (a, αPTyr) or anti-p85 PI 3-kinase pAb (B). Bands were visualized by the ECL system. Lane 1, WL; lane 2, PC. Each lane represents 10 × 10^6 cell equivalents. WL represents ~700,000 cell equivalents.

Fig. 4. p85α PI 3-kinase preferentially associates with doubly phosphorylated CD3-ε-ITAM peptides. 20 × 10^6 Jurkat cells per point were lysed with 1% Nonidet P-40 lysis buffer. Cell lysates were precleared twice with lysine-Sepharose and incubated with the affinity matrices 1.5 μg CD3 (lane 3), 3 μg CT26 (lane 4), and 3 μg CT26P (lane 5). Proteins bound to beads were separated on 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with anti-phosphotyrosine RC20 (a, αPTyr) or anti-p85 PI 3-kinase pAb (B). Bands were visualized by the ECL system. Lane 1, WL; lane 2, PC. Each lane represents 10 × 10^6 cell equivalents. WL represents ~700,000 cell equivalents.
Requirements for p85\textalpha PI 3-kinase/CD3-\epsilon-ITAM interactions. A, 10 × 10^6 Jurkat cells/point were lysed with 1% Nonidet P-40 lysis buffer. Cell lysates were precleared with lysine-Sepharose and incubated with the CT26P affinity matrix with (lanes 3–6) or without (lane 2) soluble phosphotyrosine as a competitor at 80 mM (lane 3), 20 mM (lane 4), 10 mM (lane 5), and 1 mM (lane 6). Bound complexes were subjected to Western blot with anti-p85\textalpha PI 3-kinase and detected by the ECL system. Lane 1, WL; lane 2, 20 × 10^6 Jurkat cells/point were lysed with 1% Nonidet P-40 lysis buffer. Cell lysates were precleared with lysine-Sepharose and incubated with the CT26P-beads in the presence (lanes 2 and 7–9) of the following competitors: 80 mM Tyr(P) (lane 3), 80 mM Thr(P) (Thr(P) (lane 4), 80 mM Ser(P) (Ser(P) (lane 5), and 50 mM phenylphosphate (PhenylP, lane 6). After a 1-h incubation, some samples (lanes 7–9) were washed and incubated for 15 min with 2 mM NaCl (lane 7), radioimmune precipitation buffer (RIPA) (lane 8), and 1 mM urea (lane 9). Protein bound to beads were separated on 12.5% SDS-PAGE gel, transferred to PVDF membranes, and probed with anti-p85\textalpha PI-3 K pAb and detected by the ECL system.

B, Specific high affinity interactions between individual SH2 domains of p85\textalpha PI 3-kinase and phosphorylated tyrosine residues (Y_{P}^{PO4}) have been shown to require methionine as the third amino acid residue C-terminal of the Y_{P}^{PO4} (Y_{P}^{PO4}-XXM), whereas SH2 domains of ZAP-70, Shc, and Fyn require the consensus binding sequence Y_{P}^{PO4}-XX(I/L) present in CD3-\epsilon-ITAM (38, 39). SH3 domains of Fyn could bind to two proline-rich motifs within the p85\textalpha PI 3-kinase (34, 40), and the SH3 domain of p85\textalpha PI 3-kinase could bind to the N-terminal proline-rich region of Shc (41) or Cbl (42). Therefore, any of these molecules would be a candidate for an intermediate between phosphorylated CD3-\epsilon-ITAM and p85\textalpha PI 3-kinase. It is also important to note that phosphorylation of both tyrosine residues within the CD3-\epsilon-ITAM could create a tighter binding site for proteins containing SH2 domains in tandem (such as ZAP-70 or p85\textalpha PI 3-kinase) than for single SH2-containing proteins (such as Fyn, Lck, Shc, etc.). Therefore, direct binding of p85\textalpha PI 3-kinase to CD3-\epsilon-ITAM cannot be ruled out.

Western blotting analysis was performed to determine whether various SH2-containing proteins such as p85\textalpha PI 3-kinase, ZAP-70, Shc, Lck, Cbl, and Grb-2 could bind selectively to doubly phosphorylated CD3-\epsilon-ITAM peptide as compared with nonphosphorylated CD3-\epsilon-ITAM peptide. As an additional control, a nonphosphorylated peptide corresponding to the entire cytoplasmic tail of CD3-\epsilon was used. Fig. 6 shows that the interactions of the SH2-containing proteins with the CD3-\epsilon-ITAM were quite selective, depending upon the phosphorylation state of the peptide used. Thus, proteins with two SH2 domains in tandem such as p85\textalpha PI 3-kinase and ZAP-70 bound exclusively to doubly phosphorylated CD3-\epsilon-ITAM peptide (Fig. 6, A and B, respectively), whereas proteins with a single SH2 domain bound both phosphorylated and nonphosphorylated peptides although with different efficiencies (Fig. 6). Thus, CD3-\epsilon-ITAM—Specific high affinity interactions between individual SH2 domains of p85\textalpha PI 3-kinase and phosphorylated tyrosine residues (Y_{P}^{PO4}) have been shown to require methionine as the third amino acid residue C-terminal of the Y_{P}^{PO4} (Y_{P}^{PO4}-XXM), whereas SH2 domains of ZAP-70, Shc, and Fyn require the consensus binding sequence Y_{P}^{PO4}-XX(I/L) present in CD3-\epsilon-ITAM (38, 39). SH3 domains of Fyn could bind to two proline-rich motifs within the p85\textalpha PI 3-kinase (34, 40), and the SH3 domain of p85\textalpha PI 3-kinase could bind to the N-terminal proline-rich region of Shc (41) or Cbl (42). Therefore, any of these molecules would be a candidate for an intermediate between phosphorylated CD3-\epsilon-ITAM and p85\textalpha PI 3-kinase. It is also important to note that phosphorylation of both tyrosine residues within the CD3-\epsilon-ITAM could create a tighter binding site for proteins containing SH2 domains in tandem (such as ZAP-70 or p85\textalpha PI 3-kinase) than for single SH2-containing proteins (such as Fyn, Lck, Shc, etc.). Therefore, direct binding of p85\textalpha PI 3-kinase to CD3-\epsilon-ITAM cannot be ruled out.

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DISCUSSION

Tyrosine phosphorylation of ITAMs within the CD3-\epsilon and TCR-\zeta chains is crucial for the recruitment of protein-tyrosine kinases and effector molecules into the TCR-CD3 complex. In the present study, the ability of p85\textalpha PI 3-kinase to bind the CD3-\epsilon-ITAM was analyzed. In vivo studies show that the p85\textalpha PI 3-kinase regulatory subunit associates with the CD3-\epsilon intracellular region in Jurkat cells and Jurkat cells stably transfected with the cDNA encoding a CD3-\epsilon chimera (Fig. 1B, lanes 1 and 5). Such interaction occurs despite a different basal level of CD3-\epsilon or CD3-\epsilon tyrosine phosphorylation (Fig. 1A, lanes 1 and 5). However, CD3-\epsilon or CD8-\epsilon ligation with OKT3 or OKT8 increases the amount of p85\textalpha PI 3-kinase associated with CD3-\epsilon or CD8-\epsilon. Likewise, in COS-7 cells, co-transfection of the cDNA encoding the CD3-\epsilon chimera with the cDNA of mouse p85\textalpha PI 3-kinase results in stable association of p85\textalpha PI 3-kinase with CD8-\epsilon, although the chimera is not tyrosine-phosphorylated. However, in these cells, triple transfection of cDNAs coding CD8-\epsilon, p85\textalpha PI 3-kinase, and Fyn results in additional binding of p85\textalpha PI 3-kinase to CD8-\epsilon and increased CD8-\epsilon tyrosine phosphorylation. Overall, these results seem to indicate that although p85\textalpha PI 3-kinase may bind to the intracellular domain of CD3-\epsilon independently of its tyrosine phos-
clonal antibody (Grb-2 (previously shown that single point mutations along the CD3-cytoplasmic tail of CD3-phosphatases are fully active, single tyrosine-phosphorylated (4, 6, 28). In COS-7 cells, the data show that Fyn induces strong tyrosine phosphorylation, Fyn and Lck appear to be directly implicated in the tyrosine phosphorylation of the CD3 chains when both p85 SH2 domains are involved (45). A similar spacing of the tyrosines within CD3-phosphorylated Y170F or Y181F chimeras in which one of the tyrosine-phosphorylation state, it preferentially binds to tyrosine-phosphorylated CD3-e. What is more important, we find a dramatic receptor-induced association of PI 3-kinase with the intracellular domain of CD3-e.

Although it is unclear how TCR ligation leads to initiation of tyrosine phosphorylation, Fyn and Lck appear to be directly implicated in the tyrosine phosphorylation of the CD3 chains (4, 6, 28). In COS-7 cells, the data show that Fyn induces strong tyrosine phosphorylation of wild-type CD3-e chimera and weak phosphorylation of Y170F or Y181F chimeras in which one of the tyrosines within CD3-e-ITAM is substituted by phenylalanine (Fig. 3A). These results were surprising, since it has been previously shown that single point mutations along the CD3-e-ITAM do not affect the ability of Fyn to phosphorylate the cytoplasmic tail of CD3-e expressed as a chimera with the vesicular stomatitis virus cytoplasmic glycoprotein (43). A possible explanation for these apparently contradictory results is that we have done our experiments in vivo, whereas Timson-Gauken et al. (43) used an in vitro assay to detect CD3-e-associated Fyn kinase activity. In the latter case, it is likely that little CD3-e-associated tyrosine phosphatase activity was found, and Fyn could easily tyrosine-phosphorylate CD3-e and their mutants. In contrast, in intact COS-7 cells where tyrosine phosphatases are fully active, single tyrosine-phosphorylated CD3-e-ITAMs are likely to be more easily dephosphorylated than the doubly tyrosine-phosphorylated ones.

The in vitro studies carried out with synthetic peptides allowed us to further investigate the nature of the p85α PI 3-kinase/CD3-e interaction. Thus, as it occurred in the in vivo experiments, p85 PI 3-kinase from Jurkat cell lysates binds efficiently to doubly phosphorylated CD3-e-ITAM peptides but weakly to nonphosphorylated CD3-e-ITAM (Fig. 4). p85α PI 3-kinase binding to tyrosine-phosphorylated CD3-e-ITAM is specifically abrogated by an excess of free phosphotyrosine or phenyl phosphate, whereas free phosphoserine or phosphothreonine has little effect on binding (Fig. 5). Furthermore, p85/CD3-e-ITAM interaction is resistant to the presence of high salt concentration, SDS, or urea in the medium, which are the requirements for high affinity binding of p85α PI 3-kinase to the platelet-derived growth factor-β receptor (35). These data confirm and extend previous results reported by Cambier and Johnson (17) and show that p85α binds weakly to nonphosphorylated and tightly to doubly phosphorylated CD3-e-ITAM-containing peptides. Thus, both p85α PI 3-kinase and ZAP-70 could bind to nanomolar levels of the phosphorylated CD3-e-ITAM, whereas Grb-2 could only be detected with a 100-fold higher concentration of the ITAM (Fig. 5, C–E). It is also noteworthy that a high percentage of the total cellular pool of p85α PI 3-kinase and ZAP-70 could bind the CD3-e-ITAM, whereas Shc, Lck, Cbl, and Grb-2 did so in a smaller proportion. The present data do not agree with previously published data from Osman et al. (37) in T lymphoblasts. In that study, only ZAP-70 could bind to CD3-e-ITAM, whereas Fyn, Shc, Grb-2, and p85α PI 3-kinase could not bind to CD3-e-ITAM peptides, even at a concentration 10-fold higher than that used in our own experiments. The differences with our data were particularly marked for p85α binding to phosphorylated CD3-e-ITAM peptide (CT26P), and they do not seem to be related to the RRASV sequence introduced at the C terminus of our CT26P peptide. Thus, a doubly phosphorylated CD3-e-ITAM peptide devoid of the Cys and RRASV sequences recruits similar amounts of p85α PI 3-kinase as CT26P (data not shown).

Therefore, it is likely that the differences between the results of these studies may reflect differences in the relative levels of kinases and adaptors in different populations of T cells.

Hypothetically, direct association of CD3-e-ITAM with the tandem SH2 domains of p85α PI 3-kinase could occur. However, it is well established that individual SH2 domains of p85α bind to single phosphotyrosine moieties in the consensus P\(^{Y}O\(_4\)-MX\(_{3}\)Y\(_{2}\)O\(_4\)-XXM (38), which is not present in the CD3-e-ITAM. It has been observed, for the binding of p85 to the platelet-derived growth factor receptor, that high affinity binding is only detected when both SH2 domains of p85 are able to bind to two phosphotyrosine residues on the platelet-derived growth factor receptor that are 11 residues apart (44). In this sense, it is interesting that binding of p85 SH2 domains to the phosphorylated YXXM motif activates the enzyme specifically only when both p85 SH2 domains are involved (45). A similar spacing (10 amino acids) between YEPI and YSGL binding sites occurs in the CD3-e-ITAM, and the Ile or Leu at the +3-position carboxyl terminus of the Tyr residues are the most hydrophobic amino acid residues after Met. Because of the stronger selection for Met at the +3-position, single p85 SH2 domains are expected to bind much more tightly at phosphorylated YXXM sites, yet the spacing between the tyrosine residues in the YEPI and YSGL sites of CD3-e might provide a relative high affinity binding site for the two SH2 domains of p85 PI 3-kinase. We find that mutating Tyr\(^{170}\) or Tyr\(^{181}\) within CD3-e-ITAM reduces binding of p85α PI 3-kinase (Fig. 3C). One possible model to explain these results would be that mutations of a single tyrosine of CD3-e-ITAM would leave only the potential for weak interaction of the remaining phosphotyrosine with a single p85α SH2 domain, whereas if both phos-

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**Fig. 6. A comparison of p85α, ZAP-70, Shc, Lck, Cbl, and Grb-2 binding to unphosphorylated and doubly phosphorylated CD3-e-ITAM.** 10 × 10⁴ Jurkat cells/point were lysed with 1% Nonidet P-40 lysis buffer. Cell lysates (WL, lane 1) were precleared with lysine-Sepharose (PC, lane 2) and incubated with the 0.75 µM CD3 (lane 3), 1.5 µM CT26 (lane 4) and 1.5 µM CT26P (lane 5) affinity matrices. Proteins bound to beads were analyzed by 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with anti-p85 PI 3-kinase pAb (A), anti-ZAP-70 (B) anti-Shc pAb (C), anti-human Lck kinase (CT) polyclonal antibody (D), anti-p120-c-cbl polyclonal antibody (E), or anti-Grb-2 (F). Bands were visualized by the ECL system.
phototyrosines are available, interactions with both SH2 domains of p85α would be possible, and the overall affinity of interaction would be increased significantly, allowing detection of binding. This model implicitly acknowledges that doubly phosphorylated CD3-e-ITAMs exist and that both SH2 domains of a single p85α molecule participate. If this were not so, the effect of the mutations of Tyr\textsuperscript{170} and Tyr\textsuperscript{181} on the quantity of the binding of Fyn (or Lck) to tyrosine-phosphorylated CD3-e-ITAMs could change Fyn conformation, rendering its SH3 domain more available to interact with p85α PI 3-kinase.

Our results raise the question of the biological function of PI 3-kinase binding to the activated TCR-CD3 complex. In a previous study we showed that p85α PI 3-kinase preferentially associated with the ζ chain membrane-proximal (A-ITAM) (1). In this study, we show that the binding of p85α to phosphorylated (A- and CD3-e-ITAMs) or to bring p85α to the receptor so that it can be phosphorylated by an associated tyrosine kinase (e.g. ZAP-70). Another interesting possibility is that the binding of both SH2 domains of p85α to these phosphorylated ITAMs could induce a conformational change in the p85α/p110 structure, leading to PI 3-kinase activation. Such mechanism has been shown to be involved in the activation of PI 3-kinase, when its tandem SH2 domains are engaged on phosphorylated peptides (53, 54). The presence of two ζ-ITAMs on each ζ- dimer and two CD3-e-ITAMs on the CD3-e-CD3-γ and CD3-e-CD3-β pairs provides at least four binding sites for p85α in a single TCR-CD3 complex, in the case of no steric hindrance. Docking of several molecules of p85α PI 3-kinase onto a single receptor could perhaps facilitate the activation of this important signaling molecule by adjacent tyrosine kinases or contact with other effector molecules.

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