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Differential and Multiple Binding of Signal Transducing Molecules to the ITAMs of the TCR-ζ Chain

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Abstract A biotin-streptavidin-based technique was developed for high affinity, unidirectional, and specific immobilization of synthetic peptides to a solid phase. Biotinylated 23-mer carboxamide peptides corresponding to the three immunoreceptor tyrosine-based activation motifs (ITAMs) of the T cell antigen receptor associated ζ-chain (TCR-ζ) in their bis-, mono-, or unphosphorylated forms were used to study the binding of cellular proteins from human Jurkat T cells to these signal transduction motifs. The protein tyrosine kinase ZAP-70 bound specifically to all bisphosphorylated peptides but not to the mono- or unphosphorylated peptides. In contrast, Shc, phosphatidylinositol 3-kinase (PI3K), Grb2, and Ras-GTPase activating protein (GAP) bound with different affinities to the bis- or monophosphorylated peptides, while the Src family protein tyrosine kinase (PTK) Fyn did not bind specifically to any of the tested peptides. The different preferences of the studied signaling molecules for distinct ITAMs, and in particular the binding of some of them preferentially to monophosphorylated peptides, suggests that the TCR-ζ may bind multiple signaling molecules with each ITAM binding a unique set of such molecules. In addition, partial phosphorylation of the ITAMs may result in recruitment of different proteins compared to double phosphorylation. This may be crucial for coupling of the TCR to various effector functions under different conditions of receptor triggering.

Key words: ITAMs, SH2 domains, peptides, TCR-ζ, tyrosine phosphorylation

T and B lymphocytes have the ability to recognize a vast array of pathogens and foreign substances through the use of specialized clonotypic antigen-specific receptors. In response to the recognition of these antigens, signals are transduced into the cell which initiate a number of biochemical events including activation of protein tyrosine kinases (PTKs) [reviewed in Mustelin and Burn, 1993], protein serine/threonine kinases, hydrolysis of phosphoinositides, phosphatidylinositol (PI) turnover, calcium mobilization, and the transcription of previously silent genes [reviewed in Zenner et al., 1995]. As a result of these events, the triggered cells proliferate and/or differentiate into cells displaying specific effector functions [reviewed in Altman et al., 1990; Abraham et al., 1992; Mustelin, 1994].

The T cell antigen receptor (TCR) has separate antigen-binding and signal transduction subunits. The antigen-binding TCR α and β heterodimer forms a complex with the invariant chains of CD3γ, CD3δ, CD3ε, and the ζ-ζ dimer [Frank et al., 1990]. In contrast to the short cytoplasmic parts of the α- and β-chain (5 amino acids), the large cytoplasmic domains (40–113 amino acids) of the invariant chains are responsible for coupling the antigen-binding TCR α and β chains to the intracellular signaling machinery. The signal transduction functions of the invariant chains were revealed by studies with mutant cell lines or with chimeric receptors in which the cytoplasmic domains of TCR-ζ or CD3ε were linked to the extracellular and transmembrane domains of other receptor proteins [Wegener et al., 1992; Romeo et al., 1992; Letourneur and Klausner, 1992]. An 18–19 amino acid sequence motif in the cytoplasmic portion of the TCR-ζ, CD3γ, CD3δ, and CD3ε chains has been identified that is both necessary

Abbreviations used: Fmoc, 9-fluorenylmethyl-oxycarbonyl; Fyn, fibroblast *yes* related kinase; GAP, Ras-GTPase activating protein; Grb2, growth factor receptor-bound protein 2; ITAM, immunoreceptor tyrosine-based activation motif; PI3K, phosphatidylinositol 3-kinase; PTK, protein tyrosine kinase; Shc, Src homologous and collagen; SH2, Src homology region 2; TCR, T cell antigen receptor; TCR-ζ, TCR associated ζ-chain; ZAP-70, ζ chain-associated protein tyrosine kinase.

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and sufficient for coupling the receptor to signaling events [Letourneur and Klausner, 1992; Irving et al., 1993]. The minimal functional segment of the motif consists of paired tyrosines and leucines in the consensus sequence (D/E)XXYXXL(X)$_{7-8}$YXX(L/I). These motifs have been referred to as the immunoreceptor tyrosine-based activation motif (ITAM) and are conserved in a number of receptor molecules [Reth, 1989]. They are found in three copies in the TCR-ζ chains, here referred to as ITAMζ$_1$, ITAMζ$_2$, and ITAMζ$_3$, and in one copy each in the CD3γ, CD3δ, and CD3ε chains [reviewed in Weiss, 1993].

T cell activation via the TCR results in activation of multiple PTKs [Mustelin and Burn, 1993] and in the phosphorylation of numerous substrates [Hsi et al., 1989], including the tyrosine residues in the ITAMs of the TCR-ζ, CD3γ, CD3δ, and CD3ε receptor subunits [Baniyash et al., 1988; Straus and Weiss, 1993]. Although the exact stoichiometry of phosphorylation of the individual ITAMs during T cell activation has not yet been determined, the phosphorylated ITAMs might provide docking places for SH2-containing effector molecules in vivo [Birge and Hanafusa, 1993; Songyang et al., 1994]. ZAP-70 (ζ chain–associated protein tyrosine kinase) [Iwashima et al., 1994; Isakov et al., 1995], the Src family kinase Fyn [Timson-Gauen et al., 1992; Gassmann et al., 1992], phosphatidylinositol 3-kinase (PI3K) [Exley et al., 1994], or adapter molecules such as Shc [Ravichandran et al., 1993] are likely candidates for such interactions. Thus, the ITAMs might provide a crucial coupling element between receptor-mediated antigen recognition and the intracellular signaling apparatus.

The presence of multiple ITAM sequences within a single oligomeric receptor such as the TCR/CD3 complex raises the question of whether individual ITAMs may function as docking regions for (1) distinct effector molecules in order to activate different signaling pathways or (2) multiple copies of a particular molecule in order to amplify the signal that arrives at the receptor. There is indirect evidence supporting both notions.

In the present study we describe the synthesis of biotinylated bisphosphorylated, monophosphorylated, and unphosphorylated peptides corresponding in sequence to each of the three ITAMs of the TCR-ζ chain. The synthetic, biotinylated peptides were exploited to anchor the peptides in a specific orientation to streptavidin-coated agarose beads. Using these reagents together with T cell lysates of human Jurkat T cells, we analyzed the potential of individual ITAMs to interact with signaling molecules such as ZAP-70, Shc, Grb2, PI3K, GAP, and Fyn.

MATERIALS AND METHODS

Synthesis and Characterization of ITAM-Containing Peptides

Peptides derived from the TCR-ζ chain (ITAMζ$_1$, ITAMζ$_2$, and ITAMζ$_3$) were obtained by solid phase peptide synthesis on a Milligen 9050 continuous flow synthesizer (Biosearch, Novato, CA) using an adapted software package. Individual ITAM-containing biotinylated 23-mer carboxamide peptides (Table I) were prepared as described below. A threefold excess of Fmoc (9-fluorenylmethyl-oxycarbonyl) amino acid derivatives was applied for TPTU [1,1,3,3,-tetramethyl-2-(2-oxo-1(2H)-pyridyl-uronium-tetrafluoroborate)] activation [Knorr et al., 1989] and a 20% solution of piperidine in DMF (*N,N*-dimethylformamide) were used for Fmoc removal. Fmoc–aminocaprylic acid was introduced as a spacer at the N-terminus. In the case of the nonphosphorylated control peptides, d-biotin was coupled to the spacer after removal of the Fmoc moiety. In the case of the phosphorylated peptides, Fmoc-Tyr was incorporated in the appropriate position in the sequence. Peptides, phosphorylated at tyrosine residues, were obtained according to the global phosphorylation procedure described earlier [Kitas et al., 1991]. Phosphorylation was achieved after introduction of Fmoc–aminocaprylic acid using a five- to tenfold excess of bis(benzyloxy) (diisopropyl amino) phosphine/tetrazole in acetonitril under argon atmosphere, followed by iodine oxidation in the presence of 2,6-dimethyl pyridine in THF (tetrahydrofuran). The phosphorylation reaction proceeded for 1–1.5 h and was repeated after removal of reagents under argon. Thereafter, the Fmoc-group was cleaved and d-biotin was coupled in the last step of solid phase synthesis.

For ITAMζ$_1$ and ITAMζ$_2$ peptides final cleavage was performed in 95% TFA (trifluoroacetic acid) containing 2.5% thiophenol and 2.5% water for 1.5–2 h at room temperature. For ITAMζ$_3$ peptides reagent K [King et al., 1990] was applied for 1.5–2.5 h at room temperature. Purification was performed on a preparative reverse phase HPLC column (Waters, Milford, MA),

MA), Delta pak column; Merck RP-18 (Merck, Darmstadt, Germany), 10 μm, 25 × 250 mm, Lichrospher-100Å) applying a gradient from 0.1% TFA to 100% ethanol (flow: 20 ml/min). The peptides were characterized by analytical HPLC (Merck RP-18, 5 μm, 4 × 125 mm, Lichrospher-100Å) using an acetonitril gradient (0–80% acetonitril in 30 min; flow: 1 ml/min) and ion spray mass spectroscopy (ABI, Foster City, CA).

Antibodies and GST-Fusion Proteins

The following antisera and monoclonal antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and used in the Western blotting experiments: polyclonal rabbit antiserum specific for human ZAP-70; polyclonal rabbit antiserum specific for human Shc; monoclonal antibodies specific for Grb2/sem-5; polyclonal rabbit antiserum specific for rat PI3K (cross-reacts with human); polyclonal rabbit antiserum specific for human GAP; and polyclonal rabbit antiserum specific for human Fyn.

The GST-fusion proteins of PI3K and Grb2 and the affinity-purified rabbit polyclonal antibody for GST were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The GST-PI3K fusion protein used consists only of the aminoterminal SH2 domain of PI3K (amino acids 333–430). The GST-Grb2 fusion protein consists of the full-length Grb2 protein (amino acids 1–217).

Cell Lines and Cell Extraction

The human Jurkat T cell line, the Jurkat $CD45^{-/-}$ cell line, and the human CEM/C3 cell line were cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and the antibiotics penicillin and streptomycin at a concentration of 100 μg/ml each. Cells were harvested and collected by centrifugation, washed once with phosphate buffered saline (PBS), and lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40 plus phosphatase and proteinase inhibitors: 200 μg/ml PMSF, 400 μM Na_3VO_4, 2 μg/ml aprotinin and leupeptin) on ice for 15 min. After centrifugation at 20,000*g* for 30 min at 4°C the cleared supernatants of the cellular lysates were used for peptide binding assays.

Peptide Binding Assays

The peptides (10 μg in 50 μl TBS) were incubated with 60 μl of streptavidin-agarose beads (Sigma, St. Louis, MO) on a rotating wheel at 4°C for 1 h. After washing with TBS (150 mM NaCl, 50 mM Tris, pH 7.5) the beads were preincubated with 4% BSA in TBS for 15 min and washed three times with TBS, followed by incubation with the cleared supernatants of the cellular lysates (5×10^7 cell equivalents were used in each experiment) on a rotating wheel at 4°C for 2 h. The beads were washed once in lysis buffer, twice in TBS, and then boiled in SDS-containing gel sample buffer for 5 min. The eluted proteins were analyzed by reducing SDS-PAGE and Western blotting using the antisera and antibodies described above. The blots were developed by the enhanced chemiluminescence technique (ECL kit; Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Binding Assay With GST-Fusion Proteins

The peptides (10 μg in 50 μl TBS) were incubated with 60 μl of streptavidin-agarose beads (Sigma) on a rotating wheel at 4°C for 1 h. After washing with TBS (150 mM NaCl, 50 mM Tris, pH 7.5) the beads were preincubated with 4% BSA in TBS for 15 min and washed three times with TBS, followed by incubation with the purified GST-fusion proteins (0.2 μg in 200 μl TBS, 1% NP40, 10% glycerol) on a rotating wheel at 4°C for 1 h. The beads were washed three times in TBS and then boiled in SDS-containing gel sample buffer for 5 min. The eluted proteins were analyzed by reducing SDS-PAGE and Western blotting using rabbit anti-GST antibodies. The blots were developed by the enhanced chemiluminescence technique (ECL kit; Amersham) according to the manufacturer's instructions.

RESULTS

Synthesis and Characterization of the Biotinylated Peptides

Three nonphosphorylated, one monophosphorylated, and three bisphosphorylated biotinylated, ITAM-containing 23-mer carboxamide peptides (Table I) were synthesized as described in Material and Methods. The ITAMζ_1 peptide corresponds to the amino-terminal (membrane proximal), ITAMζ_2 to the middle, and ITAMζ_3 peptide to the carboxy-terminal ITAM, contained in the cytoplasmic domain of the ζ-chain. The peptides were designed to carry the two critical tyrosine residues and a short flanking sequence at the N- and C-terminus. In addition, an N-terminal caprylic acid spacer and a biotin tag were incorporated (Table I).

TABLE I. Amino Acid Sequences of the Biotinylated 23-Mer Carboxamide Peptides Corresponding to the Three ITAMs of the TCR-ζ*

ITAMζ_1	biotinyl-C8-QQGQNQLYNELNLGRREEYDVLD-NH_2
ITAMζ_1PP	biotinyl-C8-QQGQNQLYNELNLGRREEYDVLD-NH_2
ITAMζ_2	biotinyl-C8-KNPQEGLYNELQKDKMAEAYSEIG-NH_2
ITAMζ_2PP	biotinyl-C8-KNPQEGLYNELQKDKMAEAYSEIG-NH_2
ITAMζ_3	biotinyl-C8-GKGHDGLYQGLSTATKDTYDALH-NH_2
ITAMζ_3P	biotinyl-C8-GKGHDGLYQGLSTATKDTYDALH-NH_2
ITAMζ_3PP	biotinyl-C8-GKGHDGLYQGLSTATKDTYDALH-NH_2

*C8, caprylic acid spacer; Y, phosphorylated tyrosine residue.

The synthesis of the peptides was followed by semiquantitative UV tracing of the Fmoc deprotection peak. Biotinylation was performed as a last step in the preparation of the phosphopeptides to avoid oxidation during iodine treatment (oxidation: phosphorus III to phosphorus V). Analysis of the crude product indicated quantitative phosphorylation since unphosphorylated peptide was detected only in trace amounts. Thus, the repeated phosphorylation with excess of phosphoramidite proved to be a reliable procedure for generation of biotin-labeled, bisphosphorylated peptides with a size longer than 20 residues.

All peptides were purified to near homogeneity by preparative reverse phase HPLC as described in Material and Methods. The yield of purified ITAMζ_1 peptides with respect to crude material was 31% for the nonphosphorylated peptide and 11% for the bisphosphorylated ITAMζ_1PP peptide. Purified ITAMζ_2 peptides were obtained in 9.4% (nonphosphorylated ITAMζ_2) and 8.4% (bisphosphorylated ITAMζ_2PP) yield. In the case of the ITAMζ_3 peptides, the yield amounted to 10.8% (nonphosphorylated ITAMζ_3), 18.2% (monophosphorylated ITAMζ_3P), and 11.3% (bisphosphorylated ITAMζ_3PP). For all peptides, the expected ion series was observed in the ion spray mass spectra and analytical HPLC reflected purification to near homogeneity (data not shown). A typical result of an analytical reverse phase HPLC trace (Fig. 1A) and the corresponding ion spray mass spectrum (Fig. 1B) of the bisphosphorylated ITAMζ_1PP peptide is shown.

Specific Binding of Proteins to the ITAMs

To examine the interactions of cellular proteins with the synthetic phosphopeptides and the corresponding unphosphorylated peptides, the biotinylated peptides were immobilized on streptavidin-agarose beads and incubated with lysates of human Jurkat T cells. Bound proteins were released by boiling in SDS-containing gel sample buffer and analyzed by SDS-PAGE and Western blotting using various specific antibodies.

The SH2 domain–containing ZAP-70 binds specifically to the bisphosphorylated peptides ITAMζ_1PP, ITAMζ_2PP, and ITAMζ_3PP (Fig. 2). This interaction seems to be specific as demonstrated in peptide competition experiments (data not shown) and is in agreement with previously reported experiments [Isakov et al., 1995]. These qualitative results indicate that ZAP-70 binds with different relative affinities to the three bisphosphorylated peptides. The detected binding was the strongest for ITAMζ_1PP followed by ITAMζ_2PP and ITAMζ_3PP, in agreement with results reported previously [Isakov et al., 1995]. In contrast, the monophosphorylated ITAMζ_3P and the unphosphorylated ITAMζ_1, ITAMζ_2, and ITAMζ_3 did not recruit ZAP-70 from the cellular lysates, indicating that phosphorylation of the carboxyterminal tyrosine residue within the ITAM is not sufficient for binding of ZAP-70 to the peptides. Densitometric analysis of the Western blot results shown in Figure 2 and calculation of the yield of binding revealed that approximately 35% of the ZAP-70 protein present in the lysate binds to ITAMζ_1PP.

A similar set of experiments was performed using Western blotting with antibodies specific for the adapter protein Shc. The results shown in Figure 3 demonstrate that Shc (both 46 and 52 kDa forms) interacts with the bisphosphorylated ITAMζ_1PP, monophosphorylated ITAMζ_3P, and bisphosphorylated ITAMζ_3PP, whereas bisphosphorylated ITAMζ_2PP and the unphosphorylated ITAMζ_1, ITAMζ_2, and ITAMζ_3 do not lead to any detectable recruitment of Shc from the cellular lysates. Approximately 25% of the Shc protein present in the cellular lysate was bound to the bisphosphorylated ITAMζ_1PP as determined by

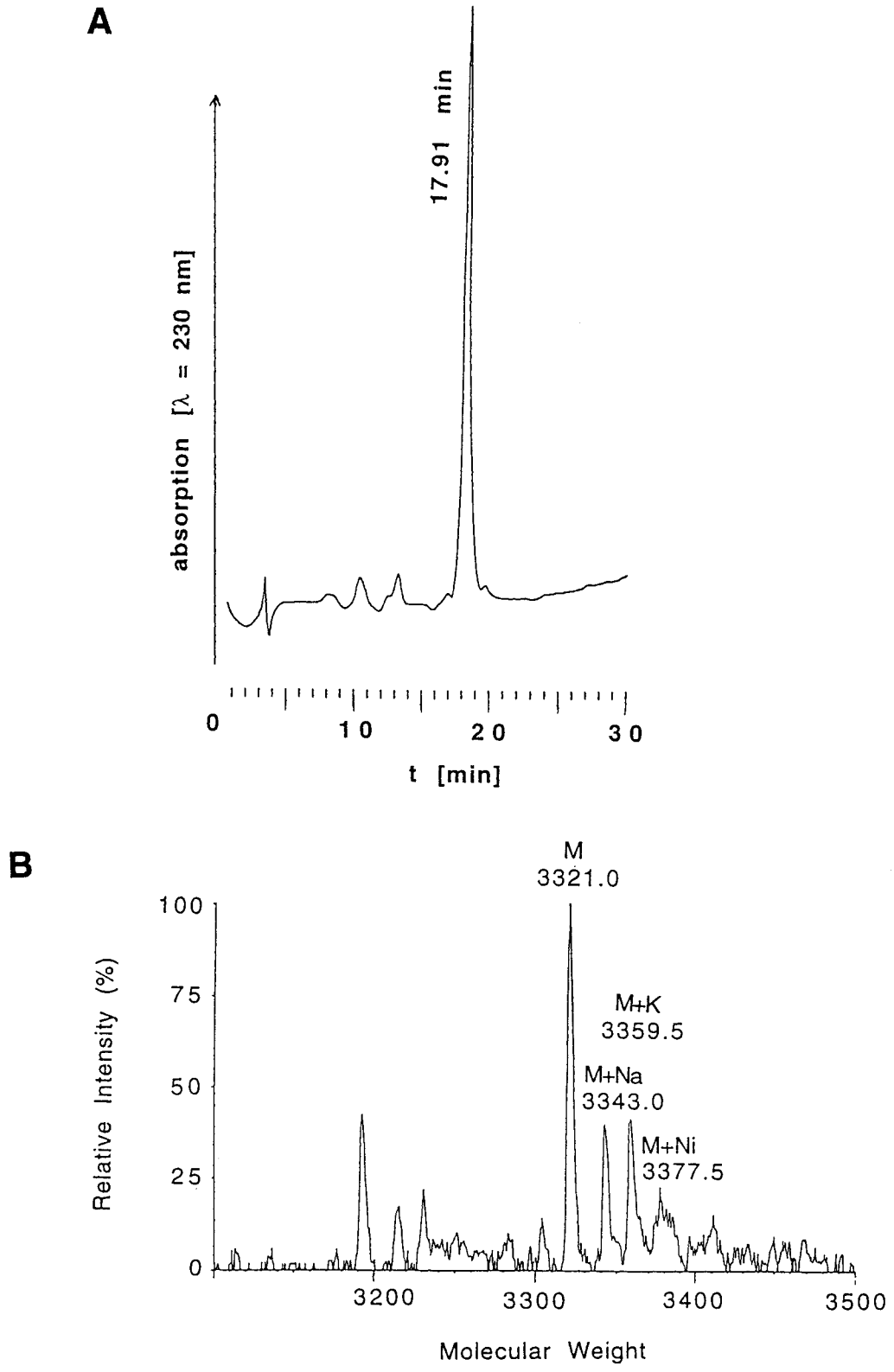

Fig. 1. Characterization of the synthetic peptide ITAMζ_1PP corresponding to the bisphosphorylated form of ITAMζ_1 of the TCR-ζ chain. **A:** Analytical reverse phase (RP-18) HPLC trace. **B:** Ion spray mass spectrum.

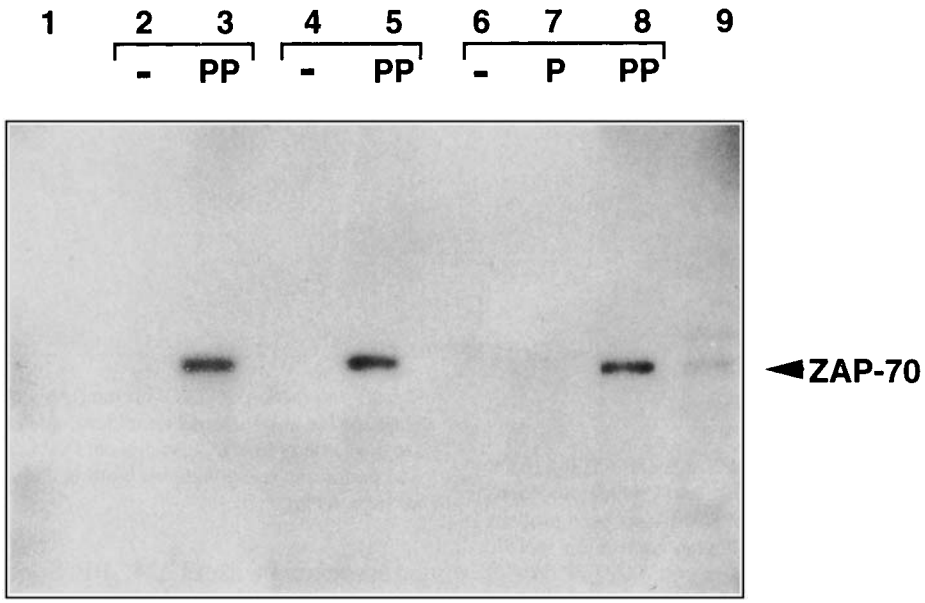

Fig. 2. Association of ZAP-70 with ITAMs of the TCR-ζ chain. Biotinylated peptides were coupled to streptavidin-agarose beads and probed with lysates of Jurkat T cells. Analysis of ITAM-associated proteins was performed using Western blotting and antiserum specific for human ZAP-70. *Lane 1:* Control with streptavidin-agarose beads without any peptide. *Lane 2:* Unphosphorylated ITAMζ_1. *Lane 3:* Bisphosphorylated ITAMζ_1PP. *Lane 4:* Unphosphorylated ITAMζ_2. *Lane 5:* Bisphosphorylated ITAMζ_2PP. *Lane 6:* Unphosphorylated ITAMζ_3. *Lane 7:* Monophosphorylated ITAMζ_3P. *Lane 8:* Bisphosphorylated ITAMζ_3PP. *Lane 9:* Lysate of Jurkat T cells. Lanes 1–8 represent 2×10^7 cell equivalents each, and lane 9 represents 2×10^6 cell equivalents.

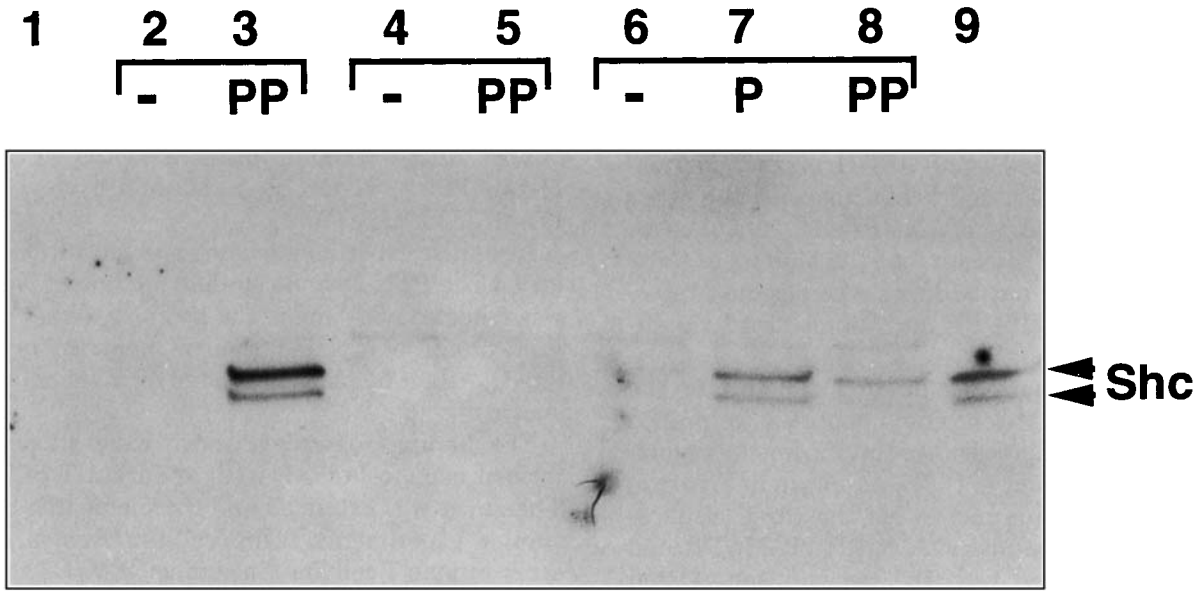

Fig. 3. Association of Shc with ITAMs of the TCR-ζ chain. The samples, experimental conditions, and lane identifications are the same as in Fig. 2. Analysis of ITAM-associated proteins was performed using Western blotting and antiserum specific for human Shc. Note that bisphosphorylated ITAMζ_1PP (*lane 3*), monophosphorylated ITAMζ_3P (*lane 7*), and bisphosphorylated ITAMζ_3PP (*lane 8*) but not bisphosphorylated ITAMζ_2PP (*lane 5*) associate with Shc.

densitometric analysis of the Western blot results. Interestingly, the affinities for the bisphosphorylated ITAMζ_1PP and monophosphorylated ITAMζ_3P were higher than for the bisphosphorylated ITAMζ_3PP.

In a next series of experiments we probed the membranes with antibodies specific for Grb2, a 25 kDa adapter protein displaying two SH3 and one SH2 domain (Fig. 4). We found that Grb2 interacts specifically with bisphosphorylated

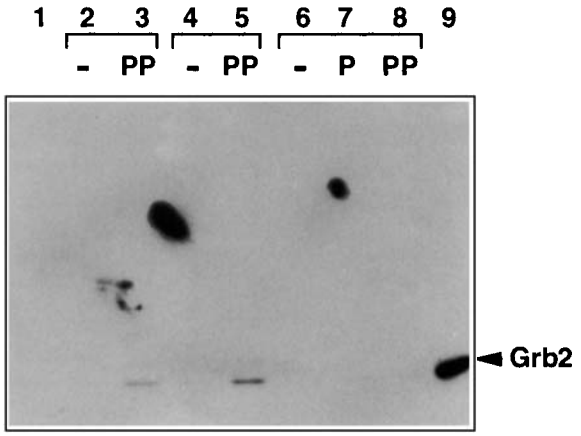

Fig. 4. Association of Grb2 with the ITAMs of the TCR-ζ chain. The samples, experimental conditions, and lane identifications are the same as in Fig. 2. Analysis of ITAM-associated proteins was performed using Western blotting and antiserum specific for Grb2. Note that only bisphosphorylated ITAMζ_1PP (*lane 3*) and bisphosphorylated ITAMζ_2PP (*lane 5*) interact with Grb2.

ITAMζ_1PP and bisphosphorylated ITAMζ_2PP, whereas no association of Grb2 could be detected using the monophosphorylated ITAMζ_3P, bisphosphorylated ITAMζ_3PP, or unphosphorylated ITAMζ_1, ITAMζ_2, or ITAMζ_3 peptides (Fig. 4). Only a small fraction (approximately 1%) of Grb2 present in the cellular lysate was precipitated with ITAMζ_2PP. To characterize the mode of interaction of Grb2 with the various ζ-chain motifs, GST-fusion proteins of Grb2 (full-length) were used. Binding of GST-Grb2 was only detected with ITAMζ_2PP but not with the other peptides including ITAMζ_1PP (data not shown). These results indicate that the binding of Grb2 to ITAMζ_2PP may be direct, whereas the interaction with ITAMζ_1PP may be mediated via another protein.

To investigate the binding of PI3K to the different ITAM-containing peptides, we probed the blots with antibodies specific for the regulatory p85 subunit of PI3K. As shown in Figure 5, this protein interacts with the three bisphosphorylated peptides ITAMζ_1PP, ITAMζ_2PP, and ITAMζ_3PP and with the monophosphorylated ITAMζ_3P. No interaction was detected with the unphosphorylated peptides ITAMζ_1, ITAMζ_2, and ITAMζ_3. Thus, in contrast to ZAP-70, PI3K may need only one phosphorylated tyrosine residue within the ITAM for binding. The following hierarchy represents the relative affinities of PI3K for the individual peptides: ITAMζ_1PP $\geq$ ITAMζ_2PP $>$ ITAMζ_3PP $\geq$ ITAMζ_3P $>>>$ ITAMζ_1, ITAMζ_2, ITAMζ_3. Eleven percent of the PI3K present in the cellular lysate was found to

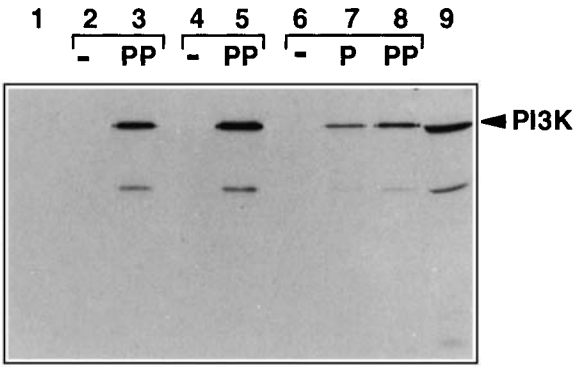

Fig. 5. Interaction of PI3K with the ITAMs of the TCR-ζ chain. The samples, experimental conditions, and lane identifications are the same as in Fig. 2. Analysis of ITAM-associated proteins was performed using Western blotting and antiserum specific for human PI3K.

be associated with ITAMζ_1PP. Specificity of binding was demonstrated by peptide competition experiments (data not shown). The results of experiments in which GST-PI3K fusion proteins were probed with the peptides indicated that PI3K interacts directly with the respective peptides.

Probing of the membranes with antibodies specific for GAP revealed that this protein associates with all bisphosphorylated ITAMs and the monophosphorylated ITAMζ_3P but not with the unphosphorylated peptides ITAMζ_1, ITAMζ_2, and ITAMζ_3 (data not shown). The relative affinity for binding of GAP to the various peptides is ITAMζ_2PP $\geq$ ITAMζ_1PP $>$ ITAMζ_3P $\geq$ ITAMζ_3PP $>>>$ ITAMζ_1, ITAMζ_2, ITAMζ_3.

In similar experiments using specific antibodies for the PTK Fyn, no specific binding of Fyn was detected with any of the peptides, either in their unphosphorylated, their monophosphorylated, or their bisphosphorylated form (data not shown).

The findings described above were all performed using cellular lysates of Jurkat T cells. The study was extended and complemented by similar experiments using cellular lysates of other human T cell lines including CEM/C3 and Jurkat CD45$^{-/-}$ cells (data not shown). All results obtained were similar and confirmed the results documented above with lysates of Jurkat T cells.

DISCUSSION

We have used a biotin-streptavidin-based technique for high affinity and unidirectional immobilization of peptides. This approach was used to investigate the association of cellular proteins

with the three ITAMs of the TCR-ζ chain. Bis-, mono-, and unphosphorylated forms of the ITAM-containing peptides were synthesized and used in the present study. Previous experiments have used either nonselective binding through available amino groups in the peptide to reactive beads (e.g., Affigel 10/15) or bacterially expressed fusion proteins containing larger portions of the ζ-chain. In the former case, a significant fraction of the peptides may bind in unfavorable conformations, and critical amino acid residues may be blocked (e.g., by binding through the amino group of asparagine or glutamine residues next to the phosphorylated tyrosine). Bacterially expressed fusion proteins, on the other hand, are invariably produced without phosphotyrosine and have to be phosphorylated in vitro [Isakov et al., 1995]. This can cause many difficulties, including a low stoichiometry of phosphorylation or the inadvertent phosphorylation of other tyrosine residues in the protein. Thus, our approach using solid phase peptide synthesis and global phosphorylation strategies in combination with d-biotin tagging represents a considerable improvement, in both reliability and specificity, over previously described techniques.

We have found that a number of important signal transducing molecules including ZAP-70, Shc, Grb2, PI3K, and GAP specifically associate with phosphorylated peptides corresponding to the three ITAMs of the TCR-ζ chain. The binding of ZAP-70 via its two SH2 domains to the bisphosphorylated ITAMs is well documented [Isakov et al., 1995; Timson et al., 1994] and was studied first to verify the accuracy of our approach. Indeed, ITAMζ_1PP, ITAMζ_2PP, and ITAMζ_3PP all bound ZAP-70, while the unphosphorylated peptides and the monophosphorylated ITAMζ_3P did not. The relative affinity of binding was highest for the ITAMζ_1PP and lowest for ITAMζ_3PP, in agreement with results published earlier [Isakov et al., 1995]. Thus, in a triggered T cell, an intact TCR-ζ-ζ homodimer may bind up to six ZAP-70 molecules, provided that in the ζ-ζ dimer all twelve tyrosine residues are phosphorylated. This multiple binding of ZAP-70 to the bisphosphorylated ITAMs could be involved in ZAP-70–mediated signal amplification after TCR/CD3 receptor triggering.

In our experiments Shc has been shown to interact with the bisphosphorylated ITAMζ_1PP and ITAMζ_3PP and the monophosphorylated ITAMζ_3P (see Fig. 3). The single SH2 domain of Shc reportedly prefers phosphotyrosine (PTyr) followed by (I/E/Y/L)-X-(I/L/M) [Songyang et al., 1994], a preference reasonably compatible with the sequence following all six tyrosines in the ITAMs. Thus, it seems likely that Shc binds to the ITAMs via its SH2 domain in a direct manner, but it remains less clear why it prefers ITAMζ_1PP and ITAMζ_3P over the two bisphosphorylated ITAMζ_2PP and ITAMζ_3PP. A possible explanation is that we perform our experiments with T cell lysates under conditions where all cellular proteins are allowed to compete for binding. Thus, it is possible that more Shc binds to ITAMζ_3P compared to ITAMζ_3PP because ZAP-70 competes efficiently only for the latter.

Binding of Grb2, PI3K, and GAP seems to be clearly dependent on tyrosine phosphorylation of the respective ITAMs. Since the SH2 domains of Grb2, PI3K, and GAP prefer ligands that differ critically in amino acid sequence from the ITAMs [Songyang et al., 1993, 1994], it can be speculated that the binding of these signaling molecules to the various phosphorylated ITAMs involves additional proteins. For example, the SH2 domain of Grb2 is highly selective for PTyr followed by asparagine at the +2 position [Songyang et al., 1994], a feature that is not found in any of the ITAMs. Therefore, it is likely that Grb2 binds to a PTyr-X-N-X sequence in another molecule, which, in turn, binds to the ITAM via an SH2 domain. One such possible intermediate molecule is Shc, which is known to bind Grb2 in a tyrosine phosphorylation-dependent manner [Ravichandran et al., 1993; Lowenstein et al., 1992]. In our experiments, Grb2 bound to ITAMζ_1PP and ITAMζ_2PP, while Shc bound ITAMζ_1PP but did not bind ITAMζ_2PP. Thus, Grb2 might bind to ITAMζ_1PP via the adapter protein Shc in an indirect fashion, whereas the binding of Grb2 to ITAMζ_2PP might be direct. This idea is supported by our findings that the GST-Grb2 fusion protein binds to ITAMζ_2PP but not ITAMζ_1PP. Interestingly, it has been shown in the case of the hepatocyte growth factor (HGF) that Grb2 binds either directly to the phosphorylated cytoplasmic domain of the receptor or indirectly via the Shc protein [Pelicci et al., 1995]. Thus, under physiologically relevant conditions, Grb2 may bind to ITAMζ_1PP via the Shc adapter protein and to ITAMζ_2PP in a phosphorylation-dependent and direct manner, a hypothesis that is supported by our results with the GST-Grb2 fusion proteins.

The two SH2 domains of the p85 subunit of PI3K have a strong preference for PTyr residues followed by methionine at position +3 [Songyang et al., 1993]. Again, the ITAMs do not meet this criteria, suggesting indirect binding. However, our results with the GST-fusion proteins of PI3K suggest that the binding might be in a direct manner. The exact requirement for the SH2 domain of GAP is not well understood. The molecule(s) mediating binding of PI3K and GAP to the ITAMs remains speculative. A role for the Src family PTKs, which have been shown to associate with both PI3K [Prasad et al., 1993] [Vogel and Fujita, 1993] and GAP [Amrein et al., 1992] and the TCR-ζ chain [Gassmann et al., 1992], seems unlikely since Fyn was not specifically detected to associate with any of the peptides tested.

Taken together, our results indicate that the three ITAMs of the TCR-ζ (i.e., ITAMζ_1, ITAMζ_2, and ITAMζ_3) differ from each other in their ability to associate with cellular proteins. At present it is not known to which extent the different ITAMs are phosphorylated under physiologically relevant conditions after TCR/CD3 triggering, nor do we know which amount of a particular protein has to be associated with the individual ITAMs to display a biologically relevant function. The apparent shift observed on SDS-polyacrylamide gels of the 16 kDa ζ-chain band to multiple higher Mr forms (up to 21 kDa and 23 kDa) is indicative of various degrees of phosphorylation of the ζ-chain ITAMs, suggesting that partially phosphorylated ζ-chain species are present after TCR/CD3 receptor stimulation. Intriguingly, it was recently shown that stimulation of TCR by an antagonist peptide (properly presented on MHC molecules), which causes anergy, correlated with a decrease of the 23 kDa form of the TCR-ζ chain and absence of ZAP-70 phosphorylation [Madrenas et al., 1995]. We speculate that this difference in TCR-ζ phosphorylation may result in association with a nonidentical set of cellular proteins. This may profoundly affect the coupling to intracellular signaling pathways and thereby affect the biological outcome of receptor triggering. Thus, dependent on the nature of the signal received by the TCR, the ITAMs may become differentially phosphorylated leading to differential and multiple binding of signal transducing molecules to the ITAMs of the TCR-ζ chain resulting in either signal amplification or in activation of different pathways.

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