Engagement of the T cell antigen receptor initiates signal transduction involving tyrosine phosphorylation of multiple effector molecules and the formation of multimolecular complexes at the receptor site. Adapter proteins that possess SH2 and SH3 protein-protein interaction domains are implicated in the assembly of cell activation-induced signaling complexes. We found that Crk adapter proteins undergo activation-induced interaction with the \( \zeta \)-chain associated protein (ZAP-70) tyrosine kinase in the human T cell line, Jurkat. Incubation of various glutathione S-transferase fusion proteins with a lysate of activated Jurkat cells resulted in selective association of ZAP-70 with Crk, but not Grb2 or Nck, adapter proteins. In addition, tyrosine-phosphorylated ZAP-70 co-immunoprecipitated with Crk from a lysate of activated Jurkat cells, and ZAP-70 association with GST-Crk was observed in a lysate of activated human peripheral blood T cells. Association between the two molecules was mediated by direct physical interaction and involved the Crk-SH2 domain and phosphotyrosyl-containing sequences on ZAP-70. The association required intact Lck, considered to be an upstream regulator of ZAP-70, because it could not take place in activated JCaM1 cells, which express normal levels of ZAP-70 but are devoid of Lck. Finally, glutathione S-transferase-Crk fusion proteins were found to interact predominantly with membrane-residing tyrosine-phosphorylated ZAP-70 that exhibited autophosphorylation activity as well as phosphorylation of an exogenous substrate, CFB3. These findings suggest that Crk adapter proteins play a role in the early activation events of T lymphocytes, apparently, by direct interaction with, and regulation of, the membrane-residing ZAP-70 protein tyrosine kinase.

Engagement of the T cell antigen receptor (TCR)\(^1\) triggers signal transduction pathways that directly regulate T cell activation and differentiation (1, 2). Although many of the individual components of the TCR-linked signaling pathway are physically separated in resting cells, upon engagement of the receptor, they reassemble into functional complexes at the site of contact with major histocompatibility complex-presenting peptide antigen on the surface of the antigen-presenting cells (3). The reassembly process directs enzymes, their corresponding substrates, and additional effector molecules to the receptor site, in a temporally and spatially regulated manner, which ensures an efficient signaling leading to cell activation. The same mechanism ensures that the level of signaling in unstimulated cells remains below the critical threshold required for cell activation.

The earliest detectable biochemical event in activated T cells is the phosphorylation of multiple protein substrates on tyrosine residues. Therefore, protein tyrosine kinases (PTKs) that mediate this activity play a critical role in the early phases of the activation response by up-regulating critical enzymes and promoting the assembly of the multimolecular complexes. Non-receptor PTKs that mediate these functions in T cells include the Src family members, Lck and Fyn, and the Syk family members, ZAP-70 and Syk (1, 2). A fraction of Fyn and Lck, which are constitutively associated with the TCR and CD4/CD8 cytoplasmic tails, respectively, appears to phosphorylate critical tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) on distinct TCR subunits (4). ZAP-70 and Syk then recruit to the phosphorylated ITAMs and interact with two phosphotyrosyl residues within an ITAM via their tandem SH2 domains. Only then can ZAP-70 undergo tyrosine phosphorylation and become enzymatically active (5, 6). The subsequent tyrosine phosphorylation of additional cellular substrates, including LAT, phospholipase C\(\gamma\)1, Vav, SLP-76, Shc, Cbl, and Pyk2, function to couple the receptor to its signaling pathways and regulate the activation response.

Members of the group of adapter proteins, which include Crk, Grb2, Nck, Grap, and Shc, consist primarily of SH2 and SH3 protein-protein interaction domains. They are involved in the control of various cellular processes linked to cell growth and differentiation. Crk adapter proteins have been implicated in signaling pathways leading to cell growth (7), migration (8), differentiation (9), apoptosis (10), and transformation (11). Although the physiological role of Crk proteins is largely unknown, studies have shown their involvement in signaling via diverse membrane receptors, including those of integrins (12), interleukins (13), and growth factors (13–15). Furthermore, Crk proteins play a role in signaling via antigen receptors in B (16–18) and T (19–22) lymphocytes. Recent data suggest that the involvement of Crk proteins in various signaling pathways is mediated by their ability to up-regulate the activity of small GTP-binding proteins, such as Ras (15, 23, 24), Rap1 (25, 26), and Rho (27), and activate a selective mitogen-activated pro-
tein kinase cascade which is controlled by c-Jun NH2-terminal kinase (23, 28).

Crk proteins have also been reported to interact with a variety of cellular proteins. These include tyrosine-phosphorylated proteins, such as the multidomain docking proteins, Cas (Crk-associated substrate, p130; see Ref. 29) and HEF1 (human enhancer for filamentation 1; see Ref. 30), tyrosine kinase receptors such as the PDGF-R (31), HEK2 (32), and Ret (33), and additional proteins including paxillin (p70; see Ref. 34), Cbl (p120; see Ref. 19), and IRS-1 (insulin receptor substrate-1; see Ref. 35), most of which interact with the Crk SH2 domain. Furthermore, Crk can interact via its SH3 domain with protein-rich sequences in the guanine nucleotide exchange factors, C3G (36) and Sos (24), in Abl (37) and Arg PTKs (38), in line-rich sequences in the guanine nucleotide exchange factors, C3G (36) and Sos (24), in Abl (37) and Arg PTKs (38), in

For in vitro binding assays, bead-adsorbed GST or GST fusion proteins (5 μg/sample) were incubated with cell lysates at 4 °C on a rotator for 1 h. The beads were then washed 3 times in lysis buffer, and bound proteins were either eluted and subjected to SDS-PAGE under reducing conditions followed by immunoblotting or tested in an in vitro kinase assay.

**Cell Culture and Stimulation—**Human leukemic Jurkat T cells, Jurkat-TAg cells which stably express the simian virus 40-derived large T antigen, and Jurkat-derived mutant cell lines, JCaM1 and J4501, that are defective in expression of Lck or CD45, respectively, were maintained at a logarithmic growth phase in complete RPMI (RPMI 1640, 10% FCS, 4 mM l-glutamine, 100 units/ml penicillin, 50 μg/ml streptomycin (all from Biological Industries, Beit Haemek, Israel), and 5 × 10−5 M β-mercaptoethanol (Sigma)) in 75-cm2 growth-area tissue culture flasks (Cell-Cult, Sterlin Limited, Feltham, UK) in an atmosphere of 7.5% CO2 at 37 °C. Peripheral blood lymphocytes (PBL) were obtained by histopaque gradient centrifugation of heparinized blood from healthy volunteers. Enriched population of preactivated and rested PBL T cells were obtained by cell culture (1 × 106/ml) in 10% fetal calf serum-containing complete RPMI in the presence of 5 μg/ml phytomenadione in 75-cm2 growth-area tissue culture flasks (50 ml/ flask). Human recombinant IL-2 (20 units/ml) was added after 72 h of culture, and cells were maintained in culture for 6 more days by the addition of IL-2 (20 units/ml) once every 2 days.

Jurkat or PBL T cells (10 × 109/ml) were stimulated with freshly prepared 1% pervanadate (10 mM Na3VO4 containing 1% H2O2) for 30 min at 37 °C. Ab-mediated cross-linking of the TCR/CD3 complex was performed by incubating Jurkat or PBL T cells (10 × 109/ml) with 100 μg/ml of C305 mAb, or OKT3 mAb, respectively, for 10 min on ice. A secondary cross-linking rabbit anti-mouse Ig Ab was then added for 10 min on ice, followed by transfer to 37 °C and incubation for 2 min.

**Mammalian Expression Vectors and Transient Transfection of Cells—**The CRK-II cDNA in pcDL-Sr296/Rbg/IIG mammalian expression vector (pYCRk-II) was a gift of Dr. Matsuda (42). For transfection, the Jurkat-TAg cells were washed three times in supplement-free RPMI 1640, resuspended at 5 × 107 cells/ml in unsupplemented medium, and aliquoted into 0.4-ml-gap Gene Pulser cuvettes (Bio-Rad) (2 × 107 cells/400 μl/cuvette). Plasmid DNA (10 μg/group) was added, and the cells were electroporated using a Bio-Rad Gene Pulser (250 volts, 950 microfarads). The cells were then cultured in 13 ml of complete RPMI 1640 in T25 tissue culture flasks for 48 h.

**Preparation of Cell Lysates and Immunoprecipitation—**Cell lysates were prepared by resuspension of cells in a lysis buffer containing 25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 10 μg/ml each of leupeptin and aprotinin, 2 μg/ml aprotinin, and 1% Triton X-100, followed by a 20-min incubation on ice. Lysates were centrifuged at 13,000 × g for 30 min at 4 °C, and the nuclear free supernatants were mixed with equal volumes of 2× SDS sample buffer, vortexed, incubated at 100 °C for 5 min, and analyzed by SDS-PAGE. Cytosolic and particulate fractions were prepared by resuspending the cells in a buffer containing 25 mM Tris/HCl (pH 7.5), 2 mM EDTA, 10 mM β-mercaptoethanol, 10 μg/ml each of leupeptin and aprotinin, and 2 μg/ml aprotinin, and repeatedly aspirating them through a 1-ml syringe with a 26-gauge needle for 20 s. Cell lysates were centrifuged at 400 × g for 5 min; nuclear pellets were removed, and lysates were recentrifuged at 13,000 × g. Supernatants (cytosolic fractions) were transferred to a second set of microcentrifuge tubes, Triton X-100 was added up to a 1% final concentration, and samples were either mixed with 5× SDS sample buffer (4.1, v/v), or used for immunoprecipitation. Pellets were washed once in buffer A, resuspended in buffer A plus 1% Triton X-100 (in the original volume used for the lysis), incubated for 30 min on ice, and centrifuged at 13,000 × g for 20 min. Supernatants (particulate fractions) were either mixed with 5× SDS sample buffer (4.1, v/v) or used for immunoprecipitation.

Immunoprecipitation was performed by using an optimal dilution of polyclonal antisera or mAbs that were preabsorbed on protein A-Sepharose beads for 2 h at 4 °C. Excess Abs were removed by 3 washes in cold phosphate-buffered saline, and Ab-coated beads were incubated with cell lysates for 2–3 h at 4 °C. Immune complexes were precipitated by centrifugation followed by extensive washing in a lysis buffer. Immunoprecipitated proteins were either fractionated by SDS-PAGE and immunoblotted or subjected to an in vitro kinase assay.

**Electrophoresis and Immunoblotting—**Samples of cell lysates, GST fusion protein, GST fusion protein-bound molecules, or Ab immunoprecipitates were resolved by electrophoresis on 10% acrylamide gels using Bio-Rad Mini-PROTEAN II Cell. Proteins in the gels were either stained with Coomassie Brilliant Blue (Sigma) or blotted onto nitrocel-
lulose membranes (Schleicher & Schuell) at 100 V for 45 min in a Bio-Rad Mini Trans-Blot transfer cell. After 1 h blocking at 37 °C with 3% bovine serum albumin in phosphate-buffered saline, nitrocellulose membranes were incubated with the indicated primary Abs, followed by incubation with HRP-conjugated sheep anti-mouse, or donkey anti-rabbit, Ig, or with HRP-conjugated protein A (Amersham Pharmacia Biotech). Immunoreactive proteins were visualized using an ECL reagent (Amersham Pharmacia Biotech) and autoradiography.

For Western Analysis—To determine direct interaction of either Crk-II or selected Crk-II domains with the electrophoresed, nitrocellulose-bound proteins, the SDS-PAGE and blotting were performed as described above, followed by blocking of the membranes with phosphate-buffered saline containing 3% bovine serum albumin and 0.1% Tween 20. Membranes were then incubated overnight at 4 °C with a blocking buffer containing 10 μg/ml of the indicated GST fusion protein, or GST, as a negative control. Bound GST proteins were detected by incubation of the membrane with a mouse anti-GST mAb for 1 h, followed by an HRP-conjugated sheep anti-mouse Ig and ECL development.

ZAP-70 Kinase Assay—ZAP-70 immunoprecipitates or cell lysate proteins adsorbed to immobilized GST fusion protein were washed twice in a lysis buffer followed by an additional wash in a kinase reaction buffer (25 mM HEPES, pH 7.3, 0.1% Nonidet P-40, 10 mM MnCl₂, 1 mM Na₃VO₄, 50 mM NaF). They were then resuspended in a reaction buffer, with or without 1 μg of CFP35. Kinase reaction, in a total volume of 15 μl, was initiated by the addition of 5 μCi of 10 μM [γ-³²P]ATP (at 3000 Ci/mmol) and incubated for 10 min at 37 °C. Reaction was terminated by the addition of 5X SDS sample buffer (4:1, v/v); samples were vortexed and boiled for 5 min, and phosphoproteins were resolved by SDS-PAGE on 10% acrylamide gels. Phosphoproteins were blotted onto nitrocellulose membranes and visualized by autoradiography by exposure to Kodak XAR-5 x-ray film at −70 °C with an intensifying screen.

RESULTS

Crk Adapter Proteins Associate with ZAP-70 from Activated Jurkat T Cells—Adapter proteins that consist of SH2 and SH3 domains play critical roles in the assembly of multimolecular signaling complexes during the early phases of cell activation response. We analyzed the involvement of Crk adapter proteins in the regulation of T cell activation, and because many of the protein-protein interaction events are regulated by tyrosine phosphorylation of signaling molecules, we questioned whether Crk proteins interact with tyrosine-phosphorylated proteins in activated T cells. Lysates of non-activated or pervanadate-stimulated human Jurkat T cells were incubated with bead-immobilized GST-Crk fusion proteins. After separation of the bound proteins by SDS-PAGE, the proteins were transblotted to nitrocellulose membranes, and tyrosyl phosphoproteins were identified using phosphotyrosine-specific mAbs. All three Crk adapter proteins were found to pull down numerous protein bands that reacted with anti-phosphotyrosine (Fig. 1A). One of the observed protein bands, at the range of 70 kDa, corresponded to the molecular mass of ZAP-70 PTK, which is a key enzyme in T cell activation. Stripping and reblotting of the membrane with anti-ZAP-70 Abs indicated that Crk interacted with ZAP-70 (Fig. 1A, 6th, 8th, and 10th lanes) and that the association occurred in lysates of activated but not of non-activated T cells (Fig. 1B, 6th, 8th, and 10th lanes, versus 5th, 7th, and 9th lanes, respectively). To examine whether this protein-protein interaction pattern is selective for Crk, we compared it with the pattern of tyrosyl phosphoproteins that associate with two other adapter proteins, Grb2 and Nck, which are also expressed in T cells. Although the overall pattern of tyrosyl phosphoproteins that interact with Crk and Grb2 was not drastically different, ZAP-70 was found to associate with Crk adapter proteins but not with Grb2 or Nck (Fig. 1B, 5–10th versus 11–14th lanes).

Depletion of ZAP-70 from the lysate of activated T cells by repeated absorption with bead-immobilized anti-ZAP-70 Abs confirmed that the Crk-associated 70-kDa protein is ZAP-70 and is not a distinct 70-kDa protein that reacts with the secondary anti-rabbit Ab or directly interacts with the ECL (Fig. 2). Furthermore, the GST-Crk-II fusion protein pulled down a tyrosine-phosphorylated 70-kDa protein only from wild-type Jurkat cells but not from its ZAP-70-deficient mutant, P116 (not shown).

The human ZAP-70 protein possesses 31 tyrosine residues that are potential phosphorylation sites for PTKs in pervanadate-activated T cells. One or more of these phosphotyrosyl residues may possibly mediate binding to the Crk-SH2 but may not necessarily be a potential site in TCR-activated T cells. We therefore tested whether cross-linking of the TCR on Jurkat T cells would also induce phosphorylation of ZAP-70 at a site that allows interaction with Crk. Activation of TCR on Jurkat cells was induced by incubation with a TCR γ-chain-specific mAb, C305, followed by analysis of the interaction of cell lysate proteins with various GST-Crk fusion proteins. The results demonstrated that C305 stimulation of Jurkat cells induced the association of ZAP-70 with Crk and that the binding affinity to the three Crk proteins was markedly distinct, indicating a hierarchy of Crk-II >> CrkL > Crk-I (Fig. 3A). This was based on observations of autoradiograms obtained after extended periods of exposure (not shown). Many of the protein bands observed in the anti-ZAP-70 immunoblots reflected the GST portion of the fusion proteins and their degradation products that react with Abs against the GST portion of the immunogen.

To analyze further whether ZAP-70-Crk association represents a general phenomenon in activated T cells, or perhaps an event unique for Jurkat cells or leukemic cells, we performed a similar analysis on lymphocytes from human peripheral blood. Because ZAP-70 is expressed in T, but not in B, lymphocytes, we used unseparated peripheral blood lymphocytes stimulated with polyclonal anti-CD3 (OKT3) mAbs. As in Jurkat cells, ZAP-70 from OKT3-stimulated peripheral blood T lymphocytes interacted with GST-Crk (Fig. 3B), and distinct Crk proteins exhibited a hierarchy of binding affinities identical to the one
observed in Jurkat cells (Fig. 3A). Stripping and re-blotting of the membrane with Tyr(P)-specific mAbs confirmed that a tyrosyl phosphoprotein band with a molecular mass of 70 kDa was pulled down by GST-Crk from a lysate of OKT3-stimulated, but not resting, PBL (Fig. 3C).

To demonstrate the interaction between ZAP-70 and Crk in activated T cells in a more direct way, Jurkat cell lysates were subjected to immunoprecipitation with either anti-Crk or anti-ZAP-70 Abs and blotting with the reciprocal combination of Abs. We found that Crk mAbs co-immunoprecipitated ZAP-70 from a lysate of activated but not non-activated Jurkat cells (not shown). Because of the relatively low stoichiometry of binding obtained in this assay, we repeated the immunoprecipitation in Jurkat-TAg cells that transiently overexpressed the Crk-II cDNA. As previously, anti-Crk mAbs were found to co-immunoprecipitate ZAP-70; association between the two proteins was specific for activated, but not non-activated, T cells (Fig. 4). The reciprocal immunoprecipitation with anti-ZAP-70 Abs and immunoblotting with anti-Crk did not yield conclusive results. This could be due to the fact that the anti-ZAP-70 polyclonal antiserum cross-reacts with additional proteins that interfere with the analysis and/or the possibility that anti-ZAP-70 Abs compete with Crk in binding to the same epitopes on the ZAP-70 molecule.

ZAP-70 Association with Crk Is Mediated by Direct Physical Interaction of ZAP-70 Phosphotyrosine-containing Sequences with the Crk-SH2 Domain—The apparent dependence of association between ZAP-70 and Crk on the activation stage of the T cells suggested that this transient event is regulated by a PTK that phosphorylates ZAP-70 and increases its affinity to the Crk-SH2 domain. A PTK candidate for this function is the Src family member, Lck, which was shown to function as an upstream regulator of ZAP-70 (43). In order to test the involvement of Lck in the regulation of ZAP-70 association with Crk, we used the JCaM1 cells, a Jurkat mutant subline that is genetically deficient in Lck. Anti-ZAP-70 blot (Fig. 5B, upper panel) demonstrated that JCaM1 cells possess ZAP-70 protein at levels indistinguishable from those observed in wild-type Jurkat cells. Absence of Lck resulted in a complete lack of activation-dependent tyrosine phosphorylation of ZAP-70 (Fig. 5B, upper panel), which correlates with the lack of association of ZAP-70 with GST-Crk-II (Fig. 5A).

A second mutant Jurkat subline, termed J4501, that was tested lacked the CD45 protein tyrosine phosphatase and is impaired in TCR-linked signal transduction because of the critical role of CD45 in dephosphorylating essential effector molecules during the early phase of the activation response (44). Even though tyrosine phosphorylation levels of multiple protein bands were reduced or even completely absent in acti-
The results thus far indicated that the interaction between ZAP-70 and Crk is dependent on cell activation and tyrosine phosphorylation of ZAP-70 and mediated by the Crk-SH2 domain. However, it is yet unclear whether the two proteins interact via a direct physical contact or through a third party mediator molecule which associate simultaneously with ZAP-70 and Crk. To distinguish between these two possibilities, we used a far Western blot analysis (overlay assay) in which direct binding of soluble fusion proteins to immunoblotted ZAP-70 was tested. We found that GST fusion proteins containing either the entire Crk-II molecule (Fig. 7B) or the isolated Crk-SH2 domain (Fig. 7C) directly interacted with ZAP-70 from pervanadate-stimulated Jurkat T cells. Despite the fact that similar levels of ZAP-70 were immunoprecipitated from non-activated or activated Jurkat T cells (Fig. 7D), GST-Crk-II and GST-Crk-II-SH2 interacted with ZAP-70 from activated but not from non-activated T cells (Fig. 7B and C). Neither Crk-SH3(N) nor Crk-SH3(C) interacted with ZAP-70 in this assay (not shown). The Crk-associated ZAP-70 from Activated Jurkat T Cells Is Enzymatically Active—The analysis of ZAP-70-deficient human SCID patients has established that ZAP-70 is critical for T cell activation (45–47). ZAP-70 appears to be inactive in resting cells and, upon TCR engagement, undergoes tyrosine phosphorylation and activation. In order to determine whether the Crk-associated ZAP-70 is enzymatically active, we immunoprecipitated Crk, and ZAP-70 for comparison, from activated Jurkat T cells and performed an immune complex kinase assay on the precipitate. A 70-kDa radiolabeled protein band was observed in both ZAP-70 and Crk immunoprecipitates (Fig. 8A, upper panel), suggesting the presence of catalytically active autophosphorylating ZAP-70 in the Crk immunoprecipitate. Furthermore, the Crk co-immunoprecipitating kinase phosphorylated a ZAP-70-specific exogenous substrate, CBF3 (Fig. 8A, lower panel). Proteins that were pulled down from a lysate of activated Jurkat cells, using bead-immobilized GST-Crk-SH2.
fus protein, exhibited a similar phosphorylating activity (Fig. 8B), indicating that the Crk-SH2 domain-associated ZAP-70 was enzymatically active. As expected, the active ZAP-70 interacted with the GST-ZAP-70-SH2 domains, which served as a positive control. This was due to the ability of the tyrosine-phosphorylated TCR-ζ-chain, which possesses three tandem binding sites for ZAP-70, to interact simultaneously with the fusion protein and with Jurkat cell-derived endogenous ZAP-70. In contrast, GST alone or GST-Grb2-SH2, which cannot bind ZAP-70, did not exhibit phosphorylation of either the 70- or the 42-kDa (CBF3) protein bands (Fig. 8).

Predominant Association of ZAP-70 and Crk Occurs at the Membrane Fraction—The transient activation of ZAP-70, which follows triggering of the TCR, is correlated with its translocation to the cell membrane and association with tyrosine-phosphorylated ITAM sequences on the TCR-ζ-chain and CD3 subunits (4). The association of ZAP-70 with Crk occurred only in activated T cells following phosphorylation of ZAP-70 on tyrosine residues, suggesting that this event may also take place at the level of the plasma membrane. We therefore fractionated lysates of activated Jurkat T cells and determined the presence of ZAP-70 in general, and tyrosine-phosphorylated ZAP-70 in particular, in each fraction. We also tested the ability of these molecules to associate with the GST-Crk-SH2 fusion protein. As previously, the association of ZAP-70 with the Crk-SH2 domain was found to be dependent on cell activation (Fig. 9). Furthermore, although similar levels of tyrosine-phosphorylated ZAP-70 were observed in the cytosol and particulate fractions, association with the Crk-SH2 domain occurred predominantly at the particulate fraction.

**DISCUSSION**

Crk proteins have been implicated in signaling cascades that are linked to various cell-surface receptors, including the antigen receptors on B (16–18) and T (19–22) lymphocytes. Engagement of the BCR in RAMOS cells induced the association of tyrosine-phosphorylated Cas and Cbl with the Crk-SH2 domain, predominantly in the particulate fraction of activated B cells (16). In addition, Vav was inducibly associated with Crk in BCR-stimulated tonsilar B cells (17), and C3G, which catalyzes guanosine triphosphate (GTP) exchange on Rap1 (17, 25), constitutively associated with the Crk SH3 domain. Since C3G functions as a negative regulator of Ras (48), it has been suggested that Crk proteins function by down-regulating BCR-induced Ras-dependent signaling pathways.

Analysis of T lymphocytes revealed that Crk proteins associate with Cbl (19, 20) and Cas-L (49) in an activation-dependent manner and with C3G in a constitutive manner (50). The latter study also suggested that Rap1, which operates downstream of C3G, functions as a negative regulator of the TCR-mediated IL-2 gene transcription and thereby contributes to the maintenance of T cell anergy (50).

Our present findings demonstrate that T cell-derived Crk proteins undergo cell activation-dependent association with the ZAP-70 PTK. Association between the two molecules requires tyrosine phosphorylation of ZAP-70 and is mediated by direct physical contact between the Crk-SH2 domain and ZAP-70 phosphotyrosyl-containing sequences. We also found that binding of ZAP-70 to adapter proteins in T cells is selective and...
The Crk-associated ZAP-70 from activated Jurkat T cells is enzymatically active. Jurkat cells (4×10^7/group) were incubated with 1% pervanadate for 30 min at 37 °C followed by lysis and incubation of the lysate with the indicated protein A-agarose-bound Abs (A) or the indicated bead-immobilized GST fusion proteins (B). After 1 h incubation on a rotator at 4 °C, the beads were spun down, washed three times in lysis buffer, and subjected to immune complex kinase assay in the presence of [γ-32P]ATP, with (A, lower panel) and without (A, upper panel) 1 μg of CFB3 as a substrate. Samples were boiled for 5 min and subjected to SDS-PAGE under reducing conditions, and radioactive protein bands were visualized by autoradiography. Molecular size markers (in kilodalton) are indicated on the right, and the positions of radiolabeled protein bands corresponding to ZAP-70 and CFB3 are indicated on the left. Results are representative of three experiments.

ZAP-70 PTK is a key regulator of signaling in T lymphocytes, and its absence leads to a complete loss of the ability of T cells to respond to antigenic stimuli (45–47). Activity of ZAP-70 is directly regulated by the TCR which, upon engagement with a major histocompatibility complex-bound peptide antigen, undergoes phosphorylation by tyrosine kinases. Phosphorylation occurs at ITAM sequences in the cytoplasmic tails of the TCR-ζ-chain and CD3 subunits (53, 54). These phospho-ITAMs function as scaffolds for catalytically inactive ZAP-70, and direct physical binding occurs by a cooperative interaction of the ZAP-70 tandem SH2 domains with doubly tyrosine-phosphorylated ITAMs (43, 55). Whereas TCR-bound ZAP-70 is found in both agonist-responsive active T cells and antagonist-responsive anergic T cells, ZAP-70 undergoes tyrosine phosphorylation and activation only upon productive stimulation with an agonistic peptide antigen (56, 57). Further studies demonstrated that Lck is the putative ZAP-70-phosphorylating kinase (49) and that a single tyrosine residue in the activation loop of the ZAP-70 kinase domain (Tyr493) is critical for activation of the enzyme. Therefore, phosphorylation of Tyr493 enables opening of the activation loop of ZAP-70 which becomes more accessible to potential cellular substrates (5, 6).

The findings that Crk association with ZAP-70 is activation-dependent (Figs. 1, 3, and 4), together with the observation by Iwashima et al. (43) that Lck is the upstream regulator of ZAP-70, raised the question whether Lck is involved in the regulation of ZAP-70 association with Crk. The analysis of the Jurkat mutant subline, JCaM1, which is deficient in Lck, confirmed our hypothesis and demonstrated that ZAP-70-Crk association occurs in activated wild-type Jurkat cells but not in activated Lck-deficient mutant cells (Fig. 5).

It is well established that T cell activation induces the association of ZAP-70 with TCR pITAMs, but the fraction of the total ZAP-70 proteins associated with pITAMs and the subcellular distribution of ZAP-70 in resting cells is still unclear. An overexpressed GFP-ZAP-70 was found to be diffusely distributed throughout the quiescent cell, and it accumulated at the plasma membrane upon cell activation (58). However, a large amount of ZAP-70 resided in the nucleus of quiescent cells and, upon cell activation, underwent tyrosine phosphorylation (58). In another study, the endogenous ZAP-70 was found to localize to the cell cortex in a diffuse band and exhibit similar distribution following TCR stimulation (59). This pattern of distribution was dependent on the ZAP-70 kinase domain and not on the SH2-containing regulatory region. Our studies demonstrate that about one-quarter of the cellular ZAP-70 is found in the membrane fraction of non-activated Jurkat cells and that a significant fraction of the cytosolic ZAP-70 translocate to the membrane fraction upon cell stimulation. Cell activation resulted in tyrosine phosphorylation of ZAP-70 at both the cytosolic and membrane fractions. However, the predominate tyrosine-phosphorylated ZAP-70 that interacted with Crk-SH2 was membrane-derived. The results suggest differences in the ability of phosphotyrosyl residues of cytosolic and membrane-
derived ZAP-70 to interact with the Crk-SH2 domain. This may also reflect diversity in the conformation of the molecules or differential association of ZAP-70 with other cell components. Alternatively, it is possible that despite the fact that the phosphorylation levels of membranous and cytosolic ZAP-70 are similar, the sites of phosphorylation of ZAP-70 in the two cellular fractions may differ, and only membranous ZAP-70 undergoes phosphorylation at regions that function as binding sites for the Crk-SH2 domain.

Recent findings demonstrated that the plasma membrane of cells contain detergent-insoluble rafts enriched in glycolipids and phosphatidylinositol-anchored membrane proteins (60, 61). T cell activation results in membrane compartmentalization and accumulation in the rafts of activated TCR and associated signal-transducing molecules (60, 62). The formation of activation-dependent clusters of TCR-containing multimolecular complexes at the site of interaction of T cells with the antigen-presenting cells has also been confirmed by fluorescence digital imaging (3). It is interesting to note that this process is followed by translocation of tyrosine-phosphorylated ZAP-70 to the rafts (59), questioning the possibility that the mechanism of translocation may involve, or even require, the direct interaction of phospho-ZAP-70 with Crk proteins.

Determination of sequence specificity of the peptide-binding sites of various SH2 domains demonstrated that the Crk-SH2 prefer sequences with a general motif pYXXP or, more specifically, pYDHP (where pY indicates phosphotyrosine) (63). Although an identical sequence has not been found in ZAP-70, the current data indicate that ZAP-70 phosphorylation sites for the Crk-SH2 domain. This may be involved in signaling pathways leading to T cell apoptosis, especially downstream of the TCR suggests that Crk may be in- volved in signaling pathways leading to T cell apoptosis, especially after strong activation conditions, such as those evoked by pervanadate.

The association of ZAP-70 with Crk may serve additional functions in activated T cells. Thus, simultaneous association of Crk with ZAP-70 and a ZAP-70-specific substrate will ensure high and efficient phosphorylation of substrates, even those that occur at low abundance. Studies of Src family members and Abl PTKs have shown that the SH2 domain of these PTKs preferentially bind phosphotyrosyl-containing sequences that are not phosphorylated by their own catalytic domain (66, 67). This may lead to consequent phosphorylation of the substrate proteins. However, this does not apply to ZAP-70 in which the tandem SH2 domains and the catalytic domain interact with completely different sequences (41). Therefore, it is possible that ZAP-70 interaction with Crk serves as a mechanism by which the Crk-SH3 domain selects and/or restricts the putative ZAP-70 substrates which may co-cluster with and become vulnerable to phosphorylation by ZAP-70. It is also possible that interaction of a ZAP-70-bound Crk protein with cytoskeletal elements via the SH3 domain, or other compartmentalized components in the cell, will permit anchoring of ZAP-70 and increase its concentration at selected subcellular locations where a critical minimal number of PTK molecules is required for signaling. Finally, another potential role for the interaction of tyrosine-phosphorylated ZAP-70 with the Crk-SH2 domain may be maintaining the enzyme as a phosphoprotein in its catalytically active state by protecting it from dephosphorylation by protein tyrosine phosphatases.

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ZAP-70-Crk Interaction in Activated T Cells

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