T Cell Activation Induces Direct Binding of the Crk Adapter Protein to the Regulatory Subunit of Phosphatidylinositol 3-Kinase (p85) via a Complex Mechanism Involving the Cbl Protein*

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Sigal Gelkop, Yael Babichev, and Noah Isakov‡
From the Department of Microbiology and Immunology, Faculty of Health Sciences, and the Cancer Research Center, Ben Gurion University of the Negev, Beer Sheva 84105, Israel

The Crk adapter proteins are assumed to play a role in T lymphocyte activation because of their induced association with tyrosine-phosphorylated proteins, such as ZAP-70 and Cbl, and with the phosphatidylinositol 3-kinase regulatory subunit, p85, following engagement of the T cell antigen receptor. Although the exact mechanism of interaction between these molecules has not been fully defined, it has been generally accepted that Crk, ZAP-70, and p85 interact with tyrosine-phosphorylated Cbl, which serves as a major scaffold protein in activated T lymphocytes. Our present results demonstrate a cell activation-dependent reciprocal co-immunoprecipitation of CrkII and p85 from lysates of Jurkat T cells and a direct binding of CrkII to p85 in an overlay assay. The use of bead-immobilized GST fusion proteins indicated a complex mechanism of interaction between CrkII and p85 involving two distinct and mutually independent regions in each molecule. A relatively high affinity binding of the CrkII-SH3(N) domain to p85 and the p85-proline-B cell receptor-proline (PBP) region to CrkII was observed in lysates of either resting or activated T cells. Direct physical interaction between the CrkII-SH3(N) and the p85-PBP domain was demonstrated using recombinant fusion proteins and was further substantiated by binding competition studies. In addition, immobilized fusion proteins possessing the CrkII-SH2 and p85-SH3 domains were found to pull down p85 and CrkII, respectively, but only from lysates of activated T cells. Nevertheless, the GST-CrkII-SH2 fusion protein was unable to mediate direct association with p85 from lysates of either resting or activated T cells. Our results support a model in which T cell activation dependent conformational changes in CrkII and/or p85 promote an initial direct or indirect low affinity interaction between the two molecules, which is then stabilized by a secondary high affinity interaction mediated by direct binding of the CrkII-SH3(N) to the p85-PBP domain.

Physiological activation of T lymphocytes is initiated by T cell antigen receptor (TCR) interaction with a major histocompatibility complex-bound peptide antigen on the surface of antigen-presenting cells. Engagement of the TCR triggers multiple intracellular biochemical events that operate in a sequence and transduce the extracellular signal into different subcellular compartments. This results in the induction of transcription of selected genes and translation of their corresponding mRNA, reorganization of cytoskeletal elements, modulation of expression of cell surface receptors, and secretion of lymphokines and other soluble immune mediators. These responses may lead to T cell proliferation, apoptosis, anergy, or differentiation into distinct types of effector cells. Determination of the specific differentiation pathway of engaged T cells is dependent upon the structure of the presented peptide antigen, and the nature of simultaneously engaged cell surface receptors.

Individual components of the TCR-linked signaling pathways are physically separated in resting cells, and they reassemble into functional complexes upon receptor engagement (1, 2). This mechanism enables the recruitment of enzymes and other effector molecules to specific subcellular compartments, predominantly the lipid rafts (3) at the site of the immunological synapse (4–6). Because this process occurs in a temporally and spatially regulated manner, it ensures an efficient signaling, which, under appropriate conditions that follow a productive T cell interaction, lead to cell activation and differentiation.

Adapter molecules such as Grb2, Shc, and Crk possess multiple protein-protein interaction domains, which allows them to play a critical role in the assembly of multimolecular activation complexes (7, 8). Many of these adapter proteins are involved in the regulation of cell growth and differentiation by coupling proximal biochemical events, initiated by cell surface receptor engagement, with distal signal transducing pathways. The Crk adapter protein was originally identified as a product of the v-crk oncogene in the avian retrovirus CT10 (9) and shortly thereafter in the ASV-1 avian retroviral isolate (10). It was later found to be encoded by a cellular proto-oncogene (11), and it has since been established that Crk proteins are implicated in signaling pathways regulating cell growth (12), migration (13), differentiation (14, 15), and apoptosis (16). In addition, Crk was shown to be involved in signaling pathways linked to a wide range of membrane receptors including those of integrins (17), interleukins (18), and growth factors (18–20). Although

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‡ To whom correspondence should be addressed: Dept. of Microbiology and Immunology, Faculty of Health Sciences, Ben Gurion University of the Negev, P. O. Box 653, Beer Sheva 84105, Israel. Tel.: 972-7-647-7267; Fax: 972-7-647-7626; E-mail: noah@bgumail.bgu.ac.il.

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adapter proteins such as Grb2 and Shc were found to be a positive role in the regulation of T cell activation, other adapter proteins, including Crk and Cbl, were predominantly implicated in the negative regulation of TCR-linked signaling pathways. It is possible therefore that Crk and Cbl regulate the termination of TCR-linked activation signals or are involved in biochemical processes promoting T cell suppression and induction of immune anergy (21).

Ligation of the TCR in the absence of appropriate co-stimulatory signals results in a state of long-term T cell anergy (22–25). The molecular mechanisms that maintain immunological tolerance in vivo are poorly understood, but several studies support a role for adapter proteins in the inductive phase (8, 26).

For example, Crk proteins, which are involved in signal transduction from antigen receptors in B (17–19) and T (20–23) lymphocytes, were found to associate with other negative regulatory proteins in anergic but not in responsive T cells (26). One of these proteins was identified as Cbl, which upon overexpression can abrogate TCR-dependent activation of AP-1 (27) and decrease the active pool of Syk/ZAP-70 PTK family members (28–30). Furthermore, Cbl knockout resulted in enhanced T cell signaling (31), whereas overexpression of a mutated oncogenic form of Cbl, Cbl70Z, increased the TCR-induced NF-AT-luciferase reporter activity (32, 33). It is assumed that Grb2 association with Cbl precedes Grb2 binding to Sos, thereby preventing the recruitment of Sos to the plasma membrane and the subsequent activation of Ras (34). This model is further supported by the findings that TCR ligation and the subsequent tyrosine phosphorylation of Cbl promotes Grb2 dissociation from Cbl and the recruitment of other effector molecules, including phostidylidyinositol 3-kinase (PI3K) (35, 36) and members of the Crk adapter protein family (35, 37, 38).

A second Crk-associated protein in anergic T cells was identified as the guanine nucleotide exchange factor, C3G (26), which catalyzes the exchange of GDP-to-GTP in Rap-1, an adapter protein family (35, 37, 38).

The Cbl-associated PI3K may also be involved in the negative regulation of T cell responsiveness, because overexpression of a mutated, constitutively active form of PI3K increased NF-AT activity in TCR-stimulated T cells (47). We now demonstrate that a direct physical interaction exists between Crk and the PI3K regulatory subunit, p85, in activated T cells and further characterizes the mechanism of this interaction.

**Experimental Procedures**

**Reagents**—Phytohemagglutinin, histopaque-1077, glutathione S-transferase (GST), aprotinin, leupeptin, Triton X-100 and L-α-phosphatidylinositol were from Sigma. AEBSF was from ICN Biomedicals, Inc. (Aurora, OH). Human recombinant interleukin-2 was a gift from Hoffmann-La Roche. Nitrocellulose membranes were from Schleicher & Schuell, ECL and protein A-Sepharose were from Amersham Pharmacia Biotech, and [γ-32P]ATP (3000 Ci/mmol) was from Rotem Industries, Ltd. (Beer Sheva, Israel).

**Antibodies**—Anti-phosphotyrosine (4G10) and anti-p85 mAbs were from Upstate Biotechnology Inc. (Lake Placid, NY), a mouse mAb specific to Crk-I/Crk-II was from Transduction Laboratories (Lexington, KY), and anti-Cbl and anti-GST mAb were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Affinity-purified anti-human CD3 mAb was purchased from Immunotech (Cryenton, CO) and CD28 from ATCC, Manassas, VA, and the TCRβ chain-specific mAb C305 was a gift of Dr. A. Weiss (University of California, San Francisco, CA). Rabbit anti-ZAP-70 polyclonal anti-serum was raised against GST fusion protein containing amino acids 255–345 of human ZAP-70, a gift of Dr. J. B. Bolen (Bristol-Myers Squibb Co.) (48). Horseradish peroxidase (HRP)-conjugated deep anti-mouse, or donkey anti-rabbit, anti-mouse Abs, and HRP-conjugated protein A were from Amersham Pharmacia Biotech.

**GST Fusion Proteins**—A pGEX plasmid containing the GST-CrkL was a gift of Dr. B. Druker (Oregon Health Sciences Center, Portland, OR), and plasmids containing GST fused to Crk-I, Crk-II, or individual Crk-II domains were gifts of Dr. M. Matsuda (National Institute of Health, Tokyo, Japan). Plasmids containing GST fused to the full-length p85 or individual regions of p85, as indicated above, were gifts of Dr. J. Bertoglio (INSERM Unit 461, Paris, France). pGEX plasmids were used to transform Escherichia coli DH5α cells (Life Technologies, Inc.). After induction of expression with 0.1 mm isopropyl-1-thio-β-D-galactopyranoside (Promega, Madison, WI) for 2–4 h, the bacterial pellets were resuspended in a lysis buffer containing 50 mm Tris-HCl, pH 8.0, 100 mm NaCl, 1 mm phenylmethylsulfonyl fluoride, and 1% Triton X-100 and were further disrupted by sonication. Following centrifugation at 10,000 × g for 20 min, the induced proteins were adsorbed to bead-immobilized glutathione. Soluble GST fusion proteins were obtained by elution with 2 mm reduced glutathione (Roche Molecular Biochemicals) in 50 mm Tris-HCl, pH 8.0.

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 3 h. The beads were then washed three times in a 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 were maintained at a logarithmic growth phase in complete RPMI (1640 supplemented with 5% heat-inactivated fetal calf serum, 2 mm l-glutamine, 10 mm HEPES, 40 ng/ml bovine insulin, 50 units/ml penicillin, 50 μg/ml streptomycin (all from Biological Industries, Beit Haemek, Israel), and 5 × 10–6 M β-mercaptoethanol (Sigma)) in 75 cm2 growth area tissue culture flasks (CellCult, Sterilin Limited, Feltham, UK) in an atmosphere of 7.5% CO2 at 37 °C. For Expt 1, peripheral blood lymphocytes (PBL) were obtained by histopaque-PBS centrifugation of heparinized blood from healthy volunteers. Enriched proliferation of preactivated and rested PBL T cells were obtained by cell culture (1 × 106/ml) in complete RPMI containing 10% fetal calf serum in the presence of 5 μg/ml phytohemagglutinin in 75 cm2 growth area tissue culture flasks (50 ml/flask). Human recombinant interleukin-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 interleukin-2 (20 units/ml) every 2 days. Jurkat or PBL T cells (10 × 106/100 μl) were stimulated with freshly prepared 1% pervanadate (10 μM Na3VO4 containing 1% H2O2) for 30 min at 37 °C. Ab-mediated cross-linking of the TCR/CD3 was performed by incubating Jurkat or PBL T cells (10 × 106/100 μl) with C305 mAb (100 μg/ml) or OKT3 mAb (100 μg/ml), respectively, for 30 min on ice. A secondary cross-linking rabbit anti-mouse Ig Ab was then added for 10 min on ice followed by cell transfer to 37 °C and incubation for the indicated time interval.

**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 leupeptin and aprotinin, 2 μg AEBSF, 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 used for immunoprecipitation studies or mixed with equal volumes of 2× SDS sample buffer, vortexed, incubated at 100 °C for 5 min, and analyzed by SDS-PAGE. Cytoplasmic and particulate fractions were prepared by resuspending the cell lysates in buffer A (20 mm Tris-HCl, pH 7.5, 2 mm EDTA, 0.5 mm EGTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 μg AEBSF) 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 centrifuged 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% Downloaded from http://www.jbc.org/ by guest on July 24, 2018
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 1 h at 4 °C. Excess Abs were removed by three 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 then fractionated by SDS-PAGE and immunoblotted with specific Abs.

Electrophoresis and Immunoblotting—Samples of cell lysates, GST fusion proteins, 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 nitrocellulose membranes (Schleicher & Schuell) at 100 V for 45 min in a Bio-Rad Mini Trans-Blot transfer cell. After 1 h of 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. Immunoreactive proteins were visualized using an ECL reagent and autoradiography.

Far Western Analysis—To determine direct binding of either Crk-II or selected Crk-II domains to the electrophoresed nitrocellulose-bound proteins, SDS-PAGE was performed as described above followed by blotting onto an Immobilon-N (Millipore) membrane. Protein denaturation was performed by incubation of the membrane for 1 h at 25 °C in denaturation buffer (7 M guanidine-HCl, 50 mM Tris-HCl, pH 8.3, 50 mM dithiothreitol, 2 mM EDTA, 0.25% (w/v) nonfat dry milk). Protein renaturation was performed by several washes of the membrane in cold renaturation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM dithiothreitol, 2 mM EDTA, 0.1% (w/v) Nonidet P-40, 0.25% (w/v) nonfat dry milk) and overnight incubation in renaturation buffer at 4 °C. After blocking the membrane with phosphate-buffered saline containing 3% bovine serum albumin and 0.1% Tween 20, it was incubated overnight at 4 °C with a blocking buffer containing 10 μg/ml the indicated GST fusion protein or GST, used 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 then by ECL development.

RESULTS

CrkII and the PI3K Regulatory Subunit, p85, Co-immunoprecipitate from Lysates of Activated Jurkat T Cells or Human Peripheral Blood T Lymphocytes—To identify Crk-binding proteins in activated T cells, we used GST-CrkII fusion proteins in a pull-down assay and searched for tyrosine-phosphorylated CrkII-binding proteins in a lysate of activated Jurkat T cells. We found that GST-CrkII pulled down a 70-kDa protein identified as ZAP-70 (47) and, occasionally, a faint protein band that this protein band corresponds to the PI3K regulatory subunit, p85 (not shown). To further analyze the putative association of p85 with CrkII, we stimulated Jurkat T cells either with pervanadate or by cross-linking of the TCR with the C305 mAb and tested whether p85 co-immunoprecipitates with CrkII. We found (Fig. 1A) that anti-Crk mAbs co-immunoprecipitated p85 from lysates of activated but not resting Jurkat T cells. The association of p85 with CrkII in activated T cells was further substantiated by the occurrence of reciprocal co-immunoprecipitation (Fig. 1B), even though a low level of constitutive association could be observed in lysates of resting cells. To further analyze whether cell activation-induced p85 association with CrkII represents a general physiological phenomenon, we repeated the experiment using human PBL as a source of T cells. The results (Fig. 1C) confirmed that p85 co-immunoprecipitates with CrkII from lysates of both pervanadate- and anti-CD3 (OKT3)-treated PBL, again demonstrating that a small fraction of these proteins are associated in a constitutive manner.

The time course of the effect of TCR cross-linking with C305 clonotypic mAbs on p85 association with CrkII demonstrated maximal binding within 2 min of activation, which persisted for 10 min and then gradually declined (Fig. 2). Striping and reblotting of the nitrocellulose membrane with anti-phosphotyrosine mAbs demonstrated that CrkII and, to a much lower extent, p85 underwent tyrosine phosphorylation (Fig. 2C). However, phosphorylation of both proteins, which peaked at 10 min post-stimulation, was hardly detectable after 2 min, demonstrating a disproportional relationship between the degree of phosphorylation of CrkII and/or p85 and the formation of p85-CrkII complexes. Thus, this association may either be independent of the tyrosine phosphorylation state of these proteins, or the low phosphorylation levels (which are below the sensitivity of the immunoblotting system) may be sufficient for mediating the observed p85-CrkII interaction.

Additional studies demonstrated that immunoprecipitates of CrkII from a lysate of pervanadate-stimulated Jurkat T cells, similar to p85 immunoprecipitates, exhibited in vitro PI3K activity (not shown) suggesting presence of the PI3K p110 catalytic subunit in the complex.

The Predominant Association of p85 with Crk Occurs at the
**Cytosolic Cell Fraction—**Immunoblot analysis of cytosolic and particulate fractions of Jurkat T cells revealed that more than 90% of the Crk molecules reside in the cytosol of the cell (see Fig. 3B). Accordingly, we found that the predominant association between Crk and p85 occurs at the cytosolic fraction (Fig. 3A). Analysis of Crk immunoprecipitates from the particulate fraction (right panel, Fig. 3B) revealed two major protein bands of ~40 and ~42 kDa in the lysate of resting cells and a third band with a slower mobility in the lysate of pervanadate-treated cells. Similar results have been reported in HEK-293 cells stimulated with insulin-like growth factor-I (49). Because mobility shift of a protein may reflect post-translational modification by a PTK, we tested whether the up-shifted CrkII protein band is tyrosine-phosphorylated. Reblotting of the nitrocellulose membrane with 4G10 mAbs substantiated this assumption and demonstrated that the upper protein band of CrkII in both the pervanadate- and C305-stimulated Jurkat cells is tyrosine-phosphorylated (Fig. 3). In addition, the 40- and 42-kDa CrkII protein bands in pervanadate-treated cell lysate also reacted with 4G10, suggesting that under these conditions CrkII undergoes tyrosine phosphorylation (or an additional, different post-translational modification) at multiple sites. Although a small amount of the nonphosphorylated 40-kDa form of CrkII was found in the particulate fraction of Jurkat T cells, pervanadate also induced membrane translocation of a 42-kDa tyrosine-phosphorylated form of CrkII.

**Binding of p85 to Crk Is Mediated by Direct Physical Interaction—**The apparent dependence of the p85 interaction with CrkII on the activation state of T cells suggested that this transient event is regulated by PTKs, which phosphorylate one or more of the proteins involved in the complex formation. However, the prospect that p85 interaction with CrkII precedes tyrosine phosphorylation of both proteins (see Fig. 2) implies that additional tyrosine-phosphorylated proteins may be involved in mediating the intermolecular interaction. This hypothesis is supported by studies showing that p85 and Crk co-immunoprecipitate with tyrosine-phosphorylated Cbl from activated T cells (12, 35, 37, 38, 50), alluding to the possibility that p85 and Crk interact with Cbl in a mutually non exclusive mechanism.

To determine whether p85 and Crk can mediate a direct physical interaction, we performed an overlay assay and tested the binding capability of a soluble GST-CrkII fusion protein to immunoblotted p85. A significant binding of GST-CrkII to p85 was observed in p85 immunoprecipitates from both resting and activated T cells (Fig. 4A). This observation and the low levels of tyrosine phosphorylation of p85 observed in activated T cells (Fig. 2) suggest that the CrkII-SH2 domain would not play a major role in binding to p85. This assumption was confirmed in a consecutive overlay assay using the GST-CrkII-SH2 fusion protein (Fig. 4B). Thus, although direct binding to tyrosine-phosphorylated ZAP-70 (Fig. 4F) occurred only in activated T cells and predominantly involved the Crk-SH2 domain (Fig. 4, A and B), binding of Crk to p85 occurred in activated as well as resting cells and involved the carboxyl-terminal non-SH2-containing region of Crk.
GST-p85 Increases after T cell Activation and Is Mediated by Two Independent Regions in the CrkII SH2 and SH3(N) Domains—To further analyze the role of individual domains of CrkII in binding to p85, we tested the ability of various GST-CrkII fusion proteins to pull down p85 from lysates of resting or pervanadate-treated Jurkat T cells. The full-length CrkII was found to pull down significant levels of p85 from lysates of resting Jurkat cells but was more efficient in the binding p85 of activated cells (Fig. 6). GST-CrkII-SH3(C) did not bind p85, whereas GST-CrkII-SH3(N) pulled down high levels of p85 from lysates of both resting and activated Jurkat T cells. In contrast, GST-Crk-SH2 pulled down relatively low levels of p85 but only from lysates of activated cells.

The results imply that at least two regions in the CrkII protein molecule are involved in the interaction with p85 and suggest that the Crk-II-SH2 preferentially or selectively interacts with p85 from activated T cells. In contrast, binding of the Crk-SH3(N) domain may function to stabilize the interaction and perhaps increase the overall binding affinity.

In Vitro Binding Affinity of CrkII from a T Cell Lysate to GST-p85 Increases after T cell Activation and Is Mediated by Two Independent Regions in the p85 Non-SH2-containing Amino Terminus—In an attempt to define the regions of p85 that mediate the interaction with CrkII, we performed a pull-down assay using various GST-p85 fusion proteins. The full-length p85 was found to pull down a significant amount of CrkII from a lysate of resting Jurkat T cells and about 4-fold excess of CrkII protein from a lysate of activated cells (Fig. 7, upper panel). Nevertheless, binding to CrkII was independent of the p85 tandem SH2 domains, since a GST fusion protein containing the p85 amino terminus (which includes the two SH2 domains) was completely ineffective in binding CrkII.

Two different regions in p85 that mediated in vitro binding to CrkII included the proline-BCR-proline (PBP) and the SH3 domains. Although GST-p85-PBP pulled down similar levels of CrkII proteins from lysates of resting and activated T cells, GST-p85-SH3 bound Crk almost exclusively in lysates of activated cells. These results demonstrate that p85 binding to CrkII is also mediated by two different regions. The p85-SH3 domain may determine the preferential association with p85 from activated T cells, whereas the p85-PBP region may function to stabilize this interaction and perhaps increase the overall binding avidity.
cell lysates (4/100 μg) were incubated with of GST or GST fusion proteins immobilized to glutathione-agarose beads. Bound proteins were subjected to SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose membranes. p85 proteins were then visualized by reaction with anti-p85 mAbs and development with an immunoperoxidase ECL detection system and autoradiography (with anti-p85 mAbs and development with an immunoperoxidase ECL detection system and autoradiography (upper panel)). The nitrocellulose membrane was then stripped and reblotted (lower panel) with Abs specific for Cbl. Molecular size markers (in kilodaltons) are indicated on the left. Arrowheads indicate the positions of immunoreactive CrkII and Cbl protein bands. Results are representative of three independent experiments.

**Fig. 7.** p85 binding to Crk is mediated by its non-SH2-containing amino terminal. Resting or pervanadate-treated (perVO₄) Jurkat cell lysates (4 × 10⁷ cell equivalent/group) were incubated with GST or GST fusion proteins containing the full-length p85 or the indicated regions of p85 immobilized to glutathione-agarose beads. Bound proteins were subjected to SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose membranes. CrkII proteins were then visualized by reaction with anti-Crk mAbs and development with an immunoperoxidase ECL detection system and autoradiography (upper panel). The nitrocellulose membrane was then stripped and reblotted (lower panel) with Abs specific for Cbl. Molecular size markers (in kilodaltons) are indicated on the left. Arrowheads indicate the positions of immunoreactive CrkII and Cbl protein bands. Results are representative of three independent experiments.

**Fig. 6.** In vitro association of p85 with GST-CrkII increases following cell activation and involves the CrkII SH2 and SH3(N) domains. Resting or pervanadate-treated (perVO₄) Jurkat cell lysates (4 × 10⁷ cell equivalent/group) were incubated with of GST or GST fusion proteins immobilized to glutathione-agarose beads. Bound proteins were subjected to SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose membranes. p85 proteins were then visualized by reaction with anti-p85 mAbs and development with an immunoperoxidase ECL detection system and autoradiography (upper panel). The nitrocellulose membrane was then stripped and reblotted (lower panel) with Abs specific for Cbl. Molecular size markers (in kilodaltons) are indicated on the left. Arrowheads indicate the positions of immunoreactive CrkII and Cbl protein bands. Results are representative of three independent experiments.
DISCUSSION

Crk proteins were shown to be involved in multiple signaling pathways linked to different cell surface receptors. Nevertheless, their mechanism of action in T lymphocytes following engagement of the TCR has not been studied thoroughly. In the present work we found that T cell activation results in CrkII association with the regulatory subunit of PI3K, as demonstrated by reciprocal co-immunoprecipitation studies. CrkII association with p85 reached its maximal level within 2 min of TCR cross-linking, and after about 10 min it started to decline gradually. Furthermore, T cell activation resulted in tyrosine phosphorylation of CrkII and, to a lesser degree, p85. However, maximal p85-CrkII interaction preceded the peak of phospho-
proteins. We found a direct binding of CrkII to p85, which, in contrast to the results of the immunoprecipitation, was independent of the activation state of the cells. Furthermore, direct binding to p85 appeared to be mediated by a non-SH2-containing region in CrkII. A pull-down assay using bead-immobilized fusion proteins indicated that the p85-binding region corresponds to the CrkII-SH3(N) and also demonstrated a lower binding affinity of the CrkII-SH2 to p85 in activated but not in resting T cells.

The observation that CrkII binding to p85 is mediated by a direct physical interaction is similar to the findings obtained with CrkL, a protein that is highly homologous to and a close relative of CrkII. In these studies, CrkL was found to mediate direct binding (via its SH3(N) domain) to p85 in steel factor-dependent manner. It is possible, however, that binding of the different forms of CrkII to isolated regions of recombinant p85 fusion proteins are not necessarily representative of all in vivo interactions between the endogenous CrkII and p85 native proteins.

Based on the present results we suggest that T cell activation that results in Cbl tyrosine phosphorylation at multiple sites creates two adjacent low affinity binding sites for the SH2 domains of Crk (possibly 774YDVP or 700YMTP; see Refs. 37 and 57) and p85 (731YEAM or 731YCEM; see Refs. 58–60). Binding of p85 to Cbl is then stabilized by a secondary intermolecular interaction involving the p85-SH3 domain and the Cbl-derived proline-rich motif, P58PPRPRLDDL (61, 62). The bimodal interaction of the two distinct p85 domains with Cbl induces a conformational change in the intermediate region of p85, the PBP proline-rich region, and positions it adjacent to the Cbl-bound CrkII to enable its interaction with the CrkII-SH3(N) domain. Because the predicted consensus region for the Crk-SH3(N) corresponds to PPXLPXK (62, 63), we assume that it interacts with the 299PPALPPK sequence in the PBP region of p85β, the predominant isoform of p85, which interacts with Cbl in activated Jurkat T cells (64).

Pull-down assays with GST-p85 fusion proteins revealed that two distinct regions mediate binding to CrkII. The p85-PBP region pulled down CrkII very efficiently from lysates of activated T cells, whereas p85-SH3 pulled down CrkII, almost exclusively, from lysates of activated cells. The observation that the p85-PBP domain can pull down CrkII but not the Cbl protein from a total cell lysate provides an additional support for the assumption that p85 can directly associate with CrkII in the absence of Cbl. This is further supported by the results in Fig. 9 showing the ability of p85 to co-immunoprecipitate with CrkII from a Cbl-depleted Jurkat cell lysate.

The involvement of p85-PBP and CrkII-SH3 in the reciprocal protein-protein interaction raised the possibility that these two regions bind to each other. This hypothesis was confirmed both by direct binding studies and by binding competition analysis. However, direct binding of p85-PBP to CrkII-SH3 was independent of the activation state of the T cells, in contrast to the results of the co-immunoprecipitation, which indicated that p85 association with CrkII occurs almost exclusively in activated T cells. Furthermore, although the GST-CrkII-SH2 was able to pull down p85 from a lysate of activated T cells, it was incapable of mediating direct binding. These results suggested the involvement of a third party molecule that functions as a link between p85 and CrkII.

An obvious candidate for mediating this linkage was the Cbl protein, which by itself is a major tyrosine-phosphorylated substrate in activated T cells and was shown to interact with multiple effector molecules, including Crk and p85, in an activation-dependent manner. We found that CrkII can associate directly with Cbl (via its SH2 domain) and that p85 can pull down Cbl from a lysate of activated T cells. In addition, Cbl associated with the p85-SH3 domain in lysates of both resting and activated T cells and with the p85-SH2 in a cell activation-dependent manner.

It is interesting to note that different regions of p85 pulled down distinct migratory forms of CrkII (Fig. 7, upper panel). For example, a fast migrating form of CrkII was associated with the p85-SH3 domain, whereas slower migrating forms of CrkII interacted with the p85-PBP region. The results suggest that post-translational modifications of CrkII, which are known to affect the protein migration rate on SDS-PAGE, also determine the ability of CrkII to interact with other molecules. Thus, the fastest migrating form of CrkII appears to be capable of interacting with the p85-SH3 (but not with the p85-PBP region), possibly via an indirect mechanism mediated by the simultaneous interaction of CrkII and p85 with tyrosine-phosphorylated Cbl. In contrast, the slow migrating forms of CrkII can bind to the p85-PBP region, apparently in a Cbl-independent manner. It is possible, however, that binding of the different forms of CrkII to isolated regions of recombinant p85 fusion proteins are not necessarily representative of all in vivo interactions between the endogenous CrkII and p85 native proteins.
p85-Crk Interaction in Activated T Cells

provide a good explanation for the SH3-mediated cell activation dependent interactions observed in our system.

The CrkII-SH3C appears to take no part in the formation of the Crk-Cbl-p85 trimolecular complex, suggesting that CrkI, which is devoid of a SH3C domain, can also be found in this complex. This assumption was confirmed in additional studies in which Crk-specific Abs (raised against the Crk amino terminus) detected a 28-kDa protein band that co-immunoprecipitated with Cbl. In contrast to CrkII, this protein band did not react with a CrkII-SH3C-specific Ab (not shown).

Previous studies in T cells (37) have indicated that Crk association with tyrosine-phosphorylated Cbl is mediated by the Rap1 guanine nucleotide exchange protein, C3G. These results raised the possibility that the Cbl-bound Crk protein can associate simultaneously with the C3G protein. It is possible therefore that the Cbl-bound Crk protein will lead to a association between C3G and Cbl was observed in Jurkat T cells only after Cbl overexpression (37). It is possible therefore that T cell activation and tyrosine phosphorylation of Cbl, which result in Cbl interaction with Crk and p85, will lead to a interaction of C3G with Cbl. In contrast to CrkII, this protein band did not react with Crk-specific Abs (raised against the Crk amino terminus) which is devoid of a SH3C domain, can also be found in this complex.

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T Cell Activation Induces Direct Binding of the Crk Adapter Protein to the Regulatory Subunit of Phosphatidylinositol 3-Kinase (p85) via a Complex Mechanism Involving the Cbl Protein

Sigal Gelkop, Yael Babichev and Noah Isakov

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