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TCR crosslinking promotes Crk adaptor protein binding to tyrosine-phosphorylated CD3ζ chain

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T cell antigen receptor (TCR) binding of a peptide antigen presented by antigen-presenting cells (APCs) in the context of surface MHC molecules initiates signaling events that regulate T cell activation, proliferation and differentiation. A key event in the activation process is the phosphorylation of the conserved tyrosine residues within the CD3 chain immunoreceptor tyrosine-based activation motifs (ITAMs), which operate as docking sites for SH2 domain-containing effector proteins. Phosphorylation of the CD3ζ ITAMs renders the CD3 chain capable of binding the ζ-chain associated protein 70 kDa (ZAP70), a protein tyrosine kinase that is essential for T cell activation.

We found that TCR/CD3 crosslinking in Jurkat T cells promotes the association of Crk adaptor proteins with the transiently phosphorylated CD3ζ chain. Pull down assays using bead-immobilized GST fusion proteins revealed that the Crk-SH2 domain mediates binding of phospho-CD3ζ. Phospho-CD3ζ binding is selective and is mediated by the three types of Crk, including CrkI, CrkII, and CrkL, but not by other SH2 domain-containing adaptor proteins, such as Grb2, GRAP and Nck.

Crk interaction with phospho-CD3ζ is rapid and transient, peaking 1 min post TCR/CD3 crosslinking. The results suggest the involvement of Crk adaptor proteins in the early stages of T cell activation in which Crk might help recruiting effector proteins to the vicinity of the phospho-CD3ζ and contribute to the fine-tuning of the TCR/CD3-coupled signal transduction pathways.

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1. Introduction

T lymphocytes play a central role in cell-mediated immune responses. They can mediate killing of virus infected and allogeneic cells (cytotoxic T cells; Tc), assist other immunocytes to differentiate into effector cells and respond to pathogen assaults (helper T cells; Th), downregulate responses of autoreactive T cells and terminate immune responses once antigens have been eliminated (regulatory T cells; Treg).

The ability of all types of T cells to respond to antigens depends on the T cell antigen receptors (TCRs), which are expressed on the outer cell surface and can recognize specific peptide antigens in the context of MHC proteins on the surface of antigen presenting cells (APCs). Engagement of the TCR triggers intracellular signaling cascades essential for the regulation of T development, activation, differentiation and acquisition of effector functions [1].

The TCR is composed of disulfide-linked TCRα-TCRβ chain heterodimers that are highly polymorphic and provide unique antigen specificity. In contrast, the TCR-associated CD3 complex operates to couple engaged receptors to downstream signal transduction

Abbreviations: C3G, Crk SH3 domain-binding guanine nucleotide-releasing factor; Crk, CT10 (chicken tumor virus number 10) regulator of kinase; CrkL, Crk-like; Grb2, growth factor receptor-bound protein 2; GST, Glutathione *S*-transferase; HRP, horseradish peroxidase; ITAM, immunoreceptor tyrosine-based activation motif; KLH, keyhole limpet hemocyanin; Lck, lymphocyte-specific protein tyrosine kinase; mAb, monoclonal antibody; Nck, non-catalytic region of tyrosine kinase adaptor protein 1; PTK, protein tyrosine kinases; pTyr-CD3ζ, tyrosine-phosphorylated CD3ζ; pTyr, phosphotyrosine; SH2, src homology 2; SHC, SH2 domain-containing-transforming protein C; TCR, T cell antigen receptor; WASP, Wiskott–Aldrich Syndrome protein; WCL, whole cell lysates; ZAP70, CD3ζ-chain associated protein kinase.

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pathways that regulate T cell behavior and responsiveness [1,2]. The CD3 includes non-covalently associated invariant heterodimers of γ-ε and δ-ε chains, and a single homodimer of ζ chains, all of which possess critically important immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tail [1,3,4]. Each ITAM possesses duplicate copies of the consensus sequence YXXL/I (where X is any amino acid). Upon receptor engagement, the two precisely spaced tyrosine residues undergo phosphorylation by Src-family protein tyrosine kinases (PTKs) and serve as docking sites for src homology 2 (SH2) domain-containing effector molecules that mediate and regulate signal delivery [5].

The CD3ζ chains (CD247) possess three copies of ITAM and their phosphorylation by Lck or Fyn creates multiple docking sites for the CD3ζ-chain associated protein kinase (ZAP70) that can interact with each of the three biphosphorylated ITAMs using its tandem SH2 domains [6–8]. Binding of ZAP70 to phospho-CD3ζ promotes the phosphorylation of ZAP70 and its conversion into a catalytically active enzyme [9,10], which further phosphorylates effector proteins along the signaling cascades [11].

We found that members of the CT10 (chicken tumor virus number 10) regulator of kinase (Crk) family of adaptor proteins, including CrkI, CrkII and CrkL can interact with ZAP70, following TCR ligation [12,13]. Crk binding to tyrosine-phosphorylated (pTyr)-ZAP70 promotes Wiskott–Aldrich Syndrome protein (WASP) recruitment to membrane lipid rafts, which mediates Arp2/3 complex-dependent actin polymerization [14]. In addition, Crk binding to Crk SH3 domain-binding guanine nucleotide-releasing factor (C3G) promotes T cell adhesion and migration to sites of inflammation [15,16].

The present study demonstrates that TCR ligation promotes the association of Crk adaptor proteins with the phospho-CD3ζ chain. Since the Crk-SH2 domain mediates binding to phospho-CD3ζ, the results suggest that Crk proteins may function as TCR-companion proteins that enable the temporal and transient recruitment of Crk-SH3 domain-binding proteins to the activated TCR.

2. Materials and methods

2.1. Reagents and antibodies

Mouse monoclonal antibodies (mAbs) specific for phosphotyrosine (pTyr) (4G10) were from Upstate Biotechnology Inc. (Lake Placid, NY), a mAb specific for Crk-I/Crk-II was from Transduction Laboratories (Lexington, KY), mAbs specific for CD3ζ and glutathione *S*-transferase (GST) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and a mAb specific for human CD3ε (UCHT1) was from DakoCytomation (Glostrup, Denmark). Affinity-purified mouse anti-CD3ε mAbs (OKT3) were prepared as described [15], and affinity purified Abs directed against phospho-Tyr^{142} within the third ITAM of human CD3ζ were prepared in rabbits by immunization with a 9-mer peptide (HDGLpYQGLS) conjugated to keyhole limpet hemocyanin (KLH) [17]. The ZAP70-specific antiserum was prepared by immunization of rabbits with GST-ZAP70-SH2(N + C) [13], while rabbit anti-CD3ζ antisera (#387) were obtained from Dr. L. E. Samelson (NIH, Bethesda, MD) and Dr. G. G. Garcia (University of Michigan, Ann Arbor, MI). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse and donkey anti-rabbit immunoglobulin Abs were from Amersham Pharmacia Biotech.

2.2. Cell lines, culture conditions, and cell stimulation

The Jurkat human leukemia T cell line (clone E6-1; ATCC® TIB-152™) and the ZAP70-deficient Jurkat-derived mutant, P116 cell line (provided by Dr. R. T. Abraham, Department of Immunology, Mayo Clinic, Rochester, MN) [18] were maintained at a logarithmic growth phase in complete RPMI (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 U penicillin/ml, 50 μg streptomycin/ml (all from Biological Industries, Beit Haemek, Israel) and 0.5 μM β_2-mercaptoethanol (Sigma-Aldrich)). Cell stimulation was performed by treatment with anti-CD3 Abs (OKT3) plus crosslinking with a secondary Ab for the indicated time intervals, or by 1% pervanadate treatment at 37 °C for 30 min.

2.3. Cell lysate preparation, immunoprecipitation and immunoblotting

Cell lysates were prepared by resuspension of cells in a lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml each of leupeptin and aprotinin, 2 mM AEBSF, and 1% Triton X-100), and 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, boiled for 5 min, and analyzed by SDS-PAGE. Immunoprecipitation was performed by incubation of Ab-coated beads with cell lysates for the indicated time periods at 4 °C. Immune complexes were precipitated by centrifugation followed by extensive washings in lysis buffer. Immunoprecipitates were then fractionated by SDS-PAGE and immunoblotted with the indicated Abs.

2.4. GST fusion proteins and pull-down assay

pGEX-5X vectors were from Amersham Pharmacia Biotech, and pGEX plasmids encoding GST-Crk fusion proteins and CrkII individual domains were gifts of M. Matsuda (National Institute of Health, Tokyo, Japan). A plasmid encoding GST-Grb2 (growth factor receptor-bound protein 2) was a gift of P. P. Di Fiore (University of Milan, Italy), a plasmid encoding GST-GRAP (Grb2-related adaptor protein) was a gift of G.-S. Feng [19], a plasmid encoding GST-SH2-SHC (SH2 domain-containing transforming protein) was a gift of G. Baumann [20], a plasmid encoding GST-NCK (non-catalytic region of tyrosine kinase adaptor protein 1) was a gift of K. C. Robbins [21], and a plasmid encoding GST-ZAP70-SH2(N + C) was a gift of L. E. Samelson (National Institutes of Health, Bethesda, MD).

Bacterial expression vectors were transformed into Escherichia *Coli* DH5α™ competent cells and GST-fusion proteins were prepared as described [13]. Pull-down assays were performed by incubation of bead-immobilized GST or GST fusion proteins (5 μg/sample) with cell lysates at 4 °C on a rotator for 4 h. The beads were washed (×3) in lysis buffer and bound proteins were eluted and subjected to SDS-PAGE under reducing conditions, followed by immunoblotting.

3. Results and discussion

3.1. TCR/CD3 stimulation of Jurkat T cells promote the association of Crk adaptor proteins with tyrosine-phosphorylated CD3ζ

Crk proteins, similar to other SH2 domain-containing adaptor proteins, utilize their SH2 domain to transiently interact with tyrosine-phosphorylated proteins in many cell types, including T lymphocytes. Previous studies demonstrated that T cell activation promotes the association of Crk adaptor proteins with tyrosine-phosphorylated proteins, such as Cbl, p85, and ZAP70 [22,23]. Using bead-immobilized GST-Crk fusion proteins in pull-down assays, we observed a consistent association of Crk with a low molecular mass tyrosine phosphorylated protein in lysates of activated, but not resting Jurkat T cells, and suspected that this protein might

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