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A role of kinase inactive ZAP-70 in altered peptide ligand stimulated T cell activation

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Abstract

T cell activation signals induced by altered peptide ligands (APLs) are different from those induced by the original agonistic peptide. The characteristics of the former are partial phosphorylation of TCR-ζ and no tyrosine-phosphorylation of ζ-associated protein-70 (ZAP-70). To analyze further those signaling pathways, we introduced a dominant negative (DN) form of ZAP-70 into a human $CD4^+$ T cell clone in which fully and partially agonistic peptide ligands have been well characterized. We found that some over-expressed partially agonistic ligands (OPALs) induced T cell responses without tyrosine-phosphorylation and kinase activation of ZAP-70. However, those responses were inhibited in T cells expressing DN ZAP-70, which could associate with partially phosphorylated TCR-ζ. In OPAL-stimulated T cells, PLC-γ1 was phosphorylated and it was suppressed by DN ZAP-70 expression, suggesting that the ZAP-70-TCR-ζ association mediates the activation of PLC-γ1 leading to T cell responses even in the absence of kinase activation of ZAP-70.

Keywords: Altered peptide ligand; TCR-ζ; ZAP-70; Phospholipase Cγ1; T cell activation

The engagement of the TCR with an antigenic peptide bound to class II MHC molecules activates the intracellular signaling cascade of biochemical events that trigger cytokine production, changes in the expression levels of cell surface molecules, and cell proliferation. Early signal transduction through the TCR is initiated by the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR-ζ chains by Src kinases [1–3]. Subsequently the doubly phosphorylated ITAMs of TCR-ζ chains provide binding sites for ZAP-70 through the interaction of its tandem Src homology 2 (SH2) domains to recruit ZAP-70 to the TCR-ζ [4–7]. The recruitment and activation of ZAP-70 molecules contribute to the activation of a cascade of downstream signals that are crucial for the initiation of cellular responses.

Altered peptide ligands (APLs), which have modifications in the original antigenic peptide, can be divided into different classes based on the potency for the induction of T cell responses; full agonist, partial agonist, and antagonist [8–11]. Cytokine production [12–15], the up-regulation of some cell surface molecules [16–18], and down-modulation of TCR [19–22] correlated with the capacity of each APL to induce TCR signaling. The difference in the TCR signal transduction between partial agonists and full agonists is characterized by the phosphorylation status of TCR-ζ and ZAP-70. While fully agonistic stimulation induces two forms (p21 and p23) of phosphorylated TCR-ζ and ZAP-70 phosphorylation, the partially agonistic stimulation induces the incomplete phosphorylation (only p21 form) of TCR-ζ and no tyrosine-phosphorylation of ZAP-70 [23–25]. Subsequently, the partially agonistic ligands thereby induce only a partial activation of T cells.

We previously found that stimulation with L cell clones over-expressing partially agonistic ligand (OPAL) covalently linked with HLA-DR4 induced proliferation and cytokine production of the cognate T cell clone without tyrosine-phosphorylation and activation of ZAP-70 [26].

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The question arose as to whether or not T cell activation stimulated with OPAL was independent of ZAP-70. To answer this question, we utilized the human $CD4^+$ T cell clone expressing dominant negative (DN) ZAP-70. Notably, the expression of DN ZAP-70 markedly inhibited T cell activation induced with OPAL. We therefore presume that the incompletely phosphorylated TCR-ζ chain associated with ZAP-70 plays an important role in the TCR signaling cascade leading to the observed T cell activation stimulated with OPAL.

Materials and methods

Cell lines and cell culture. A human $CD4^+$ T cell clone, T5-32 derived from *Herpesvirus saimiri* transformed T cell clone (YN5-32), was maintained in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 20% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 U/ml of recombinant human IL-2 kindly provided by Dr. Tomoko Ejima of Ajinomoto Co., Inc. YN5-32 cells recognize and respond to streptococcal peptide M12p54-68 (NRDLEQAYNELSGEA) in the context of HLA-DR4 (DRA/DRB1*0406) and were established, as previously described [16]. Mouse L cells expressing HLA-DR4 alone (L-DR4), HLA-DR4 covalently linked with either peptide M12p54-68 (M12DR4) or with its analogues Q59GDR4 or Y61VDR4 were established, as previously described [26]. The linker for activation of T cells (LAT) and the SH2 domain containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76) deficient Jurkat cell lines, JCaM2.5 and J14, were donated by Dr. Arthur Weiss.

Antibodies. The following antibodies were used in this study: anti-PLC-γ1 mAb, and anti-phosphotyrosine mAb, 4G10 (Upstate Biotechnology, Lake Placid, NY), anti-FLAG mAb (Sigma, St. Louis, MO), anti-human ZAP-70 mAb (Transduction Laboratories, San Diego, CA), anti-ZAP-70 Ab (Santa Cruz, CA), anti-phospho-PLC-γ1 (Tyr783) Ab (Cell Signaling Technology, Beverly, MA), goat anti-mouse IgG Ab (PIERCE, Rockford, IL), PE-conjugated anti-IFN-γ mAb (Immunotech, Marseille, France), PE-conjugated mouse IgG, anti-TCR-αβ mAb, and anti-CD3ε mAb (PharMingen, San Diego, CA).

Plasmids, generation of pseudovirus, and infection. The retroviral vector (pMX-IRES-GFP) and MLV-gagpol-IRES-bsr [27] were a kind gift from Dr. Toshio Kitamura of The University of Tokyo. The amphotropic envelope glycoprotein expression vector, SV-A-MLV-Env [28], was kindly provided by Dr. Nathaniel R. Landau of Salk Institute. The dominant negative (DN) ZAP-70 has amino acid residues 1–276 of ZAP-70 consisting of two tandem SH2 domains without the kinase domain followed by FLAG tag and the cDNA was subcloned into pMX-IRES-GFP. The R190K (a non-functional control of DN ZAP-70) has the same construct as that of DN ZAP-70 except for containing a single residue substitution of Arg^{190} to Lys [29,30]. Fifteen micrograms of retroviral vector (pMX-IRES-GFP) was co-transfected with 10 μg MLV-gag-pol expression vector (pGag-pol-IRES-bsr) and 10 μg SV-A-MLV-Env into 293 T cells using Lipofectamine 2000 reagent (Invitrogen). The supernatants were collected at 72 h after transfection. After cell debris had been removed by low-speed centrifugation (2000*g*, 10 min), the supernatants were then further centrifuged at 12,000*g* for 12 h at 4 °C. The pellets were suspended and were added to 1×10^6 of T5-32 in the presence of 6 μg/ml polybrene (Sigma). The expression of the recombinant proteins was monitored by GFP expression and a Western blot analysis using an anti-FLAG mAb.

Flow cytometry. T cells were stimulated by co-culturing with L cells expressing each peptide/HLA-DR4 complex in FCS free medium as previously described [26]. The surface markers were analyzed using FACScan (Becton–Dickinson, Mountain View, CA) and PE-conjugated anti-TCR-αβ mAb. To monitor IFN-γ production, intracellular staining using IntraPrep (Immunotech) was done according to the manufacturer's recommendations. T5-32 cells were stimulated with the L cells as described above in the presence of 20 μg/ml Brefeldin A (Sigma) for 5 h. The cells stained with PE-conjugated anti-IFN-γ were analyzed using FACScan and CELLQuest software (Becton–Dickinson).

Immunoprecipitation and Western blotting. For immunoprecipitation and Western blotting, T5-32 cells (1×10^7) stimulated with each L cell transfectant confluently grown in 15 cm dishes or Jurkat T cells (1×10^7) stimulated with anti-CD3ε mAb were recovered and lysed on ice for 30 min in lysis buffer (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM Na_3VO_4, 10% glycerol, and a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany)). The lysates were immunoprecipitated for 3 h at 4 °C with the indicated antibody followed by collection with protein-A beads (Pierce). The immunoprecipitates or whole cell lysates were separated by SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h in TBS (150 mM NaCl, 20 mM Tris, pH7.6) containing 5% skim milk, 0.5% bovine serum albumin, and 0.1% Tween 20, and incubated with the indicated primary antibody for 2 h at room temperature. The blots were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig or anti-rabbit Ig antibodies (Amersham Biosciences, Piscataway, NJ). In some experiments, the membranes were stripped and reprobed with respective antibodies followed by incubation with the HRP-conjugated second Ab. Blots were visualized using enhanced chemiluminescence (ECL, Amersham Biosciences).

Results

Differences in the tyrosine-phosphorylation of ZAP-70 and its association with TCR-ζ in T5-32 stimulated with L cell transfectant expressing each HLA-DR4/peptide complex

To investigate the tyrosine-phosphorylation status of ZAP-70 and its association with TCR-ζ in T cells stimulated with the OPALs (Y61VDR4 and Q59GDR4), the T cell clone T5-32 was co-cultured with mouse L cells expressing each HLA-DR4/peptide complex. The L cell transfectant over-expressing fully agonistic ligand (M12DR4) stimulated tyrosine-phosphorylation of ZAP-70 coupled with two forms (p21 and p23) of phosphorylated TCR-ζ (Fig. 1). In contrast, stimulation with L cell

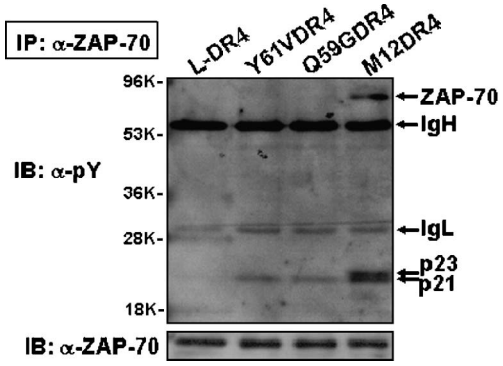

Fig. 1. The induction of tyrosine-phosphorylation of ZAP-70 after the stimulation of T cell clone (T5-32) with each L cell transfectant clone. T5-32 cells (1×10^7) were incubated with the L cell transfectant expressing each HLA-DR4/peptide complex for 5 min at 37 °C. Tyrosine-phosphorylation of TCR-ζ and ZAP-70 in the stimulated T5-32 cells was visualized after immunoprecipitation (IP) with anti-ZAP-70 mAb (α-ZAP-70) and immunoblotting (IB) with anti-phosphotyrosine mAb, 4G10 (α-pY). The positions of ZAP-70 protein, mouse immunoglobulin heavy (IgH) and light (IgL) chains of immunoprecipitating Ab, and phosphorylated TCR-ζ chains (p21 and p23) are indicated on the right. Labels on the left side of the panel indicate the approximate molecular sizes of the marker proteins.