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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

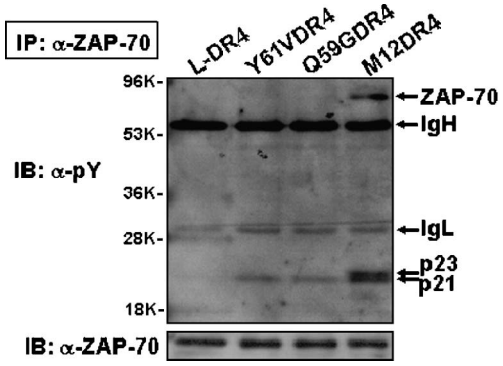

Fig. 1. The induction of tyrosine-phosphorylation of ZAP-70 after the stimulation of T cell clone (T5-32) with each L cell transfectant clone. T5-32 cells (1×10^7) were incubated with the L cell transfectant expressing each HLA-DR4/peptide complex for 5 min at 37 °C. Tyrosine-phosphorylation of TCR-ζ 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.

transfectant over-expressing Y61VDR4 or Q59GDR4 did not induce ZAP-70 phosphorylation. Interestingly, the p21 form of phosphorylated TCR-ζ was observed in the ZAP-70 immunocomplexes, thus suggesting that unphosphorylated ZAP-70 could be recruited to the TCR complex by associating with the p21 form of phosphorylated TCR-ζ (Fig. 1). L-DR4 expressing only HLA-DR4 without a covalently linked cognate peptide did not induce the phosphorylation of ZAP-70 nor its association with TCR-ζ. Depending on the phosphorylation status of ZAP-70 and the accompanying TCR-ζ, we distinguished M12DR4-stimulated activation exhibiting fully agonistic properties from either Y61VDR4- or Q59GDR4-stimulated activations exhibiting partially agonistic properties (Table 1). The partially agonistic activation pattern showed incomplete phosphorylation (p21 form) of TCR-ζ and no tyrosine-phosphorylation of ZAP-70, which is in accordance with the findings of previous reports [23–25].

DN ZAP-70 inhibited tyrosine-phosphorylation of ZAP-70 and TCR down-modulation in T cells stimulated with M12DR4

As OPAL stimulation induced neither tyrosine-phosphorylation of ZAP-70 (Fig. 1) nor kinase activation of ZAP-70, as previously reported by us [26], the T cell responses were thus suggested to be independent of ZAP-70. To examine this possibility, we expressed DN ZAP-70 and its non-functional control R190K in T5-32 cells using the retroviral system (Fig. 2A). DN ZAP-70 is a kinase domain truncated mutant, consisting of only two tandem SH2 domains, and inhibits TCR signaling stimulated with anti-TCR mAb in Jurkat cells, while R190K (DN ZAP-70 carrying Arg^{190} to Lys substitution) has no capacity to inhibit TCR signaling [29]. It was previously reported that the $FLVR^{190}E$ sequence is involved in the phosphotyrosyl binding pocket in the SH2 domain of ZAP-70 and that the Arg^{190} to Lys mutation abolished its binding to the ITAM of CD8-ζ chimeric molecule [30]. The stable expression of DN ZAP-70 or R190K did not affect the expression level of endogenous ZAP-70 (Fig. 2B).

We first examined the effects of DN ZAP-70 and R190K on the tyrosine-phosphorylation of endogenous ZAP-70 when stimulated with each TCR ligand. After 72 h of infection, T cells, in which GFP positive cells were approximately 60–65%, were stimulated and ZAP-70 was immunoprecipitated from the lysate. When T cells expressing DN ZAP-70 were stimulated with M12DR4, the phosphorylation of endogenous ZAP-70 was suppressed in comparison to that of the T cells expressing R190K or the mock vector (Fig. 3A).

T cells stimulated with peptide–MHC complexes undergo TCR down-modulation [19–22] and recently the kinase activity of ZAP-70 has shown to be involved in this phenomenon [31]. As expected, the M12DR4 stimulation induced TCR down-modulation and this was markedly suppressed by the expression of DN ZAP-70 (Fig. 3B). On the other

Table 1
Characteristics of the HLA-DR4/peptide complexes used in this study and a summary of T cell responses to L cell clones expressing each HLA-DR4/peptide complex

L cell clones	Peptide sequence covalently linked with HLA-DR4[a]	Antigenicity of peptides pulsed on PBMCs[b]	T cell responses observed in recognition of L cell clones		
			ZAP-70 Phosphorylation	Phospho-TCR-ζ form associated with ZAP 70	T cell proliferation[c]
M12DR4	NRDLEQAYNELSGEA	Full agonist	Detected	p21, p23	+++
Q59GDR4	NRDLEGAYNELSGEA	Partial agonist	Not detected	p21	++
Y61VDR4	NRDLEQAVNELSGEA	Partial agonist	Not detected	p21	+/–
L-DR4	None	Null	Not detected	Not detected	–

[a] Each L cell clone was transfected with genes encoding for HLA-DR4 α-chain and HLA-DR4 β-chain covalently linked with a linker peptide and 15-mer peptide as previously described [26]. The underlined amino acid residues are substituted amino acid residues unique to APLs derived from M12p54-63 peptide. These cells express a large amount of each single species of the HLA-DR4/peptide complexes on their cell surface.
[b] Irradiated HLA-$DR4^+$ peripheral blood mononuclear cells (PBMCs) pulsed with each peptide acted on the YN5-32 T cell clone, from which the T5-32 T cell clone had originated, as indicated in the table as we previously reported [16].
[c] According to Irie et al. [26].

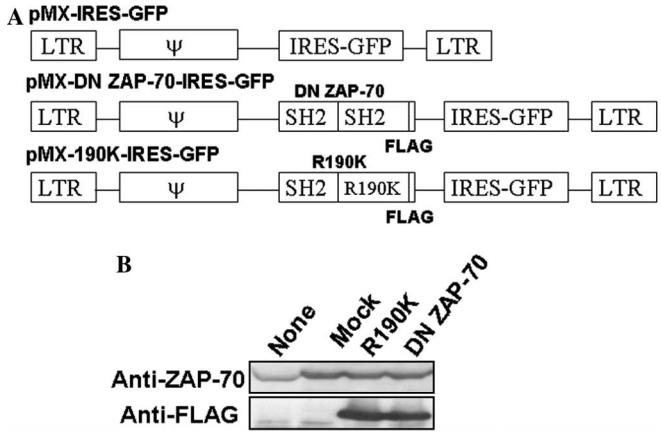

Fig. 2. The retroviral constructs used in this study and quantification of FLAG-tagged ZAP-70 mutants (lacking kinase domain) stably expressed in T cell transfectants in comparison with endogenous ZAP-70. (A) FLAG-tagged DN ZAP-70 and FLAG-tagged R190K (a non-functional control for DN ZAP-70) were subcloned into a retroviral vector containing the internal ribosome entry site (IRES) coupled to green fluorescent protein (GFP). ψ represents the psi sequence necessary for packaging full-length RNA and it also contains splice donor and acceptor sequences. (B) T cells were infected with retroviral vector alone, DN ZAP-70-IRES-GFP, or R190K-IRES-GFP. Two weeks later, 1×10^6 cells from the indicated transfectants were lysed, and the whole cell lysates were then analyzed by immunoblotting with anti-FLAG or anti-ZAP70 mAb. Because the anti-ZAP-70 mAb used recognizes kinase domain of ZAP-70, DN ZAP-70, and R190K could not be detected with the anti-ZAP70 mAb. T cells stably expressing the ZAP-70 mutants did not affect the expression level of endogenous ZAP-70.

hand, expression of R190K had no inhibitory effect on the TCR down-modulation, as observed with the expression of the mock vector. These results clearly showed that the expression of DN ZAP-70 in the T cells efficiently suppressed TCR induced activation of endogenous ZAP-70. The data support the idea that DN ZAP-70 competes with the endogenous ZAP-70 in terms of the binding to tyrosine-phosphorylated TCR-ζ, thus preventing endogenous ZAP-70 from both undergoing tyrosine-phosphorylation and the induction of its kinase activity. The binding of DN ZAP-70 but not R190K to tyrosine-phosphorylated TCR-ζ was confirmed as shown in Fig. 6.

Inhibition by DN ZAP-70 of TCR mediated IFN-γ production and TCR down-modulation in T cells stimulated with OPALs

In our previous study, the stimulation with some OPALs induced IFN-γ production in the cognate T cells without kinase activation of ZAP-70. Therefore, we investigated whether ZAP-70 was involved in the IFN-γ production in OPAL-stimulated T cells using T5-32 cells expressing DN ZAP-70 or R190K. Two weeks after retroviral infection, GFP-positive T cells were >50% (mock), >30% (R190K), and >30% (DN ZAP-70), as determined by flow cytometry. The percentage of intracellular IFN-γ-positive T cells stimulated with the M12DR4 was higher than that of T cells stimulated with Q59GDR4 (Fig. 4A). As shown in Fig. 4A, the IFN-γ production significantly decreased in the T cells expressing DN ZAP-70 in response to stimulation with the OPALs (Q59GDR4 and Y61VDR4) compared with the findings in T cells expressing either the mock control vector or the vector containing the R190K gene. The percentages of IFN-γ-producing cells in GFP-positive T cells stimulated with Y61VDR4 were 2% in DN ZAP-70 expressing T cells, 13% in R190K expressing T cells, and 12% in the mock infected T cells. While the percentages of IFN-γ-producing cells in GFP-positive T cells stimulated with

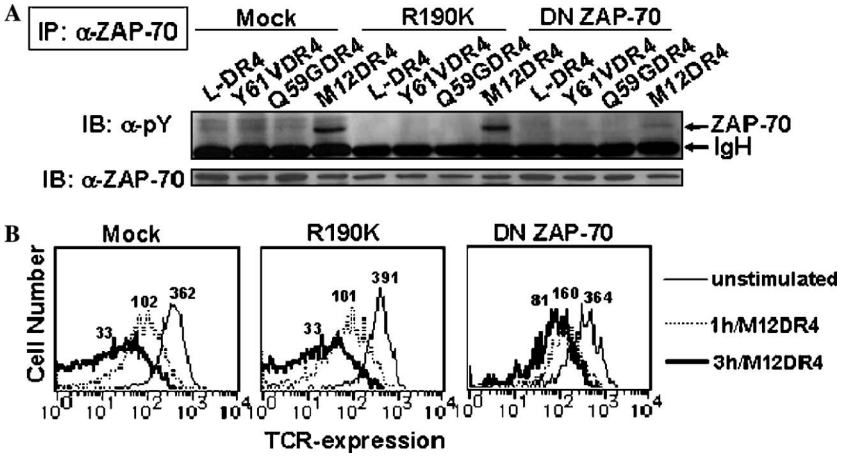

Fig. 3. The inhibitory effects of DN ZAP-70 on T cell activation. (A) The inhibition of endogenous ZAP-70 phosphorylation in T cells expressing DN ZAP-70. After 72 h of infection with retroviral vectors, 3×10^6 T cells (60% of cells were positive for GFP) were stimulated with each L cell transfectant for 10 min at 37 °C. To detect the phosphorylation of ZAP-70, ZAP-70 immunoprecipitates (IP) were analyzed by immunoblotting (IB) with anti-phosphotyrosine mAb. To confirm equal protein loading, the membranes were stripped and reprobed with anti-ZAP-70 mAb. (B) Inhibition of TCR down-modulation by DN ZAP-70. After the infection of the retroviral vectors, T cells were cultured for 2 weeks and then stimulated with L cells expressing M12p54-68/HLA-DR4 (M12DR4) for the indicated times. TCR down-modulation was analyzed using GFP positive cells. The numbers in each flow-cytometric profile indicate the mean fluorescence intensity (MFI) levels.

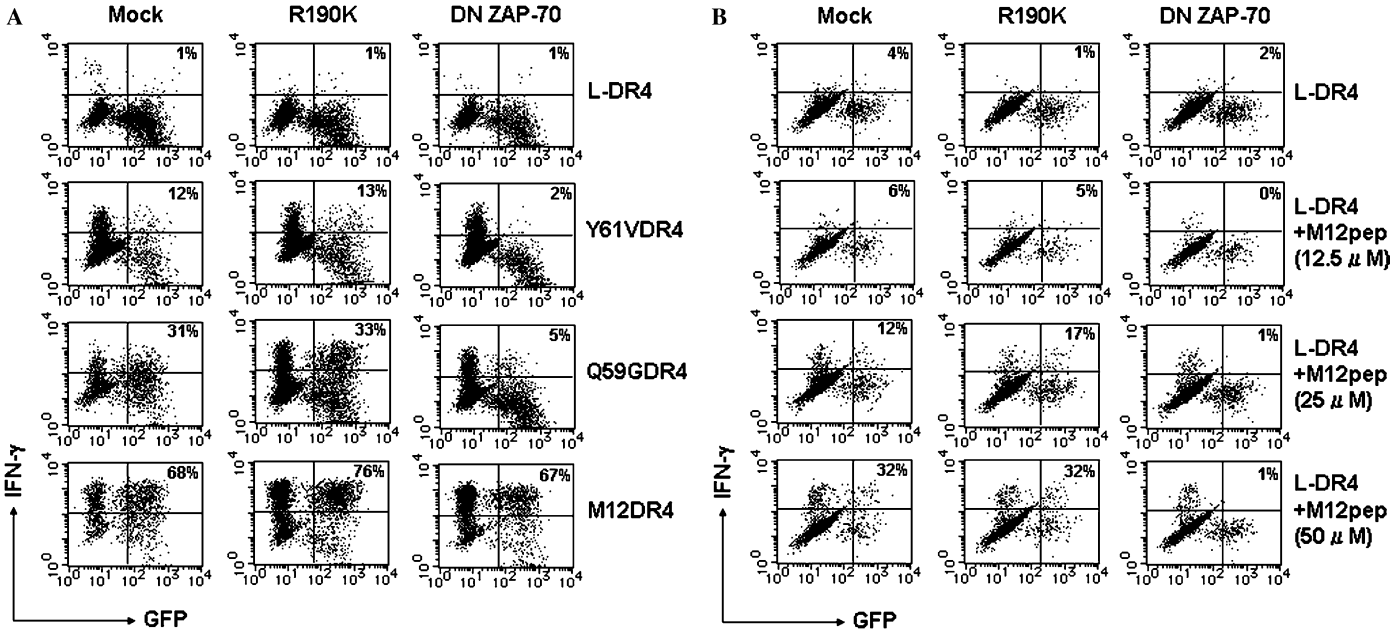

Fig. 4. Inhibition by DN-ZAP-70 of IFN-γ production in T cells stimulated with each L cell transfectant. After the infection of the retroviral vectors, T cells were cultured for 2 weeks and then were stimulated with each L cell transfectant. Intracellular IFN-γ was detected using flow cytometry after a 5 h co-culture of the T cells with each L cell transfectant in the presence of brefeldin A. (A) The effect on IFN-γ production in T cells stimulated with M12DR4 or each OPAL. (B) Effect on IFN-γ production in T cells stimulated with HLA-DR4-expressing L cells (L-DR4) prepulsed with M12p54-68 peptide. L-DR4 cells were prepulsed with the indicated dose of M12 peptide for 16 h. The percentages indicated in the given quadrants represent percentages of IFN-γ-producing cells in GFP positive T cells. The dot blots are representative of three independent and reproducible experiments.

Q59GDR4 were 33% in R190K expressing T cells, and 31% in mock infected T cells, it was markedly suppressed to 5% in the T cells expressing DN ZAP-70. The data indicate that the recruitment of kinase-inactive ZAP-70 to TCR complexes is involved in the IFN-γ production stimulated with the OPALs.

Compared with OPAL-stimulated T cells, no significant difference in the IFN-γ production was observed between DN ZAP-70 (67%) and R190K (76%) expressing T cells stimulated with M12DR4 (Fig. 4A). One reason for the absence of inhibitory effect of DN ZAP-70 on the IFN-γ production in M12DR4 stimulated T cells might be that the T cell activation stimulated with M12DR4 highly over-expressing HLA-DR4/M12p54-68 complexes is too strong to be inhibited by DN ZAP-70. To investigate the effect of DN ZAP-70 on IFN-γ production stimulated with the relatively small numbers of HLA-DR4/M12p54-68 complexes, we checked the effect of DN ZAP-70 on the T cells stimulated with L cells expressing HLA-DR4 (L-DR4) prepulsed with 12.5, 25, or 50 μM of fully agonistic peptide, M12p54-68, for 16 h at 37 °C. As shown in Fig. 4B, the IFN-γ production was abrogated in the T cells expressing DN ZAP-70 and stimulated with L-DR4 prepulsed with M12p54-68 peptide. The percentage of IFN-γ producing T cells expressing DN ZAP-70 and stimulated with the peptide-pulsed L-DR4 was similar to the background level as observed in T cells stimulated with L-DR4, whereas the IFN-γ production increased in a peptide dose-dependent manner in T cells expressing R190K or mock vector in recognition of the same TCR ligand. In our previous observation [26], L cells expressing HLA-DR4 prepulsed with 50 μM M12p54-68 induced proliferation and ZAP-70 phosphorylation of T5-32. These data support the idea that the stimulation with M12DR4 is too strong to be inhibited by the expression of DN ZAP-70.

To further investigate the effect of DN ZAP-70 on OPAL stimulation, we studied the TCR down-modulation in T5-32 cells expressing either DN ZAP-70 or R190K after TCR stimulation. The M12DR4 stimulation induced a strong TCR down-modulation as shown in Fig. 5. In contrast, the stimulation of OPALs (Q59GDR4 and Y61VDR4) induced a weak but definite TCR down-modulation. The expression of DN ZAP-70 inhibited the TCR down-modulation induced with both M12DR4 and OPAL stimulation while the expression of R190K was ineffective. These data indicate that ZAP-70 is thus involved in IFN-γ production and TCR down-modulation in T cells stimulated with OPALs.

Association of the DN ZAP-70 with tyrosine-phosphorylated TCR-ζ chain

The association of the tandem two SH2 domains of ZAP-70 with two phosphorylated tyrosines in the ITAMs of TCR-ζ chain is important in early TCR signal transduction. A previous report showed that DN ZAP-70 was bound to the hyperphosphorylated CD8-ζ chimeric molecule, suggesting that this mutant prevents

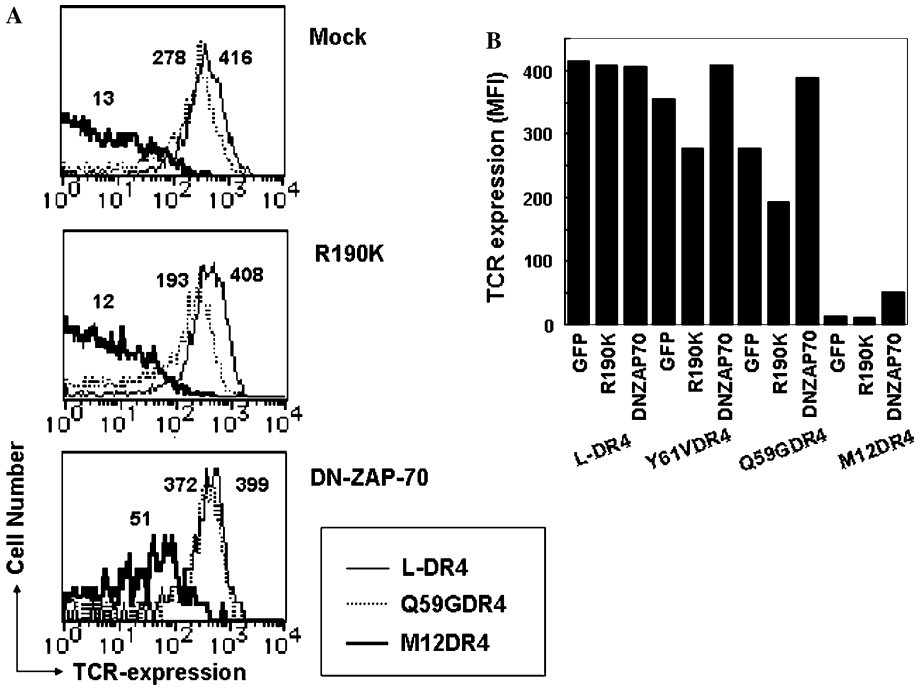

Fig. 5. Inhibition of TCR down-modulation in T cells stimulated with each L cell transfectant by DN-ZAP-70 expression. After the infection of the retroviral vectors, T cells were grown for 2 weeks and then were stimulated with each L cell transfectant. The inhibitory effect on TCR down-modulation was shown by histogram (A) and its MFI level (B). TCR was stained with anti-TCR-αβ mAb after 6 h stimulation. GFP-positive cells were gated and analyzed. The results are representative of three independent and reproducible experiments.

endogenous ZAP-70 from binding to tyrosine-phosphorylated TCR-ζ [29]. To investigate whether the DN ZAP-70 binds to the phosphorylated TCR-ζ chain in the stimulated T cells, DN ZAP-70 and R190K were immunoprecipitated with anti-FLAG mAb after 72 h of infection and the immunocomplexes were then subjected to Western blot analyses. The anti-FLAG mAb co-precipitated two forms (p21 and p23) of phosphorylated TCR-ζ with DN ZAP-70 from the lysate of T cells stimulated with M12DR4. On the other hand, similar to the result shown in Fig. 1, the anti-FLAG mAb co-precipitated only the p21 form of phosphorylated TCR-ζ from the lysate of Q59GDR4 stimulated T cells (Fig. 6). No TCR-ζ was detected in the immunocomplex from the T cells expressing R190K. These results indicate that DN ZAP-70, but not R190K, bound to the phosphorylated TCR-ζ chain

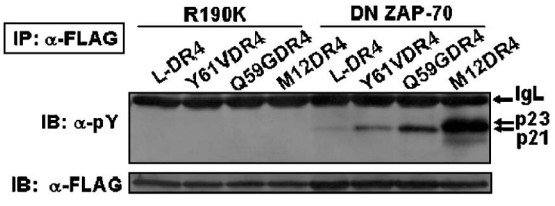

Fig. 6. The association of phosphorylated TCR-ζ with DN ZAP-70. T cells expressing R190K or DN ZAP70 were stimulated for 5 min with each L cell transfectant. R190K or DN ZAP-70 was immunoprecipitated by anti-FLAG mAb (α-FLAG) and phosphorylated forms of TCR-ζ associated with DN ZAP-70 were immunoblotted with the anti-phosphotyrosine mAb (α-pY). The blots were reprobed with anti-FLAG mAb.

and hence DN ZAP-70 prevents the recruitment of endogenous ZAP-70 to the TCR complexes. Importantly, these data suggest that the DN ZAP-70/TCR-ζ association suppresses the T cell responses stimulated not only with M12DR4 but also with OPALs.

The inhibitory effects of DN ZAP-70 on PLC-γ1 phosphorylation and PLC-γ1/ZAP-70 association in stimulated T cells

A possible explanation for the significance of the binding of kinase-inactive ZAP-70 to TCR-ζ is the recruitment of other signaling molecules to the TCR complexes. As one such candidate molecule, we investigated tyrosine-phosphorylation of PLC-γ1 and its association to ZAP-70 in the stimulated T cells. As shown in Fig. 7, M12DR4-stimulation induced an intense tyrosine-783 phosphorylation of PLC-γ1. Q59GDR4-stimulation also induced an increased tyrosine-phosphorylation of PLC-γ1 in comparison to that of the T cells co-cultured with L-DR4. The tyrosine-783 phosphorylation of PLC-γ1 was suppressed in DN ZAP-70 expressing T cells stimulated with Q59GDR4 and M12DR4. These observations suggest that DN ZAP-70 associated with tyrosine-phosphorylated TCR-ζ inhibits the tyrosine-phosphorylation of PLC-γ1 in T cells stimulated with M12DR4 and Q59GDR4.

To check the possibility that ZAP-70 could help PLC-γ1 phosphorylation by recruiting it to TCR complexes, we investigated the phosphorylation of PLC-γ1 and its association with ZAP-70. PLC-γ1 phosphorylation in M12DR4

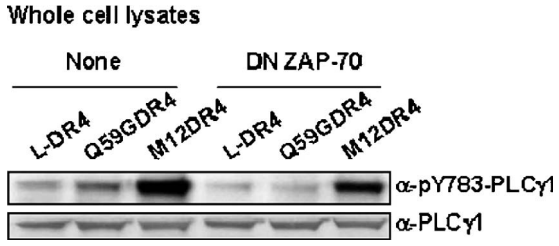

Fig. 7. The inhibitory effects of DN ZAP-70 on PLC-γ1 phosphorylation. After 72 h of infection, 3×10^6 T cells (about 50% cells were positive for GFP) were stimulated with each L cell transfectant for 10 min at 37 °C. Whole cell lysates were immunoblotted with anti-phospho-PLC-γ1 (Tyr783) Ab (α-pY783-PLC-γ1). The same blot was reprobed with anti-PLC-γ1 mAb (α-PLC-γ1) to confirm the equal loading. The data shown are representative results from three independent and reproducible experiments.

stimulated T cells was stronger than that in OPAL-stimulated T cells. Notably, ZAP-70 was co-immunoprecipitated with PLC-γ1 using anti-PLC-γ1 mAb in T cells stimulated with M12DR4, Q59GDR4, or Y61VDR4 (Fig. 8A). Although no phosphorylation of ZAP-70 was observed in the T cells stimulated with OPALs, the level of ZAP-70/PLC-γ1 association increased in comparison to that of the L-DR4 stimulated T cells. These results suggest that OPAL stimulation induces recruitment of unphosphorylated and kinase-inactive ZAP-70 and its association with incompletely phosphorylated TCR-ζ, and that ZAP-70/PLC-γ1 association results in the phosphorylation of PLC-γ1 leading to the T cell responses.

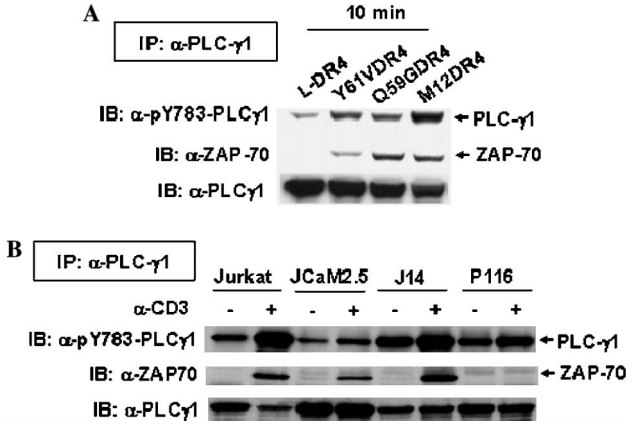

Fig. 8. The physical association of PLC-γ1 with ZAP-70 in TCR stimulated T cells. (A) The association of PLC-γ1 with unphosphorylated ZAP-70 in OPAL-stimulated T cells. T cells (5×10^6) were stimulated with each L cell transfectant for the indicated time, lysed, and subjected to immunoprecipitation using anti-PLC-γ1 mAb. As a control, T cells stimulated with L-DR4 were used. The anti-PLC-γ1 immunocomplexes were resolved by 10% SDS–PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho-PLC-γ1 (Tyr783) antibody (α-pY783-PLC-γ1). The blots were reprobed with anti-PLC-γ1 mAb. (B) The association of PLC-γ1 with ZAP-70 in Jurkat cells. Cells were stimulated with anti-CD3ε mAb or left untreated and goat anti-mouse IgG on ice for 30 min and incubated at 37 °C for 1 min. Cells were lysed and immunoprecipitated with anti-PLC-γ1 mAb. The immunoprecipitates were subjected to Western blot analysis using anti-phosphoTyr783-PLC-γ1 (top) and anti-ZAP-70 mAb (middle). The membrane was reprobed with anti-PLC-γ1 mAb (bottom).

The association of PLC-γ1 with ZAP-70 is not specific to the T5-32 cells. The association of PLC-γ1 with ZAP-70 could also be detected in Jurkat T cells stimulated with anti-CD3 mAb, but not in unstimulated cells (Fig. 8B). ZAP-70 was co-precipitated with PLC-γ1 in wild-type Jurkat cells, the LAT deficient (JCaM2.5), and the SLP-76 deficient (J14) Jurkat cell lines, but not in ZAP-70-deficient (P116) cell line using anti-PLC-γ1 mAb immunoprecipitation. These results indicate that PLC-γ1 associates with ZAP-70 in TCR stimulated T cells even in the absence of LAT or SLP-76.

Discussion

Many models of T cell activation have been proposed to explain the differential effects of T cell stimulation between the full agonist and the APLs. Our previous studies have shown that single amino acid substitutions in the antigenic peptide can affect a range of responses such as the magnitude or level of proliferation, cytokine production, and the expression of various cell surface molecules in $CD4^+$ T cell clone, YN5-32, which recognizes the streptococcal M12p54-68 peptide and its APLs in the context of HLA-DR4 [16]. To prepare large amounts of cells in order to investigate the function of signaling molecules in YN5-32 after APL stimulation, the YN5-32 T cell clone was transformed with *H. saimiri* to establish T5-32 (manuscript in preparation, H. Tsukamoto et al. and [26]). T5-32 proliferates in response to exogenous IL-2 even in the absence of feeding with irradiated PBMC pulsed with the cognate peptide. T5-32 could be maintained and expanded with an IL-2-supplemented medium. T5-32 exhibited a magnitude of reactivity to the antigenic peptides similar to that of YN5-32.

The stable introduction of DN ZAP-70 into T5-32 has heretofore not been achieved, due to the very low transfection efficiency of T5-32 and significant cytotoxicity induced by the transfection method such as electroporation. Importantly, T5-32 stably expressing DN ZAP-70 or R190K could be successfully established using our retroviral system. In M12DR4 stimulated T5-32 cells, the expression of DN ZAP-70 inhibited the tyrosine-phosphorylation of ZAP-70 and significantly suppressed the TCR down-modulation while the expression of R190K had no such effects (Fig. 3). Since DN ZAP-70 could associate with both p21 and p23 forms of phosphorylated TCR-ζ, but R190K could not, the inhibitory effects of DN ZAP-70 seemed to be due to the prevention of endogenous ZAP-70 to bind to phosphorylated TCR-ζ (Fig. 6).

Contrary to its inhibitory effect on ZAP-70 phosphorylation, no significant difference in the magnitude of IFN-γ production was observed in M12DR4-stimulated T cells expressing DN ZAP-70, R190K or the mock vector. The lack of inhibitory effects on IFN-γ production in DN ZAP-70 expressing T cells stimulated with M12DR4 (Fig. 4A) suggests the following possibility; The T cell

activation stimulated with M12DR4 highly over-expressing HLA-DR4/M12p54-68 complexes is too strong to be inhibited by DN ZAP-70. Further supporting this possibility, the expression of DN ZAP-70 in T cells markedly inhibited the response to L-DR4 cells prepulsed with the fully agonistic M12p54-68 peptide (Fig. 4B).

We also checked the IL-2 production of the stimulated T cells at a single cell level using different methods, such as intracellular staining of IL-2 and cell surface detection of secreted IL-2. Compared with IFN-γ production, the IL-2 production was quite small even in the M12DR4 stimulated T cells. Because IL-2 production was hardly detected in the stimulated T cells, we could not evaluate the inhibitory effects of DN ZAP-70 on IL-2 production. It remains to be analyzed using other sophisticated methods in the future.

ZAP-70 was also involved in the TCR down-modulation stimulated with OPALs. The extent of TCR down-modulation correlated to the capacity of each APL to induce TCR signaling [19–22]. While full agonists induce the maximal degree of TCR down-modulation, partial agonists induce a lower level of TCR down-modulation [19,20] and this phenomenon correlates with the activity of ZAP-70 [30]. The ZAP-70-deficient Jurkat cell P116 and the kinase-dead ZAP-70 containing DK33 T cell showed a reduced TCR internalization by anti-CD3 antibody stimulation. P116 reconstituted with ZAP-70 restored TCR internalization to the level achieved in wild-type Jurkat cells [30]. As expected, an intense stimulator M12DR4 can induce a strong TCR down-modulation and OPALs can induce a weak but definite TCR down-modulation (Fig. 5).

It must be noted that, although the tyrosine-phosphorylation of ZAP-70 was not detected in T5-32 T cells stimulated with OPAL, the expression of DN ZAP-70 resulted in decrease of IFN-γ production (Fig. 4A) and an inhibition of the TCR down-modulation (Fig. 5). In addition, unphosphorylated ZAP-70 associated with partially phosphorylated TCR-ζ in OPAL-stimulated T cells. These observations suggested that the association of ZAP-70 with TCR-ζ might medicate activation of other signaling molecules regardless of its kinase activity. As one such candidate molecule, we chose PLC-γ1 and examined the tyrosine-phosphorylation of PLC-γ1 in T cells expressing DN ZAP-70 stimulated with the OPAL, because (1) we provided evidence that activation of protein kinase Cμ was involved in the T cell activation stimulated with Q59GDR4, thus suggesting the production of diacylglycerol by PLC-γ1 activity, and (2) a specific inhibitor for PLC (U-73122) inhibited the Q59GDR4 stimulated T cell responses, such as IFN-γ production and T cell proliferation [26]. As a result, we found that PLC-γ1 phosphorylation induced in TCR-stimulated T cells was inhibited in the presence of DN ZAP-70 (Fig. 7).

PLC-γ1 directly binds to the phosphorylated linker for activation of T cells (LAT) in TCR stimulated T cells. However, OPAL stimulation does not induce the tyrosine-phosphorylation of ZAP-70 and LAT [26], and we observed the association of PLC-γ1 with ZAP-70 even in LAT or SLP-76 deficient Jurkat T cells stimulated with anti-CD3ε antibody (Fig. 8B). Therefore, LAT and SLP-76 seemed to be dispensable for the PLC-γ1/ZAP-70 association. Williams et al. showed that a tyrosine (corresponding to Tyr-319) phosphorylated peptide derived from interdomain B of ZAP-70 binds to a GST fusion protein with the C-terminal-side SH2 domain of PLC-γ1 [32]. In addition, even the unphosphorylated ZAP-70 peptide was also shown to be weakly associated with the PLC-γ1 C-terminus SH2 protein. Therefore, it seems feasible that unphosphorylated ZAP-70 and PLC-γ1 may be directly associated through the interdomain B of ZAP-70 and C-terminus SH2 of PLC-γ1. In our experimental system, how unphosphorylated ZAP-70 and PLC-γ1 associate remains to be elucidated in OPAL-stimulated T cells. Although the tyrosine-phosphorylation of ZAP-70 was undetectable, an increased PLC-γ1/ZAP-70 association was detected in OPAL-stimulated T cells compared with the findings in unstimulated T cells. Therefore, we presume that the PLC-γ1/ZAP-70 association correlates with the phosphorylation of PLC-γ1.

In summary, we investigated whether unphosphorylated ZAP-70 is required for the activation of T cells in response to OPAL by expression of DN ZAP-70. Our findings suggest that the association of unphosphorylated ZAP-70 with the incompletely phosphorylated TCR-ζ chain is necessary for T cell activation induced by OPAL. These observations provide a new insight into the unidentified role of ZAP-70. The further characterization of those steps in the TCR signaling pathway after APL stimulation is important to understand the activation, homeostatic proliferation, and differentiation of T cells, and the introduction of dominant negative forms of signaling molecules into T5-32 is expected to help elucidate these phenomena.

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