

学位論文
Doctor's Thesis

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

(Altered peptide ligand 刺激による T 細胞活性化における)
不活性型 ZAP-70 の重要性

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2. Summary

The protein tyrosine kinase, zeta-associated protein-70 (ZAP-70) plays an essential role in T cell receptor (TCR) mediated T cell activation. One reason why the partially agonistic altered peptide ligands do not induce full T cell activation might relate to a failure of tyrosine phosphorylation and kinase activation of ZAP-70. However, whether the induction of partial T cell responses is independent of ZAP-70 remains to be elucidated. To clarify this issue, I introduced a dominant negative (DN) form of ZAP-70 into a human CD4⁺ T cell clone of which fully and partially agonistic non-self peptide ligands have been well characterized. As a result, I found that some over-expressed partially agonistic ligands (OPALs) induced T cell responses, including IFN- γ production, CD40L up-regulation, and TCR down-modulation, without tyrosine phosphorylation and kinase activation of ZAP-70. However, those responses were markedly inhibited in T cells expressing DN ZAP-70, which associated with incompletely phosphorylated TCR- ζ , suggesting that DN ZAP-70 prevented association of endogenous ZAP-70 with TCR- ζ . The data indicate that unphosphorylated ZAP-70 associated with incompletely phosphorylated TCR- ζ is responsible for the induction of those T cell responses. Supporting this idea, OPAL stimulation induced the association of phospholipase C (PLC)- γ 1 with ZAP-70 and tyrosine-783 phosphorylation of PLC- γ 1 which was reduced in T cells expressing DN ZAP-70. These data indicate that TCR- ζ /ZAP-70 association mediates recruitment of other signaling molecules, such as PLC- γ 1 to the TCR complex to induce some kind of T cell activation even in the absence of phosphorylation and kinase activation of ZAP-70.

3. Publication list

1. J. R. Kim, A. Irie, H. Tsukamoto, and Y. Nishimura.
A role of kinase inactive ZAP-70 in altered peptide ligand stimulated T cell activation.
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2. J. R. Kim and C. H. Kim.
Association of a high activity of matrix metalloproteinase-9 to low levels of tissue inhibitors of metalloproteinase-1 and -3 in human hepatitis B-virus hepatoma cells.
International Journal of Biochemistry & Cell Biology 36: 2293-2306, 2004.
3. C. Park, J. R. Kim, J. K. Shim, B. S. Kang, Y. G. Park, K. S. Nam, Y. C. Lee, and C. H. Kim.
Inhibitory effects of streptozotocin, tumor necrosis factor- α , and interleukin-1 β on glucokinase activity in pancreatic islets and gene expression of GLUT2 and glucokinase.
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5. Abbreviations

| | |
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| APC; | antigen-presenting cell |
| APL; | altered peptide ligand |
| DN; | dominant negative |
| FCS; | fetal calf serum |
| GFP; | green fluorescent protein |
| HLA; | human histocompatibility leukocyte antigen |
| IFN; | interferon |
| IL; | interleukin |
| IRES; | internal ribosomal entry site |
| ITAM; | immune receptor tyrosine-based activation motif |
| LAT; | linker for activation of T cells |
| mAb; | monoclonal antibody |
| MFI; | mean fluorescence intensity |
| MHC; | major histocompatibility complex |
| OPAL; | over-expressed partially agonistic ligand |
| PBMC; | peripheral blood mononuclear cell |
| PBS; | phosphate-buffered saline |
| PLC; | phospholipase C |
| PTK; | protein tyrosine kinase |
| SLP-76; | the SH2-containing leukocyte-specific phosphoprotein of 76 kDa |
| SH2; | Src homology 2 |
| TCR; | T cell antigen receptor |
| ZAP-70; | zeta-associated protein-70 |

6. Introduction

6-1) Signal transduction by T cell antigen receptor for antigen

The CD4⁺ T cell-mediated immune responses depend on recognition by the T cell antigen receptor (TCR) of a specific antigenic peptide in the context of major histocompatibility complex (MHC) class II. Engagement of the TCR by an immunogenic peptide bound to class II MHC molecules mediates the intracellular signaling cascade of biochemical events that triggers cytokine production, changes in expression levels of cell surface molecules and cell proliferation. The TCR is composed of the ligand binding subunit, an $\alpha\beta$ heterodimer and the associated signaling components, including a CD3 γ - ϵ pair, a CD3 δ - ϵ pair, and a TCR ζ - ζ homodimer (Fig. 1) (1-3). The TCR $\alpha\beta$ heterodimer is responsible for ligand recognition and the associated signaling components bear the immune receptor tyrosine activating motifs (ITAMs) in their cytoplasmic domains that are phosphorylated following receptor triggering. Early signal transduction through the TCR is initiated by phosphorylation of the ITAMs of TCR- ζ chains mediated preferentially by Lck, though Fyn may play a role. Phosphorylation of the ITAMs leads to recruitment of the ZAP-70 and the doubly phosphorylated ITAMs provide binding sites for ZAP-70 through the interaction of its tandem Src homology 2 (SH2) domains (Fig. 2) (4-8). ZAP-70 is recruited to the activated TCR and associated with the phosphorylated TCR- ζ chains, facilitating subsequent their tyrosine phosphorylation and activation by Lck or Fyn. The activated ZAP-70 kinase itself is tyrosine phosphorylated, a biochemical change that is associated with regulation of its kinase activity and providing binding sites of other linker proteins. Activation of ZAP-70 molecules contributes to the activation of a cascade of downstream signals that are crucial for the initiation of cellular responses (Fig. 3).

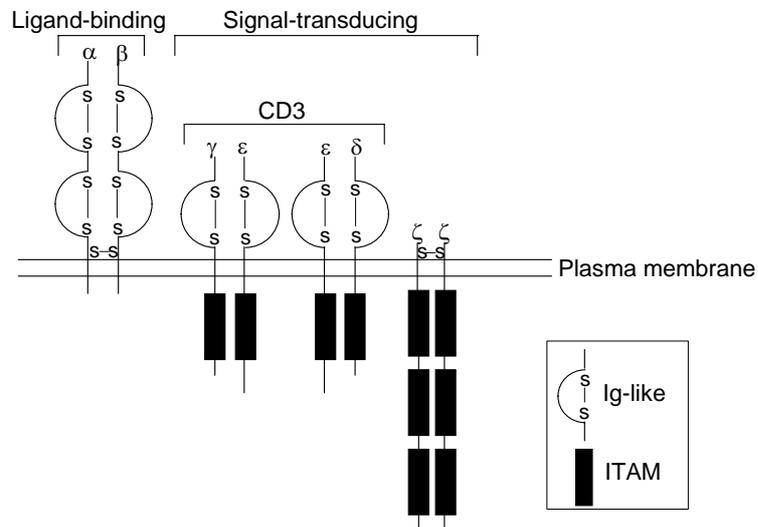


Fig. 1. The structure of TCR. The TCR $\alpha\beta$ heterodimer is antigen binding site. The signal-transducing TCR subunits (CD3 γ - ϵ , CD3 δ - ϵ , and TCR ζ - ζ) contain conserved immunoreceptor tyrosine-based activation motifs (ITAMs) within their cytoplasmic domains to which various SH2 domain containing proteins bind.

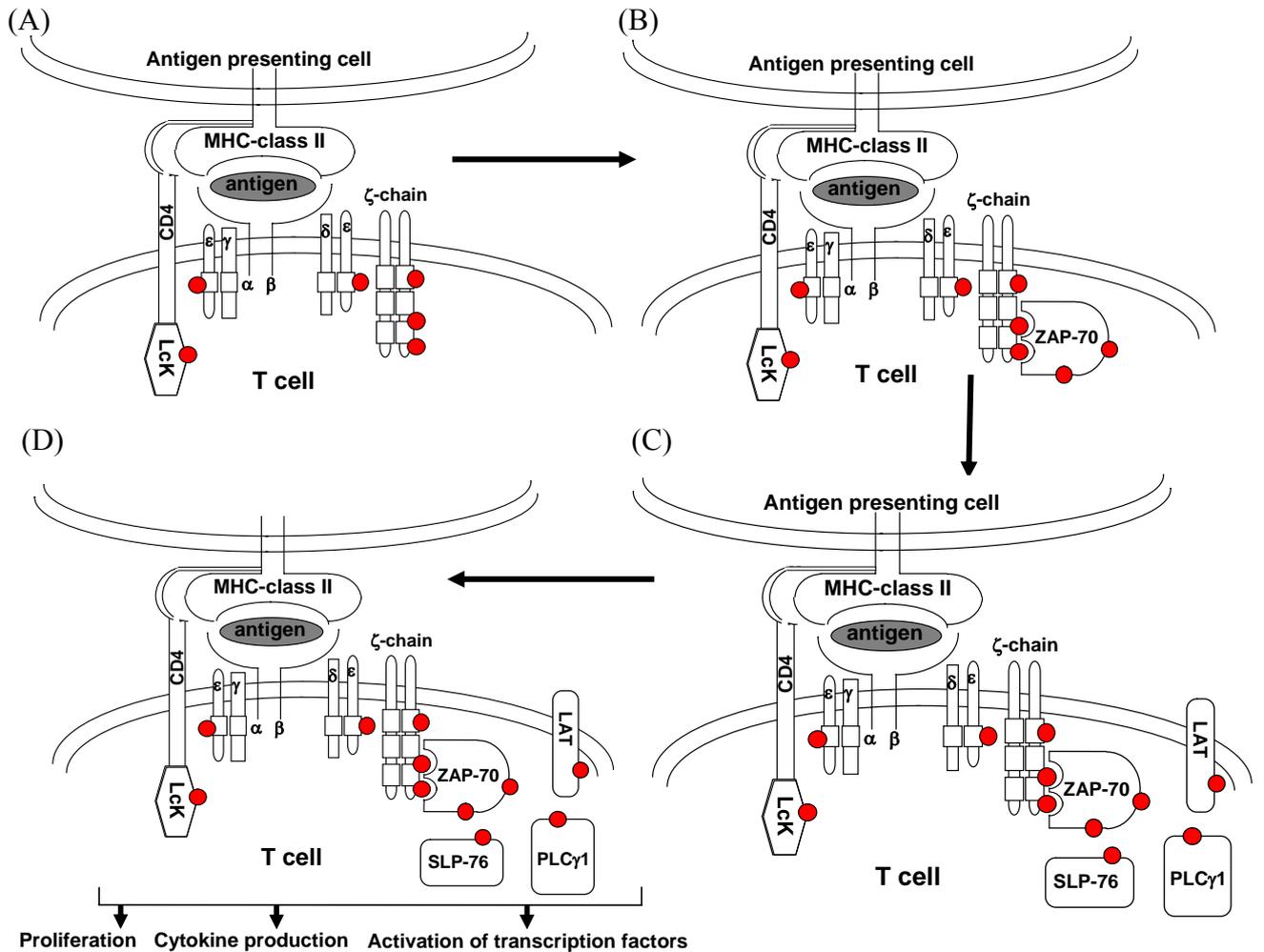
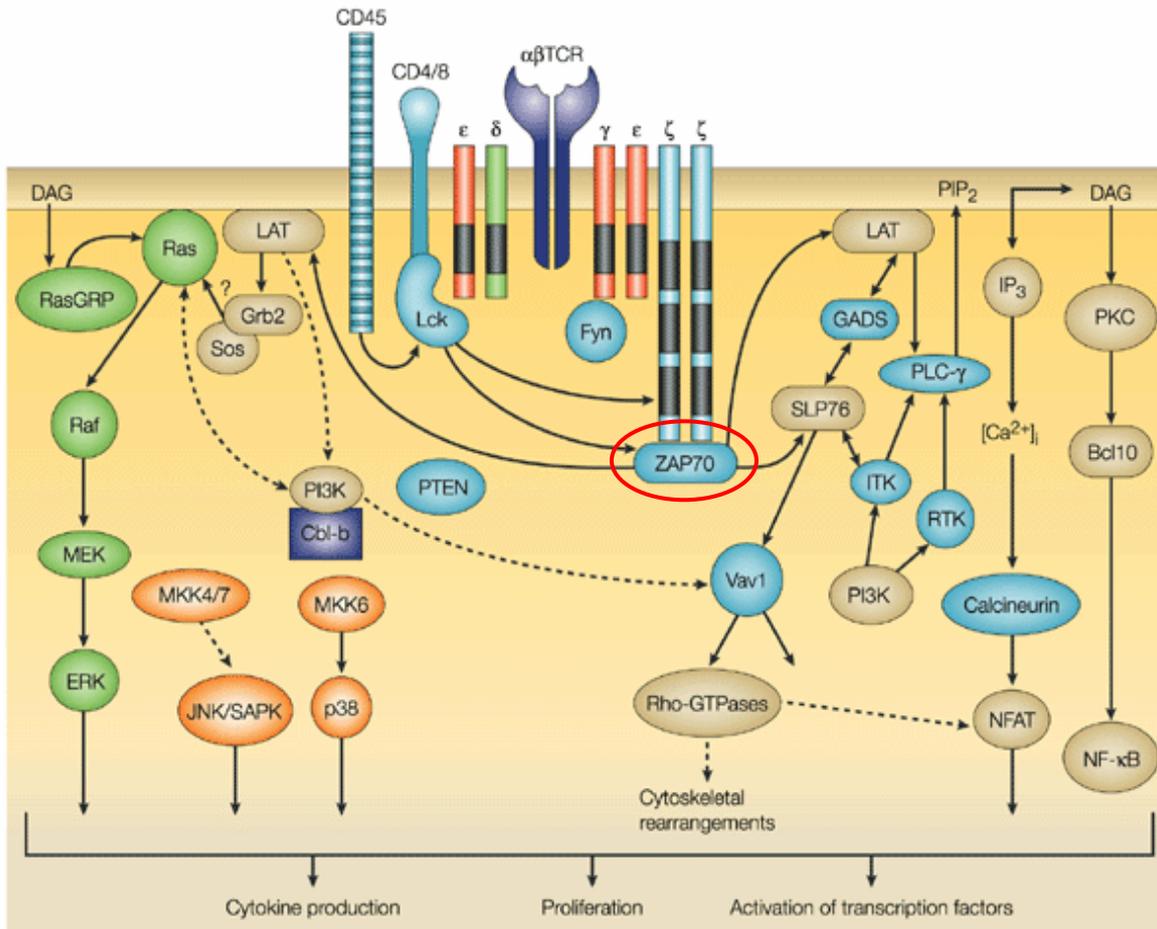


Fig.2. The steps of tyrosine phosphorylation in T cell activation after TCR stimulation. A, Antigen recognition and phosphorylation of ITAMs. TCR stimulation leads to the activation of Lck which phosphorylates the tyrosines in ITAMs. B, Recruitment and activation of ZAP-70. The tyrosine phosphorylated ITAMs provide high-affinity binding sites for the two SH2 domains of ZAP-70, resulting in the recruitment of ZAP-70 of to TCR. The binding of ZAP-70 to the ITAM facilitates the phosphorylation of ZAP-70 at Tyr493, resulting in the activation of its kinase activity, by Lck. C, Phosphorylation of other signal molecules (substrates of ZAP-70) by ZAP-70. Other tyrosine residues on ZAP-70 are subsequently phosphorylated and bind to signaling molecules, such as LAT, SLP-76, or PLC- γ 1 resulting their phosphorylation and activation D, Functional responses including activation of transcriptional factors, expression of cell surface, cytokine production, and proliferation. The substrates of ZAP-70 mediate the activation of downstream pathways resulting in activation of transcriptional factors, expression of cell surface, cytokine production, and proliferation. Red circles (●), tyrosine phosphorylated forms; Squares (□), ITAMs.



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Fig. 3. Schematic illustration of TCR signaling. This figure is cited from ref.9. A summary of signaling molecules downstream of ZAP-70. Activated ZAP-70 phosphorylates LAT which permits the binding of SH2 containing adaptor proteins such as Grb2 and Gads and these molecules are phosphorylated, which lead to activation of Ras. Activated Ras then activates mitogen-activated protein kinase (MAPK) cascades. The phosphorylation of PLC- γ catalyzes the breakdown of membrane phosphatidylinositol 4,5-biphosphate (PIP₂), generating inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). IP₃ induces the release of Ca²⁺ stored in the endoplasmic reticulum, and DAG plus Ca²⁺ activate protein kinase C (PKC) which serves to activate other enzymes, activation of transcription factors, and eventually enhanced gene expression.

[Ca²⁺], intracellular Ca²⁺ concentration; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; IP₃, inositol 1,4,5-triphosphate; MEK, MAPK/ERK kinase; MKK, MAPK kinase; PKC, protein kinase C; PLC- γ , phospholipase C- γ ; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RasGRP, Ras guanyl-releasing protein.

6-2) The structure of ZAP-70

The Syk family protein tyrosine kinases (PTKs) include Syk and ZAP-70. ZAP-70 is a 70 kd PTK expressed exclusively in T cells and natural killer cells (4-6), whereas Syk, a 72 kd PTK, is expressed in B cells, myeloid cells, and thymocytes (10). The ZAP-70 is essential for both development and activation of T lymphocytes. ZAP-70 consists of two SH2 domains that are connected by a 60-residue segment (the interdomain A) and are followed by a second connecting region (the interdomain B) and a catalytic domain (Fig. 4). The interdomain A forms coiled-coil structure, the interdomain B contains a proline-rich sequence and tyrosine phosphorylation sites, and the catalytic domain involves ATP binding sites and tyrosine phosphorylation sites. Following TCR engagement, ZAP-70 is rapidly phosphorylated on several tyrosine residues as a result of both autophosphorylation and trans-phosphorylation by Lck or Fyn (11-14).

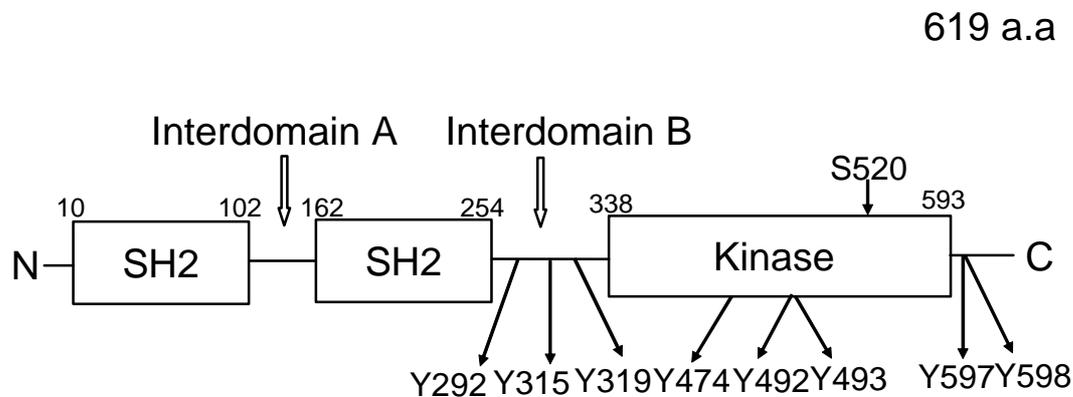


Fig. 4. The structure of ZAP-70 and its phosphorylation sites. The ZAP-70 consists of two SH2 domains and a catalytic domain. Each phosphorylation site of ZAP-70 plays regulation of catalytic activity or binding site of other adaptor molecules for their activation.

6-3) Functions and phosphorylation sites of ZAP-70

Tyrosine phosphorylation plays important roles in regulating the catalytic activity and function of ZAP-70. Tyrosine residues, Y492 and Y493 are located in the activation loop of the kinase domain and these sites are either negative or positive regulatory sites for ZAP-70 function. Y493 is phosphorylated by Lck (14). The major function of phosphorylation of Y493 is to enable the activation of the kinase activity of ZAP-70 and to allow subsequent autophosphorylation of Y492. Phosphorylation of Y492 results in the down-regulation of the kinase activity of ZAP-70 (15, 16). Thereby, Y492 and Y493 regulate enzymatic activity of ZAP-70.

Once activated, ZAP-70 becomes autophosphorylated on multiple tyrosine residues. Tyrosine phosphorylations of ZAP-70 act as a scaffold for the presentation of substrates and for the formation of multi-protein signaling complexes. Phosphorylation of Y474 is required for association of the Shc adaptor and its phosphorylation by ZAP-70 is implicated in TCR signaling to Ras activation (17). ZAP-70 function is affected by the phosphorylation of tyrosines (Y292, Y315, and Y319) located in interdomain B. Phosphorylated Y292 interacts with cbl, a ubiquitination machinery, resulting in ubiquitination and degradation (16, 18-20). In contrast to the negative regulatory Y292, Y315 and Y319 play positive roles in TCR signal transduction. Y315 has been proposed to function as a docking site for the SH2 domain of Vav1 (21), it plays a critical role in antigen receptor-mediated signaling (22), and it determines recruitment and binding of ZAP-70 to the phosphorylated ITAM (23). Phosphorylated Y319 is a binding site for SH2 domain of Lck (24), it leads the phosphorylation of LAT adaptor protein and PLC- γ 1, activation of PLC- γ 1 and Ras, Ca²⁺ mobilization, CD69 expression, the nuclear factor of activated T cells (NFAT) dependent transcription, and interleukin (IL)-2 production in T cells stimulated through TCR (25, 26). ZAP-70 bears a cluster of tyrosines YY597/598 located in close to the C-terminus. These tyrosines implicate a regulatory region modulating T cell activation by control of the cellular activity of ZAP-70 kinase, which detected by a gain-of-function mutant (27).

During T cell development, ZAP-70 plays essential role in positive and negative selection of thymocytes (28, 29). These processes are required for Y315 and Y319 (30, 31). Y315F mutation reduces the rate of positive selection and it delays the occurrence of negative selection.

Src family members have a unique N-terminal domain with a myristylated glycine, which is responsible for membrane association. In contrast to Src family PTKs, Syk and ZAP-70 are not myristylated and hence are probably not constitutively localized at the plasma membrane. Ser-520 phosphorylation of ZAP-70 plays an important role in the correct localization of ZAP-70 and in priming ZAP-70 for its recruitment and activation upon TCR stimulation (32).

6-4) T cell activation induced with altered peptide ligand

A single TCR can recognize a variety of peptide/MHC ligands (Fig. 5). Strong wild type ligands induce cytokine production, regulation of various cell surface molecules, and T cell proliferation. Subtle changes in amino acid sequence of the original antigenic peptide can generate altered peptide ligands (APLs) which can profoundly alter the response of the T cell by coordinating the selective activation of effector responses (33-37). APLs can be divided into different classes based on the potency for induction of T cell responses; full agonist, weak agonist, partial agonist and antagonist (37-41). Agonists are recognized by the TCR with a high affinity and cause T cell proliferation at low concentration. Agonists and weak agonists can maximally stimulate all effector functions of T cells, although weak agonists require a higher concentration of peptide.

Generally, partial agonists cannot induce proliferation of T cells, yet they can stimulate other effector functions such as cytokine production or modulation of cell surface markers. Antagonists inhibit responses induced by agonist. Although antagonists are inhibitory for multiple cellular responses such as proliferation and cytokine production, these ligands can act on their own to induce some alterations in gene expression and functional response in T cells. Cytokine production (42-45), up-regulation of some cell surface molecules (45-47), and down-modulation of TCR (48-51) correlated with the capacity of each APL to induce TCR signaling.

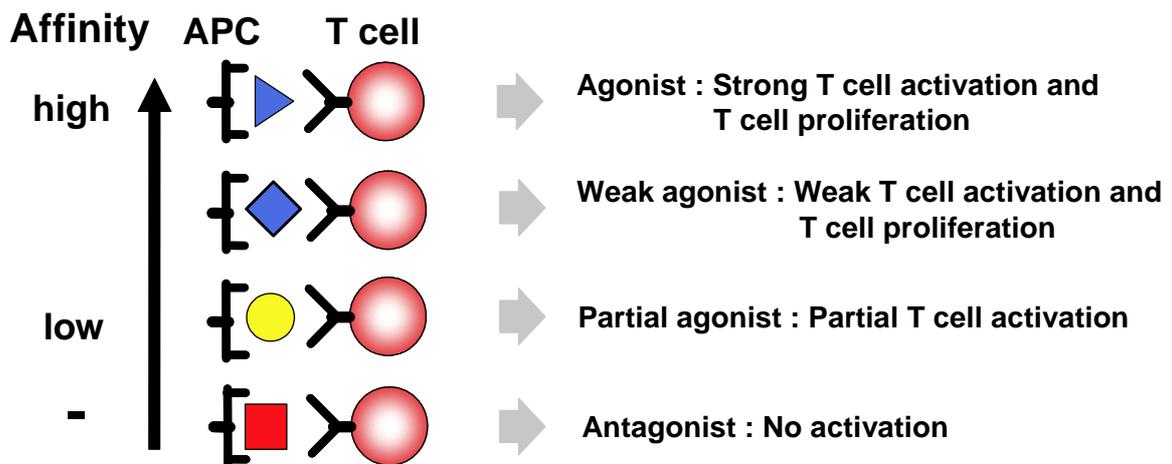


Fig. 5. Relationship between TCR and its ligand. TCR ligation by immunogenic peptide ligand (agonist) leads to efficient T cell activation which is due to its high affinity for the TCR. Agonist can induce T cell proliferation at low concentration. Weak agonist can induce T cell proliferation at high concentration. Partial agonist engagement causes inefficient T cell activation because of their lower affinity to TCR. Antagonist inhibits activation of T cell and suppresses agonist-induced T cell activation.

6-5) Differential signaling pattern between full agonism and partial agonism

Partial agonistic or antagonistic ligand signaling can lead to cellular responses that are qualitatively different from those induced by agonistic ligand signaling. Partial agonistic ligand or antagonist is involved in positive selection and differentiation of T cells in thymocytes, whereas, agonistic ligand induces apoptosis (52). Recognition of self peptide/MHC as a strong agonist by immature thymocytes results in negative selection, but positive selection of thymocytes requires some interaction between the TCR and self-peptide/MHC as a partial agonist.

The difference in TCR signal transduction between partial agonists and full agonists is characterized by phosphorylation status of TCR- ζ and ZAP-70 (Fig. 6). While fully agonistic stimulation induces two forms (p21 and p23) of phosphorylated TCR- ζ and ZAP-70 phosphorylation, the partially agonistic stimulation induces incomplete phosphorylation (only p21 form) of TCR- ζ and no tyrosine phosphorylation of ZAP-70 (53-55), resulting in no kinase activation. Subsequently, partially agonistic ligands induce only partial activation of T cells. In general, the TCR binds to partial agonist ligands with a lower affinity, and this is primarily due to an increase in the TCR-ligand off-rate. The rapid dissociation of the TCR from partial agonist ligands

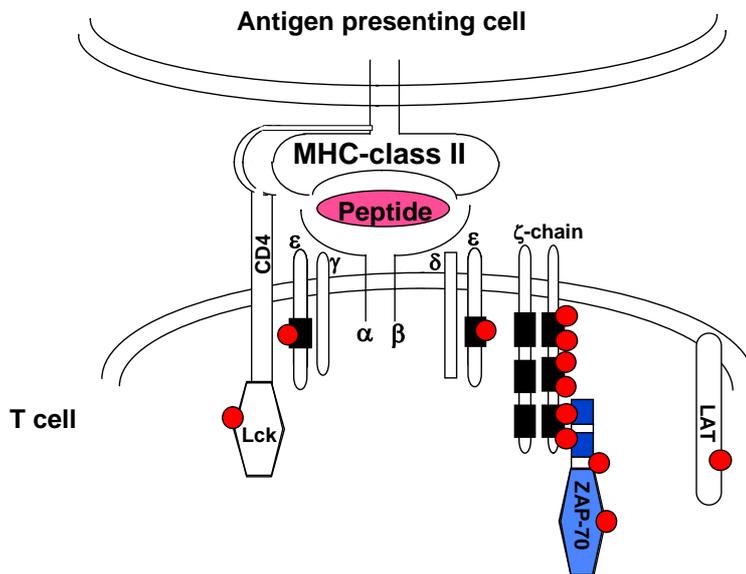
results in an incompletely phosphorylation of the TCR- ζ (56).

T cells stimulated with peptide-MHC complexes undergo TCR down-modulation (48-51) and the kinase activity of ZAP-70 is involved in this phenomenon (57). The extent of TCR down-modulation relates to the ability of each APL to induce TCR signaling. Full agonists induce the maximal degree of TCR down-modulation.

Some molecules are activated in response to TCR stimulation by both agonist and partial agonist although kinetics is different. Duration of ERK activation can be dependent on the type of TCR ligand used. ERK activation induced with full agonist is more sustained whereas partial agonist mediated signaling activates ERK only transiently (55). Agonist ligand induces a stable ternary complex on the early growth response gene 1 promoter that persists for over 30 min, whereas the ternary complex induced by partial agonist is present only for about 10 min (58). Partial agonists may fail to sustain ERK activation. The early growth factor 1 is induced by TCR signals, and this induction requires activation of ERK. The mechanism of ERK activation by partial agonists is different from the mechanism used by agonistic ligand.

Partial agonists and antagonists elicit the patterns such as partially phosphorylated TCR- ζ , the nonactivation of ZAP-70, a lower level of CD3/TCR down-regulation, and transient ERK activation.

(A) T cell activation stimulated by fully agonistic immunogenic peptide



(B) T cell activation stimulated by partially agonistic immunogenic peptide

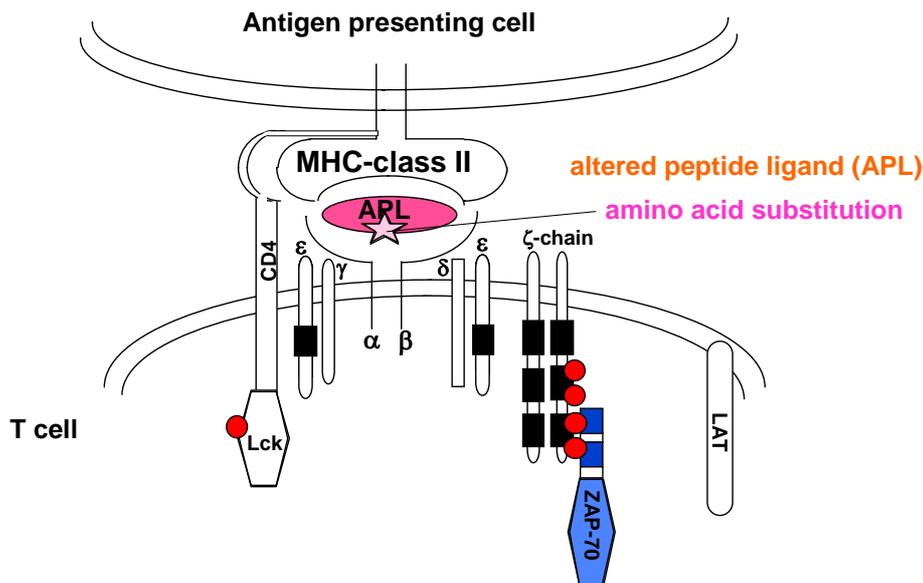


Fig. 6. Model for APL-induced partial activation of T cells. A, TCR ligation by immunogenic ligand leads to the tyrosine phosphorylation of all ITAMs of CD3 ϵ and TCR- ζ , kinase activation of ZAP-70 by its tyrosine phosphorylation, and full T cell activation. B, APL engagement leads to the incomplete tyrosine phosphorylation of TCR- ζ , unphosphorylation and kinase inactivation of ZAP-70, and partial activation. Red circles mean tyrosine phosphorylated forms. Red circles (●), tyrosine phosphorylated forms; Squares (■), ITAMs.

6-6) Objectives of this study

Previous data showed that the stimulation of the human CD4⁺ T cell clone, T5-32, with L cell clones over-expressing partially agonistic ligand (OPAL) covalently

linked with human leukocyte antigen (HLA)-DR4 did not induce tyrosine phosphorylation and kinase activation of ZAP-70 (Fig. 7), but did induce T cell proliferation and mRNA up-regulation and production of cytokines (59). The question arose as to whether or not T cell activation stimulated with OPAL was independent of ZAP-70. To answer this question, I utilized a human CD4⁺ T cell clone expressing dominant negative (DN) ZAP-70.

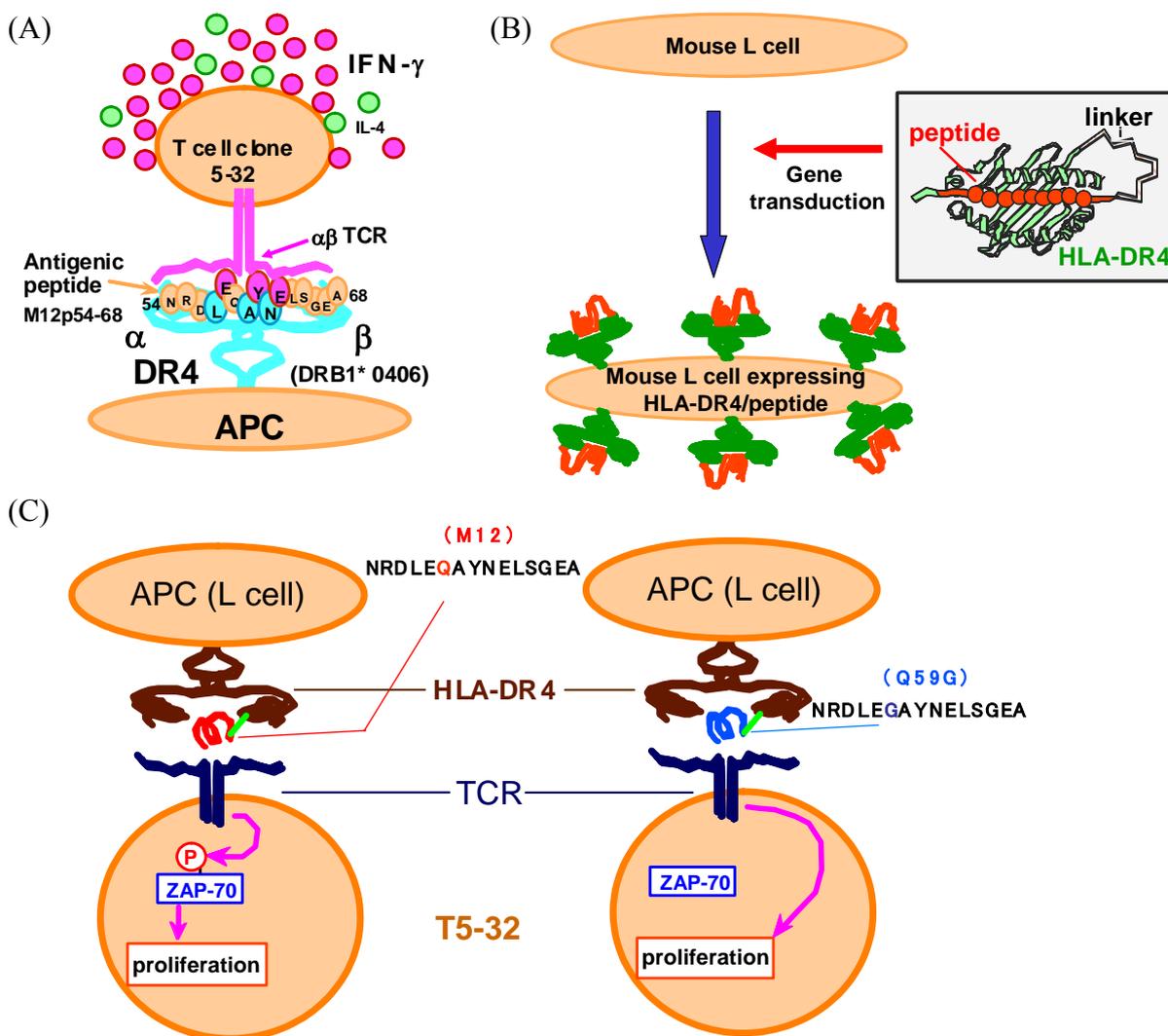


Fig. 7. The characters of a T cell clone (T5-32) and L cell transfectants used in the previous and present studies. A, T5-32 clone stimulated with streptococcal M12 peptide-pulsed peripheral blood mononuclear cell (PBMC) produces cytokine such as IFN- γ and IL-4 and proliferates. B, Generation of L cell transfectant highly over-expressing HLA-DR4/peptide. L cell transfectants express HLA-DR4 covalently linked agonistic peptide, M12 or its altered peptide ligand, Q59G on the surface. C, M12DR4 stimulated T cells induce ZAP-70 phosphorylation and proliferation. However, Q59G stimulated T cells induces proliferation without ZAP-70 phosphorylation. (59)

7. Materials and Methods

7-1) Cell lines

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 interleukin-2 (rhIL-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 described (45). T5-32 cells exhibited the similar magnitude of reactivity to antigens as did the normal YN5-32 cells. Mouse L cells expressing HLA-DR4 alone (L-DR4), HLA-DR4 which covalently linked with peptide M12p54-68 (M12DR4) or with its analogues Q59G (Q59GDR4) or Y61V (Y61VDR4) were established, as described (59) and grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. 293T cells, a human renal epithelial cell line transformed by adenovirus E1A gene product and expressed SV40 large T antigen, were also grown in DMEM-based medium, as described above. Human leukemic T cell line, Jurkat E6-1 was maintained in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The linker for activation of T cells (LAT) and the SH2-containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76) deficient Jurkat cell lines, JCaM2.5 and J14 were donated from Dr. Arthur. Weiss.

7-2) Antibodies and reagents

The following antibodies were used in this study: anti-PLC-γ1 monoclonal antibody (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 antibody (Santa Cruz, CA), anti-phospho-PLC-γ1 (Tyr783) antibody (Cell Signaling Technology, Beverly, MA), goat anti-mouse IgG (PIERCE, Rockford, IL), phycoerythrin (PE)-conjugated

anti-IFN- γ mAb (Immunotech, Marseille, France), anti-CD3 ϵ mAb UCHT-1, PE-conjugated anti-CD40L mAb, PE-conjugated anti-CD69 mAb, PE-conjugated mouse IgG, anti-TCR- $\alpha\beta$ mAb, and anti-CD3 ϵ mAb (PharMingen, San Diego, CA). IntraPrep reagent for intracellular antigen staining was from Immunotech.

7-3) Plasmids, generation of pseudovirus and infection

The retroviral vector (pMX-IRES-GFP) and MLV-gagpol-IRES-bsr (60) were a kind gift from Dr. Toshio Kitamura of The University of Tokyo. The amphotropic envelope glycoprotein expression vector, SV-A-MLV-Env (61) 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 Arg¹⁹⁰ to Lys (13, 62). Fifteen μg of retroviral vector (pMX-IRES-GFP) was co-transfected with 10 μg of MLV-gag-pol expression vector (pGag-pol-IRES-bsr) and 10 μg of SV-A-MLV-Env into 293T cells using Lipofectamine 2000 reagent (Invitrogen) (Fig. 8). The supernatants were collected at 72 h after transfection. After cell debris had been removed by low speed centrifugation (2,000 g, 10 min), the supernatants were further centrifuged at 12,000 g for 12 h at 4°C and the pellets were suspended in RPMI 1640 containing 20% FCS. The viral suspension was added to 1×10^6 of T5-32 in the presence of 6 $\mu\text{g}/\text{ml}$ hexadimethrine bromide (Sigma). Expression of the recombinant proteins was monitored by green fluorescent protein (GFP) expression and Western blot analysis using an anti-FLAG antibody. Functional analyses were carried out 2 weeks after infection.

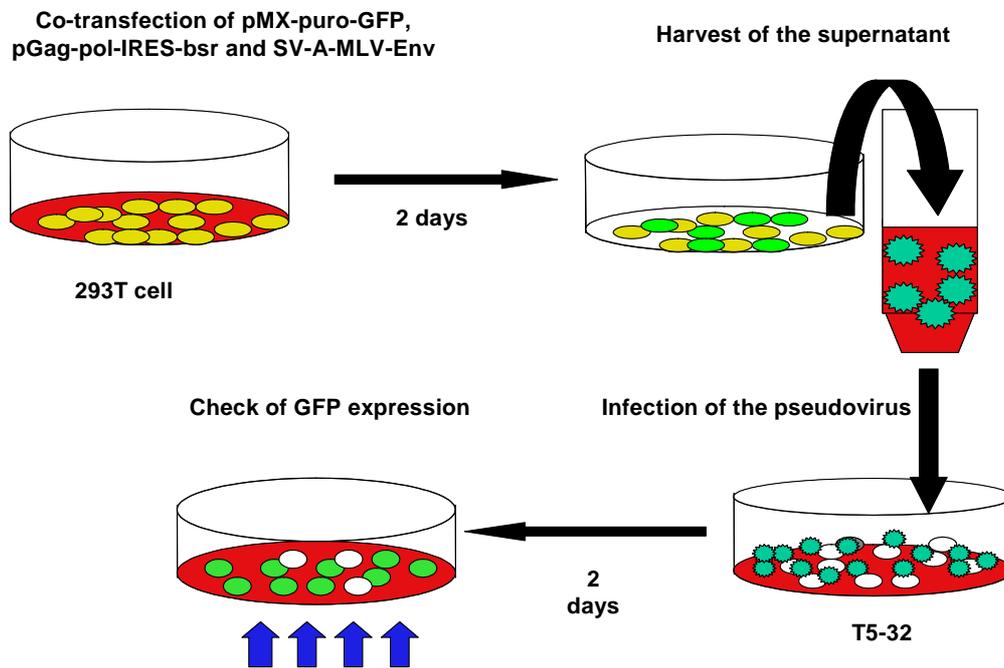


Fig. 8. Generation of pseudovirus and transduction into T5-32 cells.

7-4) Flow cytometry

T cells were stimulated by co-culturing with L cells expressing each peptide/HLA-DR4 complex in FCS free medium as described earlier (59). Surface markers were analyzed using FACScan (Becton Dickinson, Mountain View, CA) and PE-conjugated anti-CD40L mAb or anti-TCR- $\alpha\beta$ mAb. To monitor IFN- γ production, intracellular staining using IntraPrep was done according to the manufacturer's recommendations. Briefly, T5-32 was stimulated with the L cells as mentioned above in the presence of 20 $\mu\text{g/ml}$ Brefeldin A (Sigma) for 5 h. Cells were washed with ice cold phosphate buffered saline (PBS) and fixed with Reagent 1 for 15 min. After washing, the cells were permeabilized with Reagent 2 for 5 min and then incubated with PE-conjugated anti-IFN- γ for 15 min. Cells were washed with PBS and suspended with PBS containing 0.5% formaldehyde. The cells were analyzed using FACScan and CELLQuest software (Becton Dickinson).

7-5) Immunoprecipitation and Western blotting

For immunoprecipitation and Western blotting, T5-32 cells (1×10^7) stimulated with each L cell transfectant clone confluent grown in 15 cm dishes or Jurkat T cells (1×10^7) stimulated with anti-CD3 ϵ mAb were recovered and lysed on ice for 30 min in lysis buffer containing 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 1 mM Na₃VO₄, 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). Immunoprecipitates or whole cell lysates were analyzed using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked for 1h 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 2h at room temperature. Blots were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin or anti-rabbit immunoglobulin 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 antibody. Blots were visualized using enhanced chemiluminescence (ECL, Amersham Biosciences).

8. Results

8-1) Differences in the tyrosine phosphorylation of ZAP-70 and its association with TCR- ζ in T5-32 stimulated with L cell transfectant expressing each peptide/HLA-DR4 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 cell transfectant clone expressing each APL/HLA-DR4 complex, as described in the Materials and Methods. 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. 9). In contrast, stimulation with L cell 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 immune complexes, thus suggesting that unphosphorylated ZAP-70 recruited to the TCR complex could associate with the p21 form of phosphorylated TCR- ζ (Fig. 9). 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 (48-50).

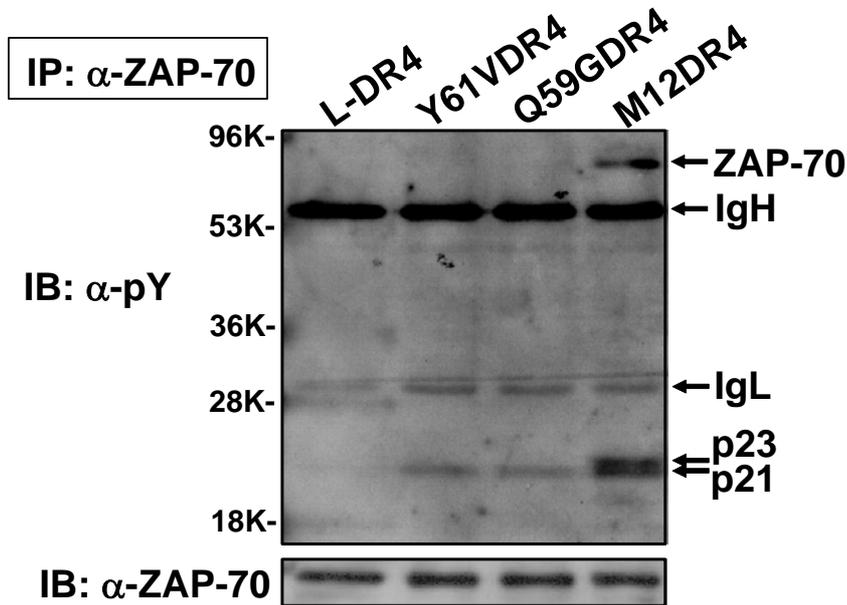


Fig. 9. The induction of tyrosine-phosphorylation of ZAP-70 after the stimulation of T cell clone (T5-32) with each L cell transfectant clone. T5-32 cells (1×10^7) were incubated with the L cell transfectant expressing each HLA-DR4/peptide complex for 5 min at 37 °C. Tyrosine-phosphorylation of TCR- ζ and ZAP-70 in the stimulated T5-32 cells were 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.

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 | NRDLE <u>Q</u> AYNELSGEA | Full agonist | Detected | p21, p23 | +++ |
| Q59GDR4 | NRDLE <u>G</u> AYNELSGEA | Partial agonist | Not detected | p21 | ++ |
| Y61VDR4 | NRDLE <u>Q</u> AVNELSGEA | 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 indicated as previously described (59). The underlined amino acid residues are substituted amino acid residues unique to APLs derived from M12p54-68 peptide. These cells express on a large amount of each single species of 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 T5-32 T cell clone was originated, as indicated in the table as we previously reported (45).

^c: According to Irie *et. al* (59).

8-2) 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. 9) nor kinase activation of ZAP-70, as previously reported by us (59), the T cell responses were thus suggested to be independent of ZAP-70. To examine this possibility, I expressed DN ZAP-70 and its non-functional control R190K in T5-32 cells using the retroviral system (Fig. 10A). 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 Arg¹⁹⁰ to Lys substitution) has no capacity to inhibit TCR signaling (62). It was previously reported that the FLVR¹⁹⁰E sequence is involved in the phosphotyrosyl binding pocket in the SH2 domain of ZAP-70 that the Arg¹⁹⁰ to Lys mutation abolished its binding to the ITAM of CD8- ζ chimeric molecule (13). The stable expression of DN ZAP-70 or R190K did not affect the expression level of endogenous ZAP-70 (Fig. 10B).

I 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. 11A).

T cells stimulated with peptide-MHC complexes undergo TCR down-modulation (48-51) and the kinase activity of ZAP-70 is involved in this phenomenon (57). As expected, the M12DR4 stimulation induced TCR down-modulation and this was markedly suppressed by the expression of DN ZAP-70 (Fig. 11B). On the other hand, expression of R190K had no inhibitory effect on the TCR down-modulation, as observed with the expression of the mock vector. These results suggest that the DN ZAP-70 in the T cells efficiently suppressed the endogenous ZAP-70 activity. 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.

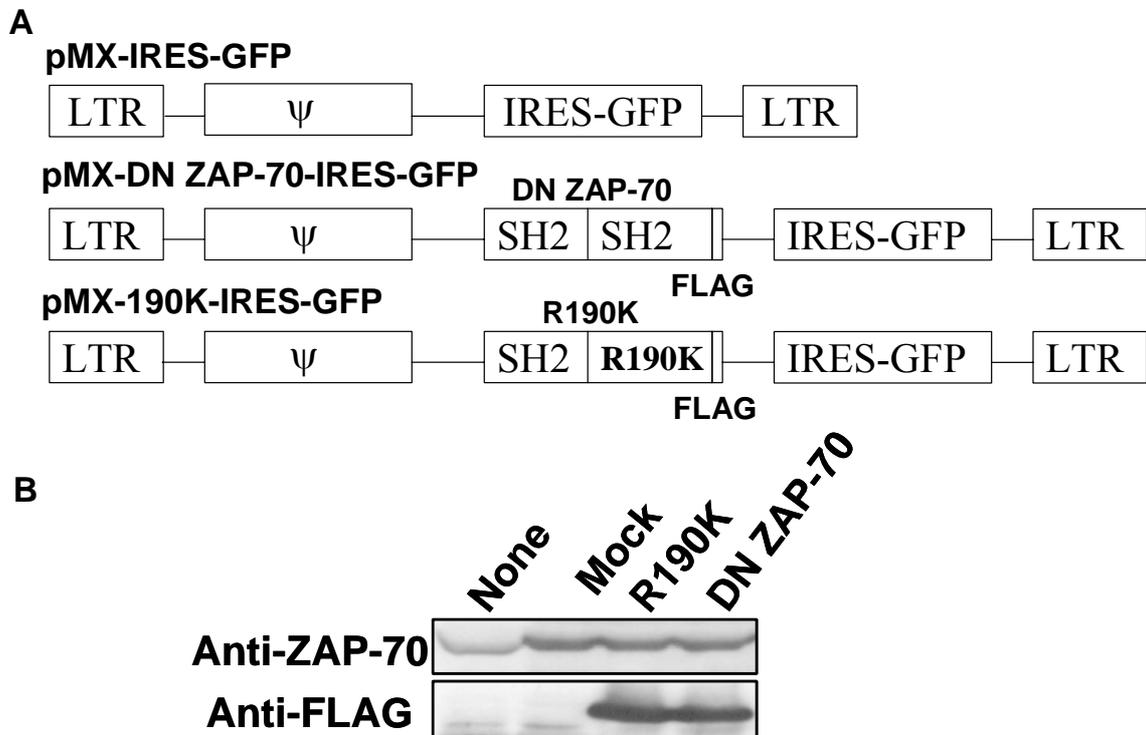


Fig. 10. The retroviral constructs used in this study and quantification of FLAG-tagged ZAP-70 mutants (lacking kinase domain) stably expressed in T cell transfectants in comparison with endogenous ZAP-70. A, FLAG-tagged DN ZAP-70 and FLAG-tagged R190K (a non-functional control for DN ZAP-70) were subcloned 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.

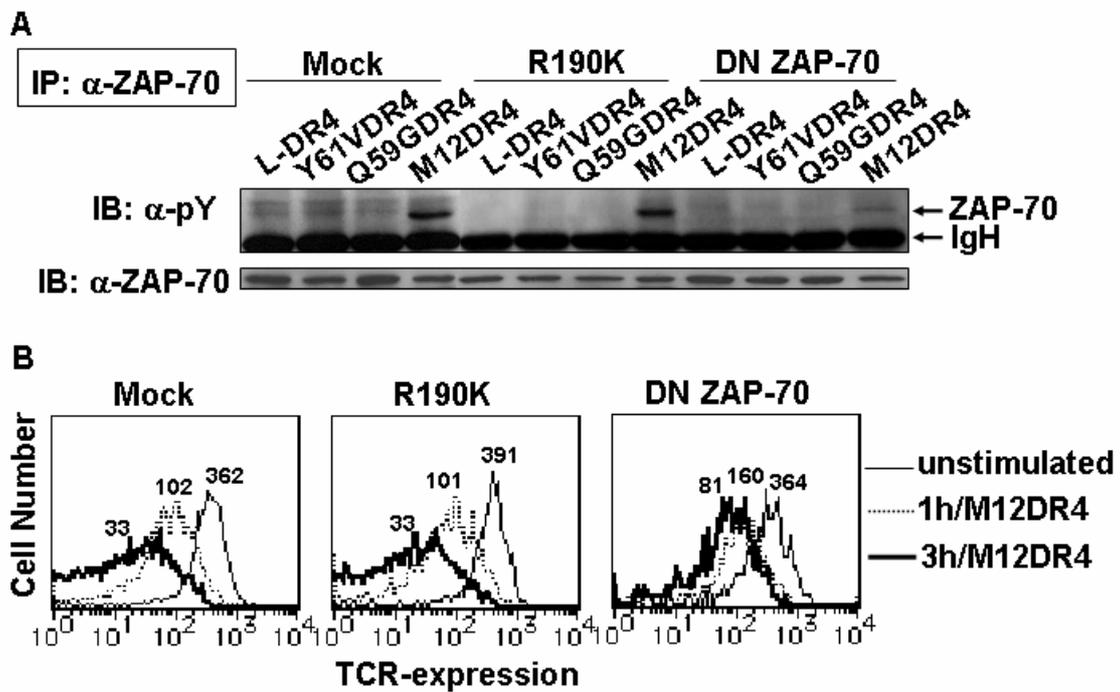


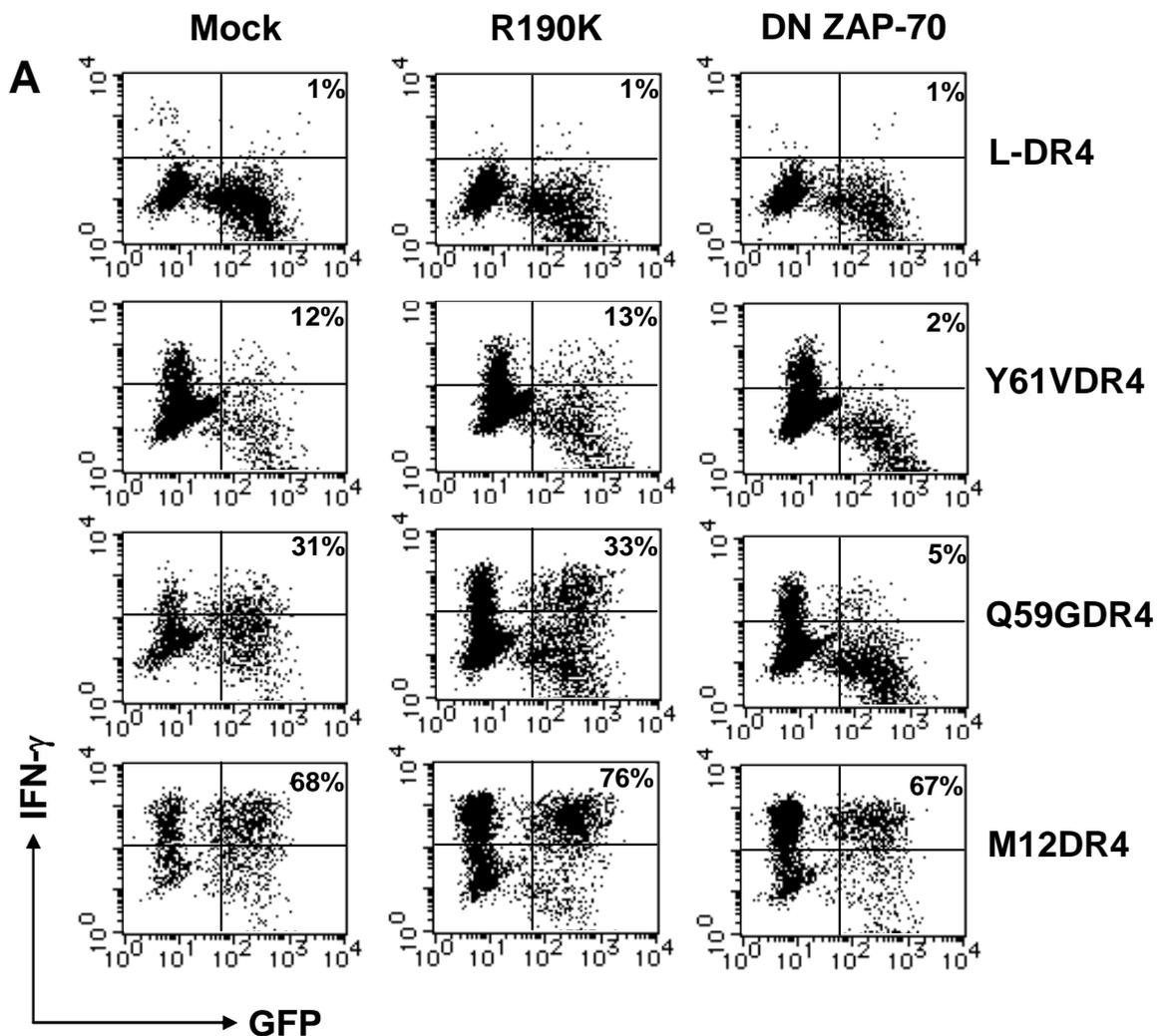
Fig. 11. The inhibitory effects of DN ZAP-70 on T cell activation. A, The inhibition of endogenous ZAP-70 phosphorylation in T cells expressing DN ZAP-70. After 72 h of infection with retroviral vectors, 3×10^6 T cells (60% of cells were positive for GFP) were stimulated with each L cell transfectant 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.

8-3) Inhibition by DN ZAP-70 of TCR mediated IFN- γ production, CD40L up-regulation, and TCR down-modulation in T cells stimulated with OPALs

In the previous study, the stimulation with some OPALs induced IFN- γ production in the cognate T cells without kinase activation of ZAP-70. Therefore, I 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. 12A). 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 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. 12A). 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, I 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 μ M of fully agonistic peptide, M12p54-68 for 16 h at 37 °C. As

shown in Fig. 12B, 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 (59), L cells expressing HLA-DR4 prepulsed with 50 μ M of 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.



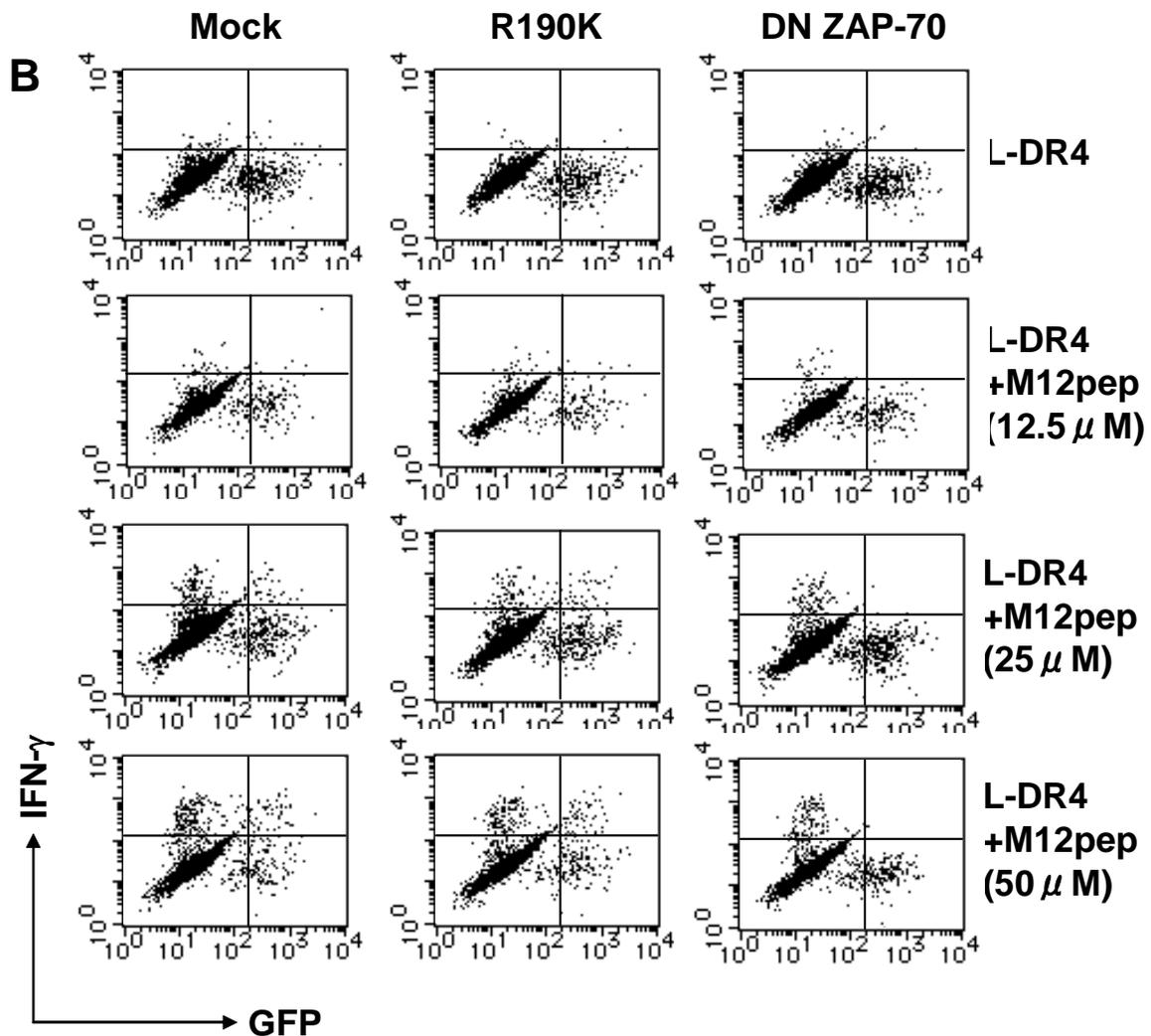


Fig. 12. Inhibition by DN-ZAP-70 of IFN- γ production in T cells stimulated with each L cell transfectant. After the infection of the retroviral vectors, T cells were cultured for 2 weeks and then were stimulated with each L cell transfectant. Intracellular IFN- γ was detected using flow cytometry after 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 representatives from three independent and reproducible experiments.

The CD40 ligand (CD40L, gp39 or CD154) is a member of the TNF gene family and it is up-regulated in activated T cells (63-67). As shown in Fig. 13, Q59G was able to induce a strong up-regulation of CD40L comparable to that induced with M12DR4. In contrast, the other OPAL, Y61VDR4, induced only small up-regulation of CD40L. The DN ZAP-70 expression effectively reduced the CD40L up-regulation in T cells stimulated with these OPALs (Q59GDR4 and Y61VDR4) while the expression of R190K was almost completely ineffective. The expression of DN ZAP-70 was less effective in the M12DR4-stimulated T cells although the inhibitory effect of DN ZAP-70 was slightly detectable. These data also indicate that the binding of ZAP-70 to TCR- ζ is involved in the CD40L up-regulation stimulated with OPAL.

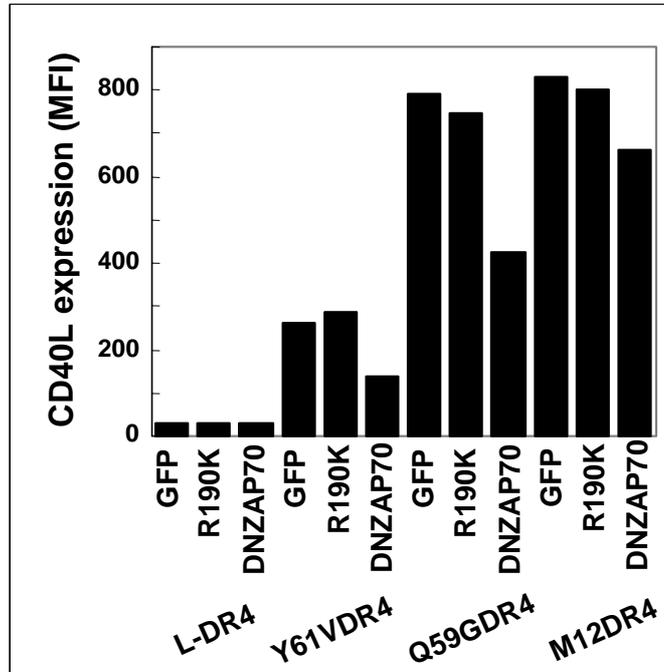


Fig. 13. Inhibitory effects of DN ZAP-70 on CD40L up-regulation. After infection of the retroviral vectors, T cells were cultured for 2 weeks and then were stimulated with each L cell transfectant. Surface CD40L was stained with PE-conjugated anti-CD40L mAb after 18h incubation, and the levels of CD40L expression are indicated as MFI of histograms.

To further investigate the effect of DN ZAP-70 on OPAL stimulation, I 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-regulation as shown in Fig. 14. In contrast, the stimulation of OPALs (Q59GDR4 and Y61VDR4)

induced a weak but definite down-modulation of TCR. 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, CD40L up-regulation, and TCR down-modulation in T cells stimulated with OPALs.

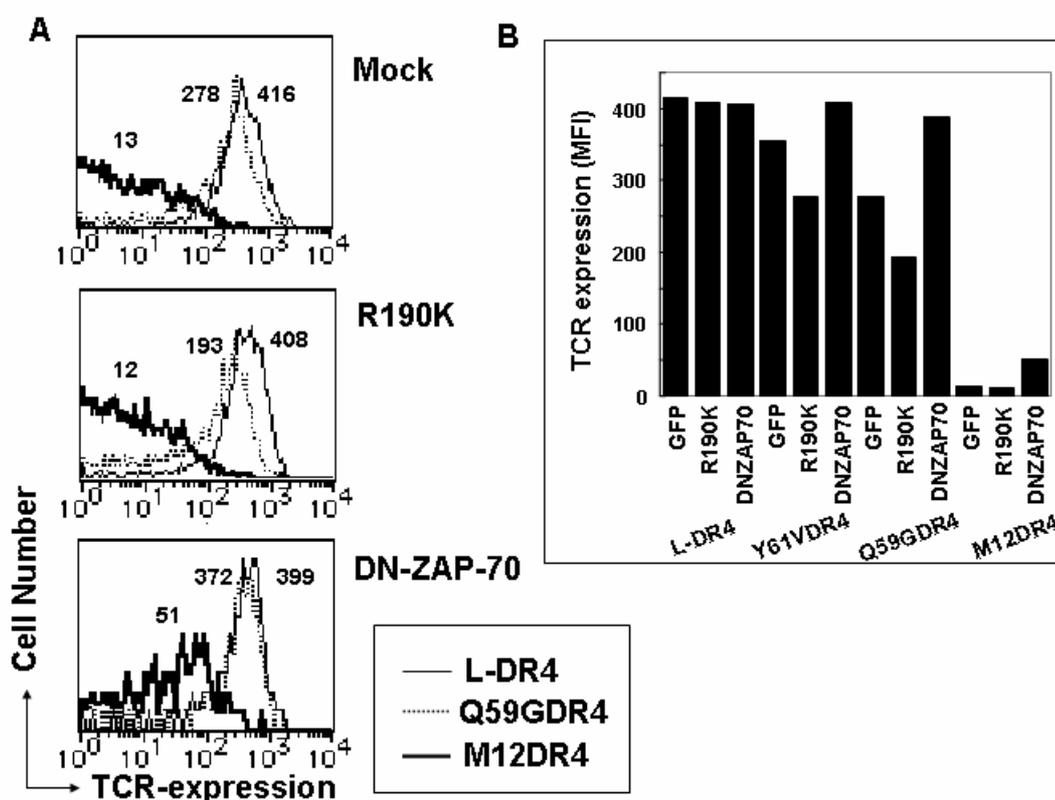


Fig. 14. Inhibition of TCR down-modulation in T cells stimulated with each L cell transfectant by DN-ZAP-70 expression. After the infection of the retroviral vectors, T cells were grown for 2 weeks and then were stimulated with each L cell transfectant. The inhibitory effect on TCR down-modulation was shown by histogram (A) and its MFI level (B). TCR was stained with anti-TCR- $\alpha\beta$ mAb after 6 h stimulation. GFP-positive cells were gated and analyzed. The results are representative of three independent and reproducible experiments.

8-4) 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 endogenous ZAP-70 from binding to tyrosine-phosphorylated TCR- ζ (13, 62). 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 findings shown in Fig. 9, the anti-FLAG mAb co-precipitated only the p21 form of phosphorylated TCR- ζ from the lysate of T cells stimulated with Q59GDR4 (Fig. 15). 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 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.

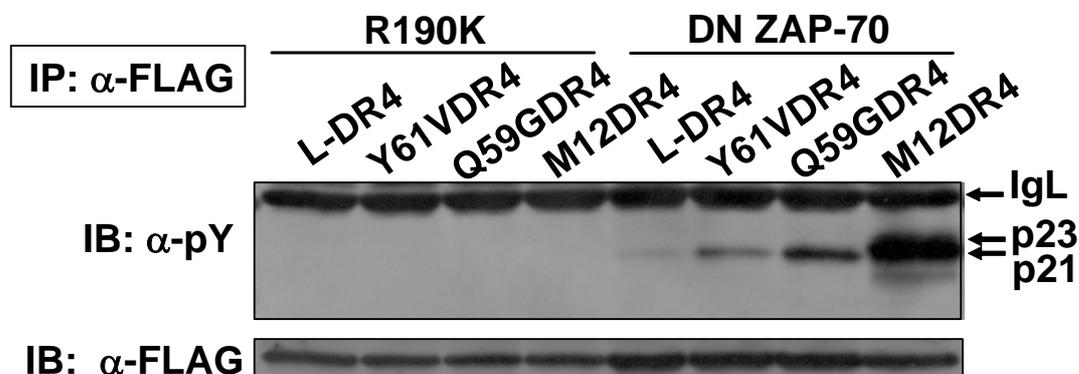


Fig. 15. The association of phosphorylated TCR- ζ with DN ZAP-70. T cells expressing R190K or DN ZAP70 were stimulated for 5 min with each L cell transfectant. R190K or DN ZAP-70 was immunoprecipitated by anti-FLAG mAb (α -FLAG) and phosphorylated forms of TCR- ζ associated with DN ZAP-70 were immunoblotted with the anti-phosphotyrosine mAb (α -pY). The blots were reprobed with anti-FLAG mAb.

8-5) The inhibitory effects of DN ZAP-70 on PLC- γ 1 phosphorylation

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, I investigated tyrosine phosphorylation of PLC- γ 1 in the stimulated T cells. As shown in Fig. 16, M12DR4-stimulation induced an intense tyrosine-783 phosphorylation of PLC- γ 1. Q59GDR4-stimulation also induced an increased tyrosine phosphorylation of PLC- γ 1 in comparison to that of the T cells co-cultured with L-DR4. The tyrosine-783 phosphorylation of PLC- γ 1 was suppressed in DN ZAP-70 expressing T cells stimulated with Q59GDR4 and M12DR4. This observation suggests that DN ZAP-70 associated with tyrosine phosphorylated TCR- ζ inhibits the tyrosine phosphorylation of PLC- γ 1 in T cells stimulated with M12DR4 and Q59GDR4.

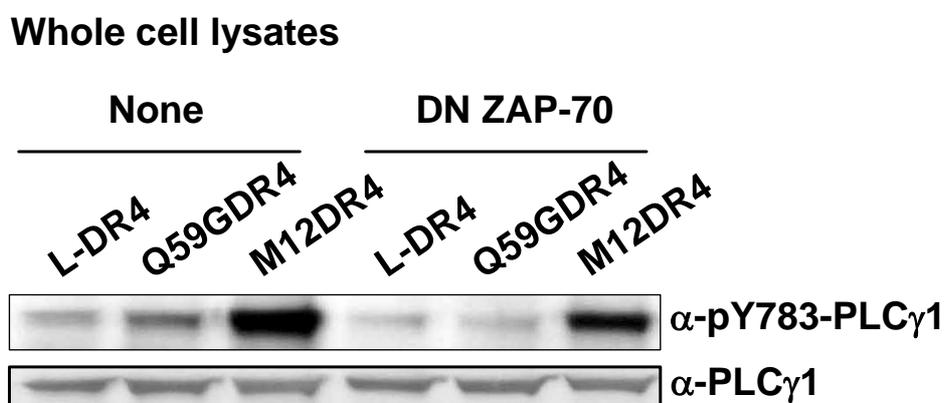


Fig. 16. The inhibitory effects of DN ZAP-70 on PLC- γ 1 phosphorylation. After 72 h of infection, 3×10^6 T cells (about 50 % cells were positive for GFP) were stimulated with each L cell transfectant for 10 min at 37 °C. Whole cell lysates were immunoblotted with anti-phospho-PLC- γ 1 (Tyr783) Ab (α -pY783-PLC- γ 1). The same blot was reprobed with anti-PLC- γ 1 mAb (α -PLC- γ 1) to confirm the equal loading. The data shown are representative results from three independent and reproducible experiments.

8-6) The association of PLC- γ 1 with ZAP-70

To check the possibility that ZAP-70 could help PLC- γ 1 phosphorylation by recruiting it to TCR complexes, I investigated the phosphorylation of PLC- γ 1 and its association with ZAP-70. PLC- γ 1 phosphorylation in M12DR4 stimulated T cells was stronger than that in OPAL stimulated T cells. Notably, ZAP-70 was co-immunoprecipitated with PLC- γ 1 using anti-PLC- γ 1 mAb in T cells stimulated with M12DR4, Q59GDR4, or Y61VDR4 (Fig. 17A). Although no phosphorylation of ZAP-70 was observed in the T cells stimulated with OPALs, the level of ZAP-70/PLC- γ 1 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- γ 1 association results in the phosphorylation of PLC- γ 1 leading to the T cell responses.

The association of PLC- γ 1 with ZAP-70 is not specific to the T5-32 cells. The association of PLC- γ 1 with ZAP-70 could be also detected in Jurkat T cells stimulated with anti-CD3 mAb, but not in unstimulated cells (Fig. 17B). ZAP-70 was co-precipitated with PLC- γ 1 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- γ 1 mAb immunoprecipitation. These results indicate that PLC- γ 1 associates with ZAP-70 in TCR stimulated T cells even in the absence of LAT or SLP-76.

8-7) Inhibition of T cell activation with PLC-specific inhibitor U-73122

Since the phosphorylation of PLC- γ 1 and its association with ZAP-70 was seen in OPAL stimulated T cells, I investigated whether the activation of PLC- γ 1 is involved in the T cell activation stimulated with OPALs. I therefore utilized the PLC-specific inhibitor U-73122 to investigate its effects on the T cell activation. The previous reports were already reported that U-73122 inhibited proliferation and IFN- γ production in T5-32 stimulated with Q59GDR4 as well as M12DR4 (59). In this study, U-73122 inhibited the up-regulations of CD69 and CD40L in T cells stimulated with Q59GDR4 or M12DR4 (Fig. 18). The inhibitory effects on the T cell responses to Q59GDR4

stimulation were more prominent with a high concentration (1 μ M) of U-73122 than those with M12DR4 stimulation. As a result, I confirmed that the activation of the PLC- γ 1 stimulated with OPAL was involved in T cell activation.

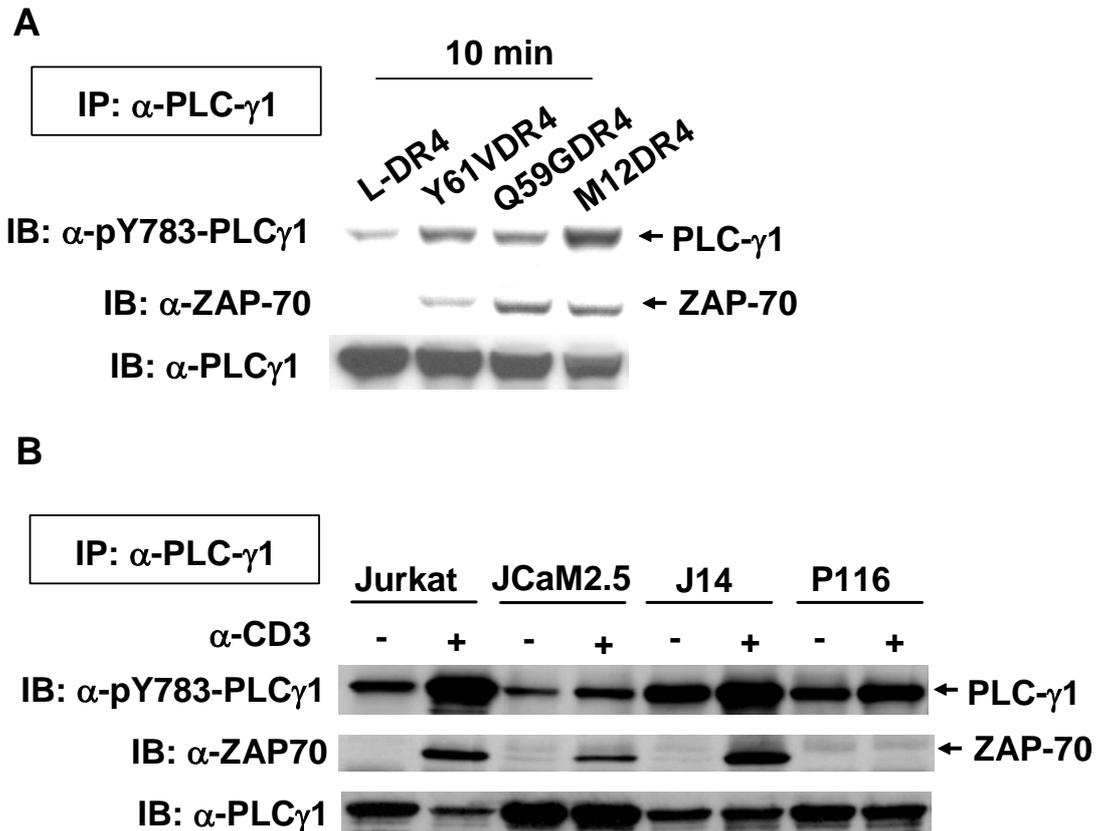


Fig. 17. The physical association of PLC- γ 1 with ZAP-70 in TCR stimulated T cells. A, The association of PLC- γ 1 with unphosphorylated ZAP-70 in OPAL stimulated T cells. T cells (5×10^6) were stimulated with each L cell transfectant for 10 min, lysed, and subjected to immunoprecipitation using anti-PLC- γ 1 mAb. As a control, T cells stimulated with L-DR4 were used. The anti-PLC- γ 1 immunocomplexes were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho-PLC- γ 1 (Tyr783) antibody (α -pY783-PLC- γ 1). The blots were reprobbed with anti-PLC- γ 1 mAb. B, The association of PLC- γ 1 with ZAP-70 in Jurkat cells. Cells were stimulated with anti-CD3 ϵ mAb or left untreated and goat anti-mouse IgG on ice for 30 min and incubated at 37 $^{\circ}$ C for 1 min. Cells were lysed and immunoprecipitated with anti-PLC- γ 1 mAb. The immunoprecipitates were subjected to Western blot analysis using anti-phosphoTyr783-PLC- γ 1 (top) and anti-ZAP-70 mAb (middle). The membrane was reprobbed with anti-PLC- γ 1 mAb (bottom).

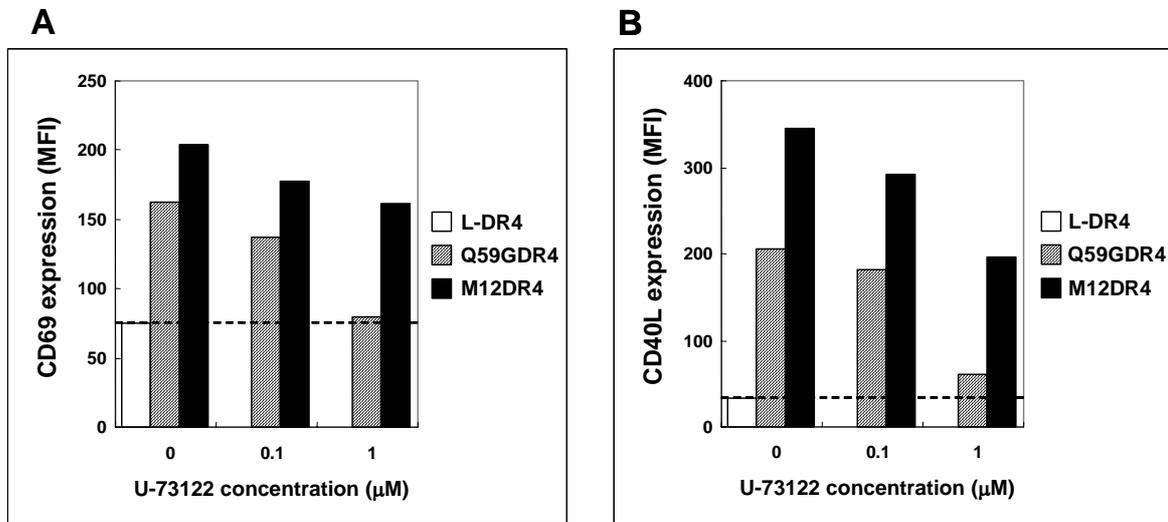


Fig. 18. The inhibition with the PLC inhibitor U-73122 of up-regulation of CD69 and CD40L induced by T cell activation. T5-32 cells (1×10^6) were pretreated with the indicated concentrations of U-73122 for 1 h in FCS free medium and co-cultured with L-DR4, Q59GDR4, and M12DR4 for 8 h. The CD4⁺ cells were gated and analyzed. A, Effects on the expression level of CD69. Surface CD69 was stained with PE-conjugated anti-CD69 mAb. B, Effects on expression level of CD40L. Surface CD40L was stained with PE-conjugated anti-CD40L mAb. The dotted lines indicate the MFI levels of the markers expressed on the T cells co-cultured with L-DR4.

9. Discussion

Many models of T cell activation have been proposed to explain the differential effects between by the stimulation of the full agonist and by the APLs. The previous studies have shown that single amino acid substitutions in the antigenic peptide can affect a range of the responses such as the 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 (45). 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 *Herpesvirus saimiri* to establish T5-32 (manuscript in preparation, H. Tsukamoto *et al.*) and as described in our previous paper (59). 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 could be successfully established using this retroviral system. To further understand the complex T cell activation, I compared the inhibitory effects of DN ZAP-70 between on M12DR4 and on OPAL (Q59GDR4 and Y61VDR4) stimulations. DN ZAP-70 could associate with both p23 form and p21 form of phosphorylated TCR- ζ resulting in its inhibitory effects (Fig. 15). 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 (Fig. 11).

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 an inhibitory effect on IFN- γ production in DN ZAP-70 expressing T cells stimulated with M12DR4

(Fig. 12A) suggests the following possibility; The T cell activation stimulated with M12DR4 highly over-expressing HLA-DR4/M12p54-68 peptide 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. 12B).

I 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, I 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 (48-51). While full agonists induce the maximal degree of TCR down-modulation, partial agonists induce a lower level of TCR down-modulation (48, 49) and this phenomenon correlates with the activity of ZAP-70 (57). 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 (57). As expected, an intense stimulator M12DR4 can induce a strong TCR down-modulation and OPALs (Q59GDR4 and Y61VDR4) can induce a weak but definite TCR down-modulation (Fig. 14).

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. 12) and CD40L up-regulation (Fig. 13), and also an inhibition of the TCR down-modulation (Fig. 14). In addition, unphosphorylated ZAP-70 associated with partially phosphorylated TCR- ζ in OPAL stimulated T cells. These observations suggested that ZAP-70 itself might have a scaffold function to recruit other signaling molecules to the TCR complex regardless of its kinase activity. As one such candidate molecule, I chose PLC- γ 1 and examined the tyrosine phosphorylation of PLC- γ 1 in T cells expressing DN ZAP-70 stimulated with the OPAL,

because 1) the previous data provided evidence that activation of protein kinase C μ was involved in the T cell activation stimulated with Q59GDR4, thus suggesting the production of diacylglycerol by PLC- γ 1 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 (59). As a result, I found that PLC- γ 1 phosphorylation induced in TCR-stimulated T cells was inhibited in the presence of DN ZAP-70 (Fig. 16).

PLC- γ 1 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 (59) and I observed the association of PLC- γ 1 with ZAP-70 even in LAT or SLP-76 deficient Jurkat T cells stimulated with anti-CD3 ϵ mAb (Fig. 17). Therefore, LAT and SLP-76 seemed to be dispensable for the PLC- γ 1/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- γ 1 (25). In addition, even the unphosphorylated ZAP-70 peptide also shown to be weakly associated with the PLC- γ 1 C-terminus SH2 protein. Therefore, it seems feasible that unphosphorylated ZAP-70 and PLC- γ 1 are thus directly associated through the interdomain B of ZAP-70 and C-terminus SH2 of PLC- γ 1. In our experimental system, how unphosphorylated ZAP-70 and PLC- γ 1 associate remains to be elucidated in OPAL stimulated T cells. Although the tyrosine phosphorylation of ZAP-70 was undetectable, an increased PLC- γ 1/ZAP-70 association was detected in OPAL stimulated T cells compared with the findings in unstimulated T cells. Therefore, I presume that the PLC- γ 1/ZAP-70 association correlates with the phosphorylation of PLC- γ 1.

I presume the existence of the following steps in the T cell response to partial agonist; (1) the incomplete phosphorylation of TCR- ζ , (2) the recruitment and binding of ZAP-70 to TCR- ζ , (3) the association of PLC- γ 1 to ZAP-70, (4) the phosphorylation of PLC- γ 1 by some other kinase such as Lck or Fyn, and (5) the activation of down stream signaling molecules by PLC- γ 1.

10. Conclusions

I investigated whether unphosphorylated ZAP-70 is required for the activation of T cell clone in response to OPAL by introduction of DN ZAP-70. My findings suggest that the association of unphosphorylated ZAP-70 with the incompletely phosphorylated TCR- ζ chain is necessary for tyrosine phosphorylation of PLC- γ 1, and that this may result in IFN- γ production, CD40L up-regulation, and TCR down-modulation in the T cells stimulated with 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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