Unique T cell proliferation associated with PKC\(\mu\) activation and impaired ZAP-70 phosphorylation in recognition of overexpressed HLA/partially agonistic peptide complexes

Atsushi Irie, Yu-Zhen Chen, Hirotake Tsukamoto, Toko Jotsuka, Masako Masuda and Yasuharu Nishimura

Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan

Altered peptide ligands (APL) induce T cell responses different from those induced by the original agonistic peptide. As shown for CD4+ T cells, partial agonists induce partial T cell activation without proliferation because of lower affinities and higher off rates to TCR than those of agonists. To determine whether overexpression of partially agonistic TCR ligands on antigen-presenting cells provides high-avidity TCR ligands, we generated L cell transfec-
tants expressing various numbers of HLA-DR4 covalently linked with APL derived from a streptococcal peptide and observed responses of the cognate T cells. Some overexpressed HLA-DR4/partially agonistic APL complexes induced T cell proliferation in a density-
dependent manner. However, tyrosine phosphorylation of zeta-associated protein-70 (ZAP-70) and linker for activation of T cells and kinase activity of ZAP-70 were not detectable. T cell proliferation stimulated with L cell transfectants was sensitive to the PKC inhibitor Gö6976, but to a lesser extent to Gö6983, suggesting the involvement of \(\mu\) isotype of PKC (PKC\(\mu\)). In vitro kinase assays revealed that PKC\(\mu\) activity was up-regulated only in T cells stimulated with L cell transfectants that induced T cell proliferation. Our data suggest the presence of a unique signaling pathway coupling TCR ligation with T cell proliferation associated with PKC\(\mu\) activation and impaired ZAP-70 activation.

Key words: Altered peptide ligand / Protein kinase C\(\mu\) / Protein kinase D / T cell activation / ZAP-70

1 Introduction

CD4+ T cells recognize antigenic peptides in the context of self-MHC class II and are activated to secrete cyto-
kines, to up-regulate expression of various cell surface molecules and finally to proliferate. Analogues of the antigenic peptides (or altered peptide ligands, APL) car-
ying single amino acid substitutions induce T cell responses different from those induced by the original peptide [1–3]. There are four known types of T cell responses to APL; full and partial agonism, simple antagonism and null. Partial agonists induce partial acti-
vation of T cells such as IL-4 production [1], H+ release [4], increase in intracellular Ca\(^{2+}\) concentrations [4–6], cell volume increase and up-regulation of T cell activa-
tion markers [7, 8] without causing proliferation, and cell survival [9].

Partial agonists cannot induce full CD4+ T cell activation, as explained by the so-called “kinetic model”. MHC complexed with partially agonistic or simply antagonistic peptides have a lower binding affinity to TCR and a larger off rate, as compared with bindings with agonistic pep-
tides [10–13]. Hence, there may be insufficient time and/or number of recruited interactions between TCR and their ligands so as not to provoke full activation signals in T cells. The question raised is whether the partial ago-
nists and simple antagonists can overcome their lower binding affinity to TCR if present in excessive amounts and become agonistic to induce T cell proliferation. To address this question, we took advantage of (1) the well-characterized human CD4+ T cell clone YN5–32, which recognizes a streptococcal peptide M12p54–68 in the
context of HLA-DR4 [6, 8, 14], and (2) mouse L cells expressing various amounts of surface HLA-DR4 molecules with covalently linked M12p54–68 or its single-amino-acid-substituted APL. Using the T cell clone and the L cell transfectants, we investigated the relationship among T cell responses, activation of signaling molecules and density of various TCR ligands in this study.

2 Results

2.1 Expression of HLA-DR4 molecules with covalently linked APL on the surface of L cell transfectants

We investigated detailed responses of the human CD4⁺ T cell clone YN5–32 to 156 single-amino-acid-substituted APL derived from streptococcal M12p54–68 [8]. We found that a number of APL induced partial activation of YN5–32 cells and stimulated increase in cell volume and up-regulation of CD markers, without inducing proliferation. We then asked whether these partially agonistic TCR ligands could induce T cell proliferation if over-expressed on APC. We generated a series of mouse L cell transfectants expressing various HLA-DR4 and M12p54–68-derived APL complexes and investigated the proliferative responses of YN5–32 cells. The APL used and their antigenicities are summarized in Table 1. Fig. 1 shows flow cytometric profiles of HLA-DR expression in each L cell transfectant. M12DR4 cells are L cells expressing HLA-DR4 with covalently linked wild-type M12p54–68. E58N, Q59G, Y61V, E63V, E63S and E63R cells are L cells expressing HLA-DR4 with covalently linked respective APL. L-DR4 cells and CD20DR4 cells express HLA-DR4 alone and HLA-DR4 with covalently linked irrelevant human CD20p26–45 with binding capacity to HLA-DR4 [15], respectively. The estimated numbers multiplied by 10⁻⁴ of HLA-DR4/peptide complexes per L cell are indicated in Fig. 1. The number of HLA-DR4/peptide complexes on the surface of L mock cells transfected with empty vectors, was assigned to be zero.

![Fig. 1. Expression profiles of HLA-DR in L cell transfectants.](image)

---

Table 1. Sequences of M12p54–68 and its analogue peptides used in this study

<table>
<thead>
<tr>
<th>peptide</th>
<th>sequence</th>
<th>antigenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M12p54–68</td>
<td>NRDLEQAYNELSGEA</td>
<td>full agonist</td>
</tr>
<tr>
<td>E58N</td>
<td>NRDLEQAYNELSGEA</td>
<td>partial agonist</td>
</tr>
<tr>
<td>Q59G</td>
<td>NRDLEQAYNELSGEA</td>
<td>partial agonist</td>
</tr>
<tr>
<td>Y61V</td>
<td>NRDLEQAYNELSGEA</td>
<td>partial agonist</td>
</tr>
<tr>
<td>E63S</td>
<td>NRDLEQAYNELSGEA</td>
<td>partial agonist</td>
</tr>
<tr>
<td>E63V</td>
<td>NRDLEQAYNELSGEA</td>
<td>partial agonist</td>
</tr>
<tr>
<td>E63R</td>
<td>NRDLEQAYNELSGEA</td>
<td>simple antagonist</td>
</tr>
</tbody>
</table>

a) Substituted amino acid residues of analogue peptides are underlined.
b) According to results on stimulatory activities of peptide-pulsed APC reported in [8].
2.2 HLA-DR4 molecules with covalently linked partially agonistic APL induced T cell proliferation that depended on expression levels

Using L cell transfectants, we examined T cell proliferative responses to HLA-DR4/peptide complexes in the absence of exogenously added peptide (Fig. 2). L-DR4 cells pulsed with these APL up to 200 μM induced no T cell proliferation [8]. In Fig. 2 and hereafter, numbers in parentheses following the clone names indicate the numbers of the HLA-DR4/peptide complex ×10⁻⁴/cell. L-DR4(65) cells prepulsed with 50 μM of M12p54–68 induced a strong T cell proliferative response, while L-DR4(65) cells alone did not. M12DR4 cells, which express HLA-DR4 covalently linked with fully agonistic M12p54–68, stimulated the plateau level T cell responses even at 2.3×10⁵/HLA-DR4/peptide complexes/cell. Interestingly, the HLA-DR4 complexed with the partially agonistic Q59G peptide induced T cell proliferation in a density-dependent manner. This ligand stimulated detectable T cell responses even at 3×10⁴/cell, and Q59G(171) cells induced full response comparable to M12DR4 cells and L-DR4(65) cells prepulsed with 50 μM of M12p54–68. The HLA-DR4 complexed with partially agonistic E63S or E63V peptide induced no T cell proliferation at a relatively lower number, 2×10⁴–6×10⁴/cell. However, when increasing the surface number (9×10⁴–2×10⁵/cell), these cells induced a weak but definite T cell proliferation (Fig. 2). HLA-DR4 complexed with other partially agonistic ligands, E58N and Y61V, did not induce T cell proliferation, even at 1×10⁵–3×10⁵/cell. Therefore, not all but some partially agonistic TCR ligands can induce T cell proliferation if present at high density, and it seems likely that each partially agonistic APL has a different potential to become agonistic. On the other hand, HLA-DR4 complexed with the simply antagonistic E63R peptide did not stimulate T cell proliferation even at 8×10³/cell (Fig. 2). CD20DR4 cells, which express a relatively large number (1.4×10⁶/cell) of HLA-DR4/CD20p26–45 complexes, had no apparent effect.

2.3 Cytokine production by T cells co-cultured with L cell transfectants

We next checked the cytokine production by YN5–32 cells stimulated with the L cells using ELISA. Full IL-2, IL-4 and IFN-γ production was observed in T cells stimulated with L-DR4(65) cells pulsed with 50 μM M12p54–68 and with M12DR4(235) cells (Fig. 3). The Q59G(171) cells induced production of these cytokines to a comparable or a slightly lesser extent. The E63V(219) cells stimulated lower levels of IL-4 and IFN-γ production, which was consistent with the weaker proliferative T cell responses (Fig. 2). Although IL-2 production was not detected in T cells stimulated with E63V(219) cells, the proliferative response seemed to be in part IL-2-dependent, since saturating amounts of neutralizing anti-IL-2 (50 μg/ml) mAb partially inhibited the proliferative response to E63V(219) cells (Fig. 3D). In addition, E63V(219) cells, as well as M12DR4(235) and Q59G(171) cells, stimulated the up-regulation of IL-2 mRNA in T cells as revealed by ribonuclease protection assay (RPA). The cytokines were not detectable in supernatants from coculture with E58N(122) or L-DR4(65) cells.

Induction of cytokine mRNA in YN5–32 cells stimulated with the L cell transfectants were analyzed using RPA. Up-regulation of IL-4, IL-5, IL-10 and IL-13 mRNA was detected in T cells co-cultured with M12DR4(235), Q59G(171) and E63V(219) cells at either 4 or 16 h after co-culture and, except for IL-4 mRNA, the up-regulation persisted for up to 48 h (Fig. 3E). Y61V(243) cells, which did not induce T cell proliferation, stimulated a slight up-regulation of cytokine mRNA while Lmock and CD20DR4(142) cells did not. These data indicate that stimulation with overexpressed HLA-DR4/partially ago-
Fig. 3. Profiles of cytokine produced by T cells stimulated with L cell transfectants. After starting T cell and L cell co-culture, IL-2 (A), IL-4 (B) and IFN-γ (C) concentrations in the culture supernatant at the indicated period were determined, using ELISA. L cells used were: L-DR4(65) only (closed triangles), M12DR4(235) (open squares), Q59G(171) (closed circles), E63V(219) (closed squares), E58N(122) (closed diamonds) and L-DR4(65) prepulsed with 50 μM M12p54–68 (open triangles). (D) Inhibitory effects of neutralizing anti-IL-2 mAb (50 μg/ml) on the T cell proliferation. (E) Three micrograms of total RNA from YN5–32 cells (1×10⁶) co-cultured with each L cell transfectant for the indicated period was subjected to RPA. Ribosomal L32 and cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal controls. Yeast tRNA was used for the background control. M12DR4(235), E63V(219) and Q59G(171) cells, which stimulated T cell proliferation, induced similar up-regulation profiles of the indicated cytokine mRNA in the T cells over the stimulation period. Although Y61V(243) cells did not stimulate T cell proliferation, they induced a definite up-regulation of IL-4, IL-5, IL-10 and IL-13 mRNA in the T cells.

nistic APL complexes that induce T cell proliferation also induced gene expression of at least IL-4, IFN-γ and IL-2, as did the M12DR4(235) cells.

2.4 Phosphorylation of zeta-associated protein-70 and linker for activation of T cells was impaired in T cells stimulated with Q59G(171) or E63V(219) cells

Some overexpressed HLA-DR4/partially agonistic APL complexes stimulated both T cell proliferation and cytokine production. However, they did not induce tyrosine phosphorylation of zeta-associated protein-70 (ZAP-70) and linker for activation of T cells (LAT) at detectable levels in the T cells. Hereafter, we used Herpesvirus saimiri (HVS)-transformed YN5–32 cells to prepare a relatively large number of clonal human T cells for biochemical experiments. As shown in Fig. 4A, phospho-ZAP-70 was only detected in T cells stimulated with M12DR4(235) cells, but not in T cells stimulated with Q59G(171) or with E63V(219) cells. Phospho-ZAP-70 was also not detected in T cells stimulated with Lmock, Y61V(243) or CD20DR4(142) cells. LAT, the immediate substrate for ZAP-70, was also tyrosine-phosphorylated only in T cells stimulated with M12DR4(235) cells (Fig. 4B). Phospho-ZAP-70 was also observed in normal YN5–32 cells co-cultured with M12DR4(235) cells or L-DR4(65) cells pulsed with M12p54–68 (200 μM) for 30 min, but neither with Q59G(171) nor with E63V(219) cells (Fig. 4C); this indicating that the ZAP-70 phosphorylation shown in Fig. 4A was neither a specific event for the HVS-transformed YN5–32 cells nor for the artificial HLA-DR4 molecules with covalently linked peptide. In parallel experiments, Q59G(171) cells induced comparable proliferative responses of YN5–32 cells with M12p54–68-pulsed L-DR4(65) and M12DR4(235) cells (Fig. 4C).

Undetectable phosphorylation of ZAP-70 did not seem to be due to retarded kinetics since phospho-ZAP-70 was not detected for up to 2.5 h after a co-culture with Q59G(171) cells in HVS-transformed YN5–32 cells (Fig. 4D). On the other hand, phospho-ZAP-70 was detected as early as 5 min of co-culture with
Fig. 4. Phosphorylation of ZAP-70 and LAT was impaired in T cells stimulated with Q59G(171) or E63V(219) cells that induce proliferative T cell responses. ZAP-70 (A) or LAT (B) immunoprecipitated from HVS-transformed YN5–32 cells co-cultured with each L cell transfectant for 5 min were subjected to SDS-PAGE and Western blots. The membranes were probed with an anti-pY mAb (A, B; upper panels) and reprobed with an anti-ZAP-70 mAb (A, lower panel) or with an anti-LAT antibody (B, lower panel). (C) Normal YN5–32 cells were co-cultured with L-DR4 cells alone or prepulsed with M12p54–68 (200 μM), M12DR4(235), E63V(219) or Q59G(171) cells for 30 min. Each T cell lysate was subjected to ZAP-70 immunoprecipitation and Western blots probed with an anti-pY mAb (top) and reprobed with an anti-ZAP-70 mAb (middle). The results of parallel experiments for T cell proliferation are shown (bottom). Phospho-ZAP-70 was detected only in T cells stimulated with M12DR4(235) cells or with L-DR4 cells prepulsed with M12p54–68. (D) T cells were co-cultured with M12DR4(235) or Q59G(171) cells for the indicated period and subjected to ZAP-70 immunoprecipitation and Western blots probed with an anti-pY mAb (upper panels) and reprobed with an anti-ZAP-70 mAb (lower panels). (E) Lysates from T cells co-cultured with respective L cell transfectants for 10 min were immunoprecipitated with an anti-ZAP-70 antibody. The kinase activity in the immunocomplexes was determined in \textit{in vitro} kinase assays using GST-LAT and GST-band 3 as substrates (top). Equal amounts of ZAP-70 in the immunocomplexes were confirmed by Western blot analyses (bottom). ZAP-70-pY: tyrosine-phosphorylated ZAP-70, LAT-pY: tyrosine-phosphorylated LAT, pGST-LAT: phosphorylated GST-LAT, pGST-band3: phosphorylated GST-band 3.

M12DR4(235) cells and persisted for up to 2.5 h. The total amount of ZAP-70 was down-regulated in T cells stimulated with M12DR4(235) cells, in relation to the co-culture period, while such down-regulation was not observed in T cells stimulated with Q59G(171) cells. In \textit{in vitro} kinase assays, only ZAP-70 immunoprecipitates from M12DR4(235) cell-stimulated T cells phosphorylated the substrates glutathione S-transferase (GST)-LAT and GST-band 3 (Fig. 4E). On the other hand, none of ZAP-70 immunoprecipitates from Q59G(171), L-DR4(65) and Y61V(243) cell-stimulated or unstimulated T cells did phosphorylate the substrates. These observations indicate that the magnitude of cytokine productions and proliferative responses, which was comparable between T cells stimulated with Q59G(171) cells and with M12DR4(235), was irrespective of ZAP-70 kinase activity.

\subsection*{2.5 Inhibition of PKC\textsubscript{\(\mu\)} abrogated both T cell proliferation and cytokine production}

Since ZAP-70-independent but PKC-dependent extracellular signal-regulated kinases (ERK) activation pathway in the ZAP-70-deficient Jurkat T cell line was
reported [16], we asked whether the responses of T cells stimulated with L cells were PKC-dependent. IFN-γ production by HVS-transformed YN5–32 cells co-cultured with M12DR4(235) or Q59G(171) cells for 4 h was inhibited by pretreatment and presence of the PKC inhibitors Gö6976, Ro318220 (bisindolmaleimide IX) and Gö6850 (GF109203X, bisindolmaleimide I) (Fig. 5A). Gö6976 is a specific inhibitor of conventional PKC, while Ro318220 and Gö6850 are the inhibitors of three groups (conventional, non-conventional and atypical) of PKC [17]. Among these PKC inhibitors, the conventional PKC inhibitor Gö6976 most potently inhibited the T cell responses, while Ro318220 was less effective and Gö6850 showed only moderate inhibition. Pretreatment and presence of these PKC inhibitors also inhibited T cell proliferation stimulated with M12DR4(235) or Q59G(171) cells (Fig. 5A). Again, Gö6976 was most potent, while Ro318220 and Gö6850 were less effective.

These contradictory observations together with recent reports that kinase activity of the μ subtype of PKC (or protein kinase D, PKD) could be inhibited by Gö6976 but was resistant to Ro318220 and Gö6850 [18] suggested that activation of PKCμ was involved in the T cell activation pathways. PKCμ was classified into the fourth group of PKC [19] or into the independent PKD family [20] because of its fewest structural similarities with other PKC. Another PKC inhibitor Gö6983, which is a potent PKC inhibitor except for PKCμ [18], exhibited effects on T cell responses similar to those of Gö6850 and much less effective than those of Gö6976 (Fig. 5B), thus suggesting the association of PKCμ activation in the T cell activation pathway. It must be noted that IFN-γ production and proliferation of T cells stimulated with Q59G(171) cells were more susceptible to treatment with Gö6976 than in case of stimulation with M12DR4(235) or L-DR4(65) cells pulsed with M12p54–68.

Since PKCμ are activated by diacylglycerol produced by phospholipase C (PLC) [21], effects of the PLC-specific inhibitor U73122 on the T cell responses were examined. Pretreatment and the presence of U73122 inhibited both IFN-γ production and T cell proliferation in a dose-dependent manner; however, T cells stimulated with Q59G(171) cells were more susceptible than those stimulated with M12DR4(235) cells or with L-DR4(65) cells pulsed with 50 μM M12p54–68 (Fig. 6A). Phosphorylation of tyrosine residue 783 of PLCγ1, which is essential for the activation of PLCγ1 [22], was observed in M12DR4(235) and Q59G(171) cell-stimulated T cells, suggesting PLCγ1 activation in those T cells (Fig. 6B). On the other hand, there was no such phosphorylation in the T cells unstimulated or co-cultured with L-DR4(65) cells.

The Src inhibitor PP2 also inhibited both IFN-γ production and T cell proliferation stimulated with the L cell transfectants, suggesting the involvement of Src kinases in the T cell activation pathways (Fig. 6A). Compared with stimulation with M12DR4(235) cells, Q59G(171) cell-stimulated T cell responses were selectively inhibited by PP2 at the concentration of 3 μM, and those stimulated with L-DR4(65) cells pulsed with 50 μM M12p54–68 showed intermediate susceptibility. The MAPK/ERK kinase (MEK)-specific inhibitor PD98059 suppressed T cell proliferation in a dose dependent manner, while effects on IFN-γ production were slight (Fig. 6A).

2.6 PKCμ activity was up-regulated only by stimulation with L cell transfectants that induced T cell proliferation

In vitro kinase assays using an anti-human PKCμ polyclonal antibody revealed that co-culture with M12DR4(235) cells induced a marked incorporation of
Fig. 6. Activation of PLC and Src were involved in the T cell responses. (A) HVS-transformed YN5–32 cells were pretreated with PLC inhibitor U73122, Src inhibitor PP2, or MEK inhibitor PD98059 as described in the legend of Fig. 5. T cells were then co-cultured with 100 µl of M12DR4(235) cells (open squares), Q59G(171) cells (closed circles) or L-DR4(65) cells prepulsed with 50 µM M12p54–68 (triangles with dashed lines). IFN-γ production (upper panels) and proliferation (lower panels) of the T cells were quantified as described in Sect. 4. T cell responses without inhibitors were assigned to be 1.0. (B) Lysate from T cells co-cultured with respective L cell transfectants for 10 min were subjected to PLCγ1 immunoprecipitation and Western blots probed with an anti-phospho-PLCγ1 (tyrosine 783) antibody. Phosphorylation of tyrosine residue 783 of PLCγ1 was observed in T cells co-cultured with M12DR4(235) and Q59G(171) cells but not in those unstimulated or co-cultured with L-DR4(65) cells (top panels). Equal amounts of PLCγ1 were confirmed by reprobing the membrane with an anti-PLCγ1 antibody (bottom panels).

radioactivity into PKCμ of the T cells, indicating strong up-regulation of the kinase activity (Fig. 7, upper left panel) [23]. Co-culture with Q59G(171) cells also up-regulated the incorporated radioactivity into PKCμ, while no such up-regulation was observed in T cells unstimulated or co-cultured with Y61V(243), Lmock and L-DR4(65) cells. The radioactivity was reduced to baseline level by the presence of 3 µM of G66976 but not in the presence of G66983 (upper right panel). The relative radioactivity of each PKCμ band normalized by the relative amount of each PKCμ protein is shown in the bottom graph.

3 Discussion

We found that T cell proliferative responses stimulated with overexpressed HLA-DR4/partially agonistic APL complexes differed depending on the partial agonist used and on the density of the complexes: E63S and E63V cells required a relatively high cell surface number (9×10^5–2×10^6) to stimulate T cell proliferation, Q59G cells induced a detectable T cell proliferation at only 3×10^4/cell and induced full responses at 9×10^5/cell, whereas E58N and Y61V cells did not stimulate T cell proliferation even at 1×10^6–3×10^6/cell.

At the interface between T cell and APC, MHC/peptide/TCR clusters are surrounded by complexes of costimulatory and adhesion molecules, and the three-dimensional supramolecular structure formed is called “immunological synapse” [24–27]. Regarding the density of MHC/peptide/TCR complexes in the cluster, a weak agonist and an antagonist form MHC/peptide complexes of lower densities (34–193 molecules/µm^2) at the central
part of the cluster than does the agonist (132–352 molecules/µm²), and the size of the cluster relative to the threshold value determines whether or not T cells begin to proliferate [25]. If the cell surface MHC/APL density becomes much higher than the MHC/peptide density observed in the physiological immunological synapse by forced overexpression, the density of TCR complexes bound to the MHC/APL complexes might be high enough to transduce full T cell activation signals. Indeed, in our experiment, a steep increase in T cell proliferative responses was observed at a ligand number of 9×10²/cell for Q59G and E63S (Fig. 2), which corresponds to 1,300 molecules/µm² on average on the surface of L cells, and is several times higher than densities of MHC/agonistic peptide complexes accumulating at the T cell contact area that stimulate T cell proliferation in the planar bilayer [25].

However, the Q59G(171) cell stimuli were not simply agonistic because they did not induce any detectable amount of tyrosine-phosphorylated ZAP-70 and LAT, such being characteristic observations for T cells stimulated with partial agonists or simple antagonists [5, 11, 28, 29]. Therefore, the Q59G(171) cell stimuli retained some potency of a partial agonist. Since the impaired phosphorylation of TCR proximal signaling molecules is considered to cause a lack of downstream signal transduction, resulting in the failure of T cell proliferation, the question is which signaling pathways are needed to induce full proliferative responses in T cells stimulated with Q59G(171) cells. ZAP-70-independent T cell activation pathways have been reported [16, 30, 31] and these observations indicate that there might be TCR-mediated T cell activation pathways that skip phospho-ZAP-70. Using P116 ZAP-70-negative Jurkat cell lines, Shan et al. [16] reported alternative ERK activation pathways stimulated with a high dose of anti-CD3 mAb. Since ERK phosphorylation in P116 cells was susceptible to the PKC inhibitors Gö6985 and Ro318220 but not to the conventional PKC inhibitor Gö6976, they proposed that non-conventional PKC mediate ZAP-70-independent ERK activation pathways, and this ruled out the involvement of PKCµ. On the contrary, the conventional PKC inhibitor Gö6976, but not Ro318220, Gö6985 and Gö6983, potently inhibited both IFN-γ production and T cell proliferation in our system. The apparent contradiction may be due to differences in the readouts, ERK1/2 phosphorylation or the proliferation and IFN-γ production.

PKCµ is present in various tissues [19, 32], including lymphocytes [23, 33, 34]. In T cells, PKCµ was activated by cross-linking of TCR complexes using an anti-CD3ε antibody and cognate TCR ligands (present study); however, the target molecule for the activated PKCµ is unknown. The MEK/ERK pathway is apparently involved in T cell activation pathways leading to proliferation since it was suppressed by treatment with PD98059. PKCµ did not seem to activate this pathway since Gö6976 had almost no effect on the ERK1/2 phosphorylation (data not shown) [16]. Therefore, the PKCµ-mediated downstream signaling pathway seems to be distinct from the MEK/ERK pathway.

Although the upstream molecules responsible for activation of PKCµ remain to be clarified, several non-conventional PKC such as PKCη [34–36], PKCε [34] and PKCθ [37] have been reported to activate PKCµ in vivo. Actually, pretreatment of A20 mouse B lymphoma and human peripheral T lymphocytes with 2.5 µM Ro318220 and 3.5 µM Gö6850, respectively, abrogated activation of PKCµ, and transfection of constitutively active forms of PKCη or PKCε induced almost maximal PKCµ activity in RBL 2H3 mast cells, suggesting that PKCµ activation was controlled by those non-conventional PKC [34]. PKCµ might have been also activated by certain non-conventional PKC in our system, since the T cell responses were abrogated by treatment with 3 µM Ro318220. Both non-conventional PKC and PKCµ as well as conventional PKC have cysteine-rich zinc finger-like motifs to which diacylglycerol binds and activates the kinases [17, 20, 21]. Inhibition of T cell responses by U73122 and phosphorylation of PLCγ1 tyrosine 783 observed in the T cells stimulated with M12DR4(235) and Q59G(171) cells indicated PLCγ1 activation. This may induce PKC activation in those T cells; however, the absence of ZAP-70 kinase activity in Q59G(171) cell-stimulated T cells raise the question as to which kinase is responsible for the PLCγ1 activation. One possibility is that Src kinases may directly phosphorylate PLCγ1 [16].

It is evident that Src kinases are involved in the Q59G(171) cell-stimulated T cell proliferation since the Src inhibitor PP2 effectively inhibited the T cell responses. Compared with stimulation with M12DR4(235) cells, Q59G(171) cell-stimulated T cell responses were selectively inhibited by PP2 at the concentration of 3 µM. These observations suggest that a smaller fraction of Src kinases is activated in the T cells, which in turn activate lesser amounts of PLC and then PKC. We were not able to directly evaluate Lck and Fyn kinase activities; however, to clarify whether the up-regulation of PKCµ is indispensable for the full T cell response, one needs to determine the active Src kinases needed for PKCµ activation and its downstream pathways. The correlation between PKCµ activation and phospho-ZAP-70 less-dependent T cell proliferation we observed also remains to be investigated.
4 Materials and methods

4.1 Materials

The CD4+ T cell clone YN5–32, which recognizes streptococcal peptide M12p54–68 (NRDLEQAYNELSGEA) in the context of HLA-DR4 (DRA/DRB1*0406), was established as described [8]. In some experiments, HVS-transformed YN5–32 cells were used. The transformed YN5–32 cells exhibited the same magnitude of reactivity to antigens as did the normal YN5–32 cells (unpublished observations). The mouse L cell transfectant L-DR4 cell clone expressing DRA/DRB1*0406 was distributed in the 11th International Histocompatibility Workshop.

Human LAT cDNA encoding the amino acid residues 118–223 and human erythrocyte band 3 protein cDNA encoding amino acid residues 1–14 [38] were ligated into pGEX4T vectors. The GST fusion proteins (GST-LAT and GST-band 3) were expressed in Escherichia coli DH5α and were purified with glutathione-agarose beads (Pharmacia).

4.2 Generation of transfectants and quantification of the expressed peptide/HLA-DR4 complexes

Synthesized sense and antisense DNA fragments encoding amino acid residues of M12p54–68 plus linker were annealed and inserted into DRB1*0406 cDNA as described [39]. The cDNA fragment was subcloned into pBJ1neo vector (provided by Dr. M. M. Davis). To generate HLA-DR4 with covalently linked APL (Table 1), mutations were introduced into the DNA sequence encoding M12p54–68. As an HLA-DR4-binding irrelevant peptide control, synthetic DNA fragments encoding CD20p26–45 (GPKPLFR RMSSLVGPTQSSF) [15] were used.

The expression vectors for HLA-DRA and peptide/linker/HLA-DRB1*0406 were co-transfected into L cells. Cells stably expressing the HLA-DR4 peptide complex were stained with FITC-conjugated anti-HLA-DR mAb (Becton Dickinson), Transfectants expressing various amounts of HLA-DR were cloned using FACScan (Becton Dickinson). Transfectants expressing various amounts of HLA-DR were cloned using FACScan (Becton Dickinson). The surface number of each HLA-DR4/peptide complex on the L cell was quantified using Quantum Simply Cellular™ microbeads (Flow Cytometry Standards).

4.3 Assays for T cell responses

Mitomycin C (Sigma)-treated (20 μg/ml for 30 min at 37°C) L cell transfectants (3.7×10^6/well) were incubated in 96-well plates for 1 day and YN5–32 cells (3×10^5/well) were added to each well. In some experiments neutralizing mouse anti-human-IL-2 mAb (50 μg/ml), rabbit anti-human-IL-4 antibody (25 μg/ml) or isotype-matched control IgG were added throughout the culture period. T cell proliferative responses were measured as reported [8]. IL-2, IL-4 and IFN-γ contents in the culture supernatant were determined using ELISA kits (BioSource, R&D Systems and Endogen, respectively). For RPA, mitomycin C-treated L cells were plated in 10-cm culture dishes (2×10^6/plate), and T cells (1×10^6) were added. After the indicated co-culture periods, T cell RNA were extracted using TRIZOL™ reagent (Life Technologies) and subjected to a commercial RPA system (Pharmingen).

In some experiments, the HVS-transformed YN5–32 cells (3×10^5/100 μl) were pretreated with indicated concentrations of PKC inhibitors (Gö69876, Gö6850, Gö6983 and Ro318220; Calbiochem), PLC inhibitor (U73122, Calbiochem) and Src inhibitor (PP2, Sigma) for 30 min at 37°C. Then the T cells and L cells were co-cultured in 96-well plates and T cell responses were assayed as mentioned above.

4.4 Immunoprecipitation and Western blot analyses

T cells (1×10^6) were plated on the monolayer of L cells confluent grown in 15-cm culture dishes and co-cultured for indicated periods. T cells were lysed using lysis buffer I (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% Nonidet-P40 (NP40), 1 mM NaN3, 10% glycerol and a protease inhibitor cocktail (Boehringer Mannheim)). ZAP-70, LAT and PLCγ1 were immunoprecipitated with anti-ZAP-70 mAb (Santa Cruz Biotechnologies), rabbit anti-LAT polyclonal IgG and mouse anti-PLCγ1 mixed mAb (Upstate Biotechnology), respectively, together with protein A beads (Pierce). The proteins were analyzed using 10% SDS-PAGE and transferred onto nitrocellulose membrane. Phospho-ZAP-70 and LAT were blotted with anti-phosphotyrosine (pY) mAb (4G10, Upstate Biotechnology) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Amersham). Phospho-PLCγ1 was blotted with anti-phospho-PLCγ1 (tyrosine 783) antibody (Cell Signaling) and HRP-conjugated anti-rabbit IgG antibody (Amersham). The membrane was reprobed with either anti-ZAP-70 mAb, anti-LAT antibody or anti-PLCγ1 antibody together with HRP-conjugated second antibodies. The bands were visualized by enhanced chemiluminescence (ECL, Amersham).

4.5 In vitro ZAP-70 kinase assay

The HVS-transformed YN5–32 cells co-cultured with L cell transfectants for 10 min were lysed with lysis buffer II [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM NaN3, 10 mM NaF and a protease inhibitor cocktail]. ZAP-70 was immunoprecipitated using an anti-human ZAP-70 antibody (Santa Cruz Biotechnology) and protein A beads at 4°C for 2 h. The immunocomplexes were washed twice with lysis buffer II and once with kinase buffer [30 mM Tris-HCl (pH 7.4), 10 mM MgCl2 and 1 mM DTT] and then incubated with 20 μl kinase buffer containing 2.4 μg each of PKCζ activation and impaired ZAP-70 activation.
GST-LAT and GST-band 3 and 0.6 MBq of \( \text{[}\gamma^{32}\text{P}]\text{ATP} \) (Amersham) at 30°C for 10 min. The supernatants were analyzed with 12% SDS-PAGE followed by autoradiography. ZAP-70 in the immunocomplex was subjected to 10% SDS-PAGE and total amounts of ZAP-70 were estimated on Western blots.

4.6 In vitro PKC\(\eta\) kinase assay

The HVS-transformed YN5–32 cells co-cultured with L cells for 1 h were lysed with lysis buffer II. PKC\(\eta\) was immunoprecipitated using an anti-human PKC\(\eta\) antibody (D-20, Santa Cruz Biotechnology) and protein A beads at 4°C for 2 h. The immunocomplexes were successively washed with lysis buffer II, RIPA buffer [20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM DTT, 5 mM Na\(\text{}_{2}\)VO\(\text{4}\), 0.1% SDS, 0.5% NaF, 10% glycerol and a proteinase inhibitor cocktail] and precipitated using an anti-human PKC\(\eta\) antibody (D-20, Santa Cruz Biotechnology) and protein A beads at 4°C for 2 h. The immunocomplex was subjected to 10% SDS-PAGE and transferred onto nitrocellulose membrane. The kinase activities were determined by measuring the incorporated radioactive protein was detected on Western blots and quantified using NIH image software (http://rsb.info.nih.gov/nih-image/).

Acknowledgements: We are grateful to Dr. M. M. Davis (Stanford University) for pB71 neo vector and to Dr. R. C. Desrosiers (Harvard University) for HVS and OMK cells. We also thank M. Ohara for helpful comments. This work was supported in part by Grants-in-Aid 08282104, 10770142 and 12051203 from the Ministry of Education, Science, Technology, Sports, and Culture, Japan.

References


Correspondence: Yasuharu Nishimura, Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Honjo 2-2-1, Kumamoto 860-0811, Japan Fax: +81-96-373-5314 e-mail: mxnishim@gpo.kumamoto-u.ac.jp