Allelic exclusion and differentiation by protein kinase C-mediated signals in immature thymocytes

Alison M. Michie*, Jae-Won Soh†, Robert G. Hawley§, I. Bernard Weinstein†, and Juan Carlos Zúñiga-Pflücker*§

*Department of Immunology, University of Toronto, Toronto, ON, Canada M5S 1A8; †Herbert Irving Comprehensive Cancer Center, Columbia University, New York, NY 10032; and §Hematopoiesis Department, Holland Laboratory, American Red Cross, Rockville, MD 20855

Pre-T cell receptor (preTCR)-derived signals mediate the transition of thymocytes from the CD4−CD8− double-negative (DN) to CD4+ CD8+ double-positive (DP) stage of T lymphocyte development. This progression, termed β-selection, is limited to thymocytes that have generated a functional TCR-β chain able to associate with pTα to form the preTCR complex. Formation of the preTCR complex not only induces differentiation, survival, and proliferation of DN thymocytes; it also inhibits further TCR-β gene rearrangement through an ill-defined process known as allelic exclusion. The signaling pathways controlling this critical developmental checkpoint have not been characterized. Here we demonstrate that formation of the preTCR complex leads to the activation of protein kinase C (PKC), and that activation of PKC is necessary for the differentiation and expansion of DN thymocytes. Importantly, we also show that allelic exclusion at the TCR-β gene loci is enforced by PKC-mediated signals. These results define PKC as a central mediator of both differentiation and allelic exclusion during thymocyte development.

On their way to becoming functional and self-tolerant αβ T cells, immature thymocytes must first successfully generate a T cell receptor-β (TCR-β) chain that is able to associate with pTα to form the pre-T cell receptor (preTCR) complex (1, 2). PreTCR-derived signals mediate the transition of thymocytes from the CD4−CD8− double-negative (DN) to the CD4+CD8+ double-positive (DP) stage of T cell development by inducing the survival, proliferation, differentiation, and TCR-β gene allelic exclusion of immature thymocytes. This progression, termed β-selection, appears to depend on the nonreceptor tyrosine kinases Lck/Fyn and ZAP-70/Syk (3–8), as well as the adaptor proteins SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa) and LAT (linker for activation of T cells) (9–11). Although the Ras/Raf signaling cascade promotes differentiation and proliferation of DN thymocytes (12), it fails to mediate allelic exclusion at the TCR-β gene loci (13, 14). Nevertheless, allelic exclusion depends on the presence of Lck and SLP-76 (15–17), suggesting that, contrary to the induction of differentiation and proliferation, the signals mediating allelic exclusion occur upstream of Ras/Raf activation and downstream of Lck/ZAP-70 phosphorylation of SLP-76. Therefore, a bifurcation of signals must occur before the activation of Ras/Raf, which is responsible for the induction of allelic exclusion.

As SLP-76 is a critical scaffold protein for several adaptor molecules, regulators of Ras signaling, and phospholipase C-γ1 (18, 19), we hypothesized that signals downstream of SLP-76, such as protein kinase C (PKC) activation by phospholipase C-γ1-derived second messengers (20), would be responsible for the induction of allelic exclusion. Because PKC has been shown to directly activate Ras/Raf (21, 22), our hypothesis stipulates that activation of PKC by the preTCR not only will enforce allelic exclusion but may also induce the proliferation and differentiation of DN thymocytes.

By using an experimental approach that allows for the transfection of fetal thymuses in situ (23, 24), we demonstrate that formation of the preTCR complex leads to the activation of PKC. Moreover, we show that PKC activation is essential for the differentiation and expansion of immature thymocytes. Notably, our findings also establish that allelic exclusion at the TCR-β gene loci is enforced by PKC-mediated signals. These results identify PKC as a central mediator of both differentiation and allelic exclusion during thymocyte development.

Materials and Methods

Animals. Recombination activating gene-2-deficient (RAG2) mice were bred and maintained in our animal facility (25). Timed-pregnant RAG2 mice were generated, and the fetuses were extracted at day 14 of gestation. Timed-pregnant CD1 mice were obtained from the Charles River Breeding Laboratories, and the fetuses were extracted at day 14 of gestation.

Gene-Gun Transfection and Fetal Thymic Organ Culture. Fetal thymuses were transfected with DNA/gold bombardment, as described previously (23). As previously reported, 0.5–2.0% of CD45+ cells were transfected with this technique (23). For short-term biochemical assays, the transfected fetal thymic lobes were incubated for a further 16–20 h in transfact fetal thymus organ cultures (FTOCs), then the cells were lysed, and the lysate was assayed for luciferase and β-galactosidase activities (23). For long-term developmental progression studies, the thymic lobes were incubated in FTOC for 7–10 days, during which the medium was changed once, and then were analyzed by flow cytometry (23).

Flow Cytometry and Cell Sorting. FITC-, R-phycocerythrin (PE)-, and allophycocyanin (APC)-conjugated anti-mouse antibodies were used for flow cytometric analysis (PharMingen). Staining of cells was carried out as described (23). The data were live gated by size and lack of propidium iodide uptake. For cell sorting, a Coulter Elite (Coulter) cytometer was used. Cells were prepared as described (23). Sorted cells were ≥98% pure, as determined by postsort analysis.

Generation of Retroviral Constructs and Packaging Cell Lines. Retroviral constructs were engineered by subcloning the gene of interest (a constitutively active form of PKC, PKCα-CAT; a dominant-negative form of PKC, PKCα-KR; and constitutively active Ras, RasV12) into the retroviral backbone (MIEV), 5′ of the internal–ribosomal entry site, allowing the bicistronic expression of the gene of interest and green fluorescent protein...
Fig. 1. Constitutively active PKC bypasses the requirement of TCR-β chain for the induction of thymocyte differentiation. CD4+ CD8+ thymocytes are generated in RAG+ FTOC gene gun transfected with plasmid encoding a functional TCR-β chain, a constitutively active PKC (PKC-CAT), or a constitutively active GTPase Ras (RasV12). RAG+ fetal thymuses were gene gun-transfected with control DNA (DNA loading ratio [DLR] = 250 ng), plasmid encoding a functionally rearranged TCR-β chain (DLR = 250 ng), plasmid encoding PKC-CAT (DLR = 250 ng), or plasmid encoding RasV12 (DLR = 250 ng). The transfected FTOCs were cultured for 9 days. After this time, a single-cell suspension was prepared and thymocytes were analyzed for surface expression of CD25, CD8, and CD4 by flow cytometry. The data shown are representative of five independent experiments.

Results and Discussion

CD4+ CD8+ Thymocyte Differentiation Is Mediated by PKC. Whereas the Ras/Raf signaling cascade has been shown to promote differentiation and proliferation of DN thymocytes (12–14), and because PKC has been postulated to activate Ras/Raf (21, 22), we hypothesized that PKC activation may also induce the proliferation and differentiation of DN thymocytes.

To address the above-stated hypothesis, we took advantage of an experimental approach that allows for transfection of fetal thymuses in situ (23). This approach involves the use of an accelerated DNA/particle bombardment (gene gun) delivery system to transfect FTOCs (24). In this way, we transfected (25) RAG+ mouse-derived FTOCs with a plasmid encoding a functionally rearranged TCR-β chain, as the introduction of a TCR-β transgene into RAG mice allows DN thymocytes to differentiate to the DP stage (26). In keeping with this finding, flow cytometric analysis of TCR-β-transfected FTOCs revealed a population of DP thymocytes, whereas thymocytes from mock-transfected FTOCs remained at the DN stage (Fig. 1). Moreover, we observed the appearance of a CD25+ CD8+ population and a 3-fold increase in thymic cellularity in TCR-β-transfected FTOCs relative to control FTOCs (Fig. 1). The increase in total cell number after transfection was likely because of preferential survival of the CD25+ thymocytes, which in the absence of preTCR expression have been shown to have a limited life span (1, 2). These observations reflect several hallmarks of preTCR-mediated events and indicate that the transfected thymocytes generated a preTCR complex and passed the β-selection checkpoint (1).

To directly test our hypothesis and establish whether PKC-derived signals can induce the differentiation and proliferation of DN thymocytes, we transfected RAG+ FTOCs with a plasmid encoding a constitutively active form of PKC (PKC-CAT; deletion of the regulatory N-terminal domain of PKCa) (27). This particular PKC isoform was chosen, as it has previously been shown to be expressed in thymocytes (28, 29), including the DN subset (data not shown), and has been demonstrated to play an important role in T cell activation (30, 31). Flow cytometric analysis of PKC-CAT-transfected RAG+ FTOCs revealed a population of DP thymocytes, whereas thymocytes from mock-
transfected FTOCs remained at the DN stage (Fig. 1). Notably, we also observed the appearance of a CD25^lo CD8^1 population and a 3-fold increase in thymic cellularity in PKC-CAT transfected FTOCs relative to the control (Fig. 1). These results indicate that activated PKC can induce differentiation and proliferation of DN thymocytes, supporting our notion that activation of PKC induces β-selection signals. Similar results were obtained from RAG° FTOCs transfected with a plasmid-encoding RasV12, in which the generation of DP thymocytes was observed, as well as the emergence of a CD25^lo CD8^1 population and a more than 3-fold elevation in thymic cellularity as compared with mock-transfected RAG° FTOCs (Fig. 1). Thus, thymocytes from RAG° FTOCs expressing these transfected genes undergo cellular proliferation and differentiation to the DP stage, bypassing the requirement for preTCR-dependent β-selection.

**Formation of the PreTCR Complex Induces PKC Activation.** Taking advantage of our ability to introduce multiple DNA plasmids into developing thymocytes in FTOC (Fig. 1), we coupled this transfection technique with a previously characterized reporter-plasmid system that allows for the detection of PKC activation in developing thymocytes (23). RAG° fetal thymuses were gene gun-transfected with pFR-Luc (DLR = 250 ng) and CMV-β-gal (DLR = 250 ng) and either pFA2-CREB (DLR = 75 ng) alone or together with TCR-β (DLR = 250 ng), TCR-β and dominant-negative PKC (PKC-KR, DLR = 750 ng), or TCR-β and treated with PKC-specific inhibitor bisindolylmaleimide (1 μM) or constitutively active PKC (PKC-CAT, DLR = 250 ng), as indicated. The transfected lobes were then cultured for 16–20 h. Cells were then lysed, and the lysates were assayed for luciferase and β-galactosidase activity. The data shown are an average of at least four independent experiments. The individual luciferase activities were indexed against the observed β-galactosidase activity.

![Fig. 3](image-url)

**Fig. 3.** PKC function is necessary for the differentiation of thymocytes to the CD4^- CD8^- stage. Intact fetal thymic lobes from d14 timed-pregnant CD1 mice were retrovirally infected with either (A) vector alone (MIEV) or (B) PKC-KR encoding constructs for 72 h in HOS-FTOC, then incubated in standard FTOC for 5 days. Flow cytometric analysis of CD4 vs. CD8 cell surface expression on thymocytes gated for GFP expression is shown (R1, GFP^-; R2, GFP^hi; R3, GFP^lo). The data shown are representative of four independent experiments.
of PKC (PKC-KR; point mutation in the ATP-binding region of the catalytic domain of PKCα) (27) and a TCR-β plasmid, together with the reporter plasmids. Strikingly, the increased luciferase activity observed in TCR-β-transfected FTOCs was completely abrogated in the presence of PKC-KR (60 ± 11 RLUs; Fig. 2). Furthermore, luciferase activity was severely diminished in RAG° FTOCs transfected with TCR-β and reporter plasmids that were treated with the PKC-specific inhibitor bisindolylmaleimide (1 μM) (80 ± 13 RLUs; Fig. 2). Moreover, FTOCs transfected with the PKC-CAT plasmid together with the reporter plasmids showed a dramatic 20-fold increase in luciferase activity as compared with FTOCs transfected with reporter plasmids alone (498 ± 55 RLUs; Fig. 2), demonstrating the sensitive nature of this assay system in detecting PKC activation.

Although our data suggest that the upstream kinase responsible for observed luciferase activity appears to be PKC (Fig. 2), the reporter plasmids used to measure PKC activation rely on the phosphorylation and activation of CREB. Therefore, we sought to determine whether another upstream kinase, cAMP-dependent protein kinase (PKA) (32), could elicit CREB activation after preTCR complex formation. To this end, RAG° FTOCs were transfected with a plasmid encoding a dominant-negative form of PKA (PKA-RG324D) (33) and a TCR-β plasmid, together with the reporter plasmids. Importantly, this combination of plasmids revealed almost a 20-fold stimulation in luciferase activity similar to that observed in FTOCs transfected with TCR-β alone. This PKA-RG324D construct was shown to effectively inhibit forskolin-induced elevation of luciferase activity in EL4 cells when cotransfected with the reporter plasmids pFR-Luc/pFA2-CREB (data not shown), indicating that PKA-RG324D can inhibit PKA function. On the other hand, as expected, a substantial elevation in luciferase activity was observed in RAG° FTOCs transfected with a plasmid encoding a constitutively active PKA (catalytic subunit of PKA) (34) and the reporter plasmids (2156 ± 575 RLUs). Taken together, these data indicate that, although CREB can be activated by different upstream kinases, the CREB activation observed on expression of the preTCR complex is indeed mediated by PKC.

PKC Is Necessary for Thymocyte Differentiation to the CD4+ CD8+ Stage. To determine whether the preTCR-mediated activation of PKC represents a necessary event for the differentiation and proliferation of DN thymocytes, we infected fetal thymocytes from normal mice with a retroviral construct (MIEV) (35) that allows for the bicistronic expression of GFP together with the catalytic domain of PKC (PKC-KR). Infected cells were cultured in FTOCs for 8–10 days before flow cytometric analysis. Fig. 3 shows that thymocytes infected with the MIEV retroviral construct encoding GFP alone differentiated toward the DP and SP stages of thymocyte development. In contrast, flow cytometric analysis of thymocytes infected with MIEV.PKCAT showed that GFP+ cells failed to reach the SP stage (Fig. 4). Moreover, flow cytometric analysis for αβ-TCR surface expression from thymocytes infected with MIEV.GFP revealed a population of GFP+ TCRhi CD4+ cells (Fig. 4). Notably, this population of TCRhi cells was absent in PKC-CAT-infected GFP+ CD4+ thymocytes (Fig. 4). As a control, flow cytometric analysis of GFP− cells from the two groups or from GFP+ RasV12-infected cells showed similar levels of TCR surface expression (Fig. 4 and data not shown). Moreover, we

**PKC Mediates Allelic Exclusion at the TCR-β Gene Loci.** In light of these findings, we sought to determine whether PKC-mediated signals could not only lead to the differentiation and expansion of DN thymocytes (Figs. 1 and 3) but also enforce allelic exclusion at the TCR-β gene loci. To this end, we infected fetal thymocytes from normal mice with the MIEV retroviral construct encoding PKC-CAT. Infected cells were cultured in FTOCs for 8–10 days before flow cytometric analysis. Fig. 4 shows that thymocytes infected with the MIEV retroviral construct-encoding GFP alone differentiated toward the DP and SP stages of thymocyte development. In contrast, flow cytometric analysis of thymocytes infected with MIEV.PKCAT showed that GFP+ cells failed to reach the SP stage (Fig. 4). Furthermore, flow cytometric analysis for αβ-TCR surface expression from thymocytes infected with MIEV.GFP revealed a population of GFP+ TCRhi CD4+ cells (Fig. 4). Notably, this population of TCRhi cells was absent in PKC-CAT-infected GFP+ CD4+ thymocytes (Fig. 4). As a control, flow cytometric analysis of GFP− cells from the two groups or from GFP+ RasV12-infected cells showed similar levels of TCR surface expression (Fig. 4 and data not shown). Moreover, we
observed a ~2-fold decrease in the frequency of thymocytes with a TCR lo phenotype when comparing GFP + CD4 + cells from PKC-CAT- and MIEV-infected FTOCs (Fig. 4, R1-gate). These data suggest that, similar to the results obtained in mice expressing a constitutively active Lck transgene (Lck<sup>F50S</sup>) (15, 16), PKC-mediated signals may lead to the induction of allelic exclusion and therefore result in DP thymocytes that fail to express a TCR-β chain, making them unable to form an αβ-TCR complex with which to undergo positive selection and developmental progression to become TCR hi SP thymocytes. To directly address whether activation of PKC results in the induction of allelic exclusion, genomic DNA was isolated from GFP + (lanes 1, 2, and 4) or GFP + (lanes 3 and 5) thymocytes and subjected to a PCR-based analysis to assess the rearrangement status of their TCR-β gene loci (Fig. 5). Analysis for DNA rearrangement between the TCR-β D and J gene segments showed that expression of constitutively active PKC did not interfere with this recombination event, as all DNA samples showed the expected pattern of random Dβ2 to Jβ<sub>2.1-2.6</sub> rearrangements (Fig. 5). Moreover, Vβ to DJβ rearrangements were detected in DNA from uninfected (GFP −) thymocytes (Fig. 5, lanes 1, 2, and 4) and from MIEV.GFP + (Fig. 5, lane 3) or Ras<sup>V12</sup>-infected thymocytes (data not shown). We analyzed Vβ to DJβ rearrangements involving the Vβ<sub>6</sub> gene segment, as this segment is the predominant TCR-Vβ chain expressed in thymocytes from CD1 mice, which are of the TCR-Vβ haplotype. This result is consistent with the previous findings that Ras-mediated signals are unable to induce allelic exclusion of the TCR-β gene loci (13, 14), whereas they are capable of inducing differentiation and proliferation of DN thymocytes (Fig. 1; ref. 12). Notably, we were unable to detect specific Vβ to DJβ rearrangements in DNA obtained from PKC-CAT-infected (GFP +) thymocytes (Fig. 5, lane 5). Importantly, PCR amplification of a single copy gene, βm, yielded similar amounts of product, indicating that the DNA samples used were equivalent (Fig. 5). Thus, it is unlikely that our failure to observe Vβ to DJβ rearrangements in PKC-CAT-infected cells was because of a lack of quantifiable DNA, as we could detect Dβ2 to Jβ<sub>2.1-2.6</sub> rearrangements and βm PCR products, but rather suggests that constitutively active PKC can mediate allelic exclusion at the TCR-β gene loci. These data are consistent with our flow cytometric analysis, in which TCR hi SP cells were not present among PKC-CAT-infected thymocytes (Fig. 4). However, surface expression of TCR was not completely abolished, as we were able to detect, albeit at reduced levels, TCR<sub>lo</sub>-expressing PKC-CAT-infected thymocytes. Importantly, that we observed allelic exclusion of a predominantly used Vβ gene segment by PCR serves to underscore the results obtained with the flow cytometric analysis. This finding is similar to results obtained in mice expressing a constitutively active Lck transgene (Lck<sup>F50S</sup>) (15, 16), in which TCR expression was severely diminished but not totally abrogated. Collectively, these results indicate that PKC signals can enforce allelic exclusion of the TCR-β gene loci.

Conclusions

The importance of PKC in regulating T lymphocyte activation has been well characterized (20, 36, 37). However, the exact downstream targets of PKC that contribute to T cell activation remain undefined. Moreover, the potential role of PKC during T cell development has not been elucidated. In particular, the study of mice deficient for specific PKC isoforms, such as PKC-β, -γ, and -θ, did not reveal a requirement for these isoenzymes during thymocyte differentiation (37–39). Because of a failure to detect a role for these PKCs, possibly because of a redundancy within PKC family members, we used constitutively active and dominant-negative forms of PKC. To this end, we selected PKCe, as it is one of the major PKC isoforms expressed in

![Diagram showing the role of PKC-mediated signals in enforcing allelic exclusion of the TCR-β gene loci during β-selection](image-url)
thymocytes (ref. 29 and data not shown). This approach allowed us to implicate PKC as an important mediator of thymocyte β-selection events.

A broad-based literature supports the conclusion that the specificity of an acquired immune response reflects the selection of T lymphocytes expressing clonally distributed antigen receptors. However, although preservation of allelic exclusion at the TCR gene loci is fundamental to the observed outcome, the mechanism(s) underlying this process during thymocyte development remains elusive. Although several signaling events proximal to the preTCR complex have been implicated in the regulation of allelic exclusion (15–17, 40), the downstream signaling pathways governing this event remain unknown. In particular, allelic exclusion was shown not to be mediated by the well-characterized Ras/Raf signaling cascade (12–14). Here we provide explicit evidence implicating PKC as the downstream molecule responsible for delivering allelic exclusion signals.

Our results not only demonstrate that PKC can mediate allelic exclusion; they also show that PKC is activated on preTCR complex formation in vivo, and that differentiation of DN thymocytes to the DP stage depends on PKC function. Our findings show that induction of allelic exclusion occurs downstream of PKC activation through a Ras/Raf-independent pathway, illustrating that a signal bifurcation beyond SLP-76 phosphorylation is responsible for mediating differentiation, proliferation, and survival, as well as allelic exclusion. A model outlining our findings is shown in Fig. 6. Potentially, the bifurcation point may occur after the activation of PKC, as this event has been shown to play an important role in T cell activation. In this respect, as with T cell activation, the identities of the downstream targets that ultimately induce allelic exclusion in thymocytes after PKC activation also remain undefined. Thus, these results provide direct evidence demonstrating a bifurcation of preTCR signals downstream of SLP-76 (Fig. 6), suggesting a central role for phospholipase C-γ-1-mediated PKC activation during β-selection.

We thank Michael Julius for his helpful discussion of the manuscript; Cheryl Smith and Ouyen Fong for their excellent technical assistance; and Pamela Ohashi (Ontario Cancer Institute, Toronto, ON, Canada) for her gift of the plasmid encoding constitutively active Ras (RasV12). A.M.M. is supported by a postdoctoral fellowship from the Cancer Research Institute. J.C.Z.-P. is supported by an Investigator Award from the Canadian Institute of Health Research.