Extracellular Signal–regulated Kinase (ERK) Activation by the Pre-T Cell Receptor in Developing Thymocytes In Vivo

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Summary

The first checkpoint in T cell development occurs between the CD4⁻CD8⁻ and CD4⁺CD8⁺ stages and is associated with formation of the pre-T cell receptor (TCR). The signaling mechanisms that drive this progression remain largely unknown. Here, we show that extracellular signal–regulated kinases (ERKs)-1/2 are activated upon engagement of the pre-TCR. Using a novel experimental system, we demonstrate that expression of the pre-TCR by developing thymocytes induces ERK-1/2 activation within the thymus. In addition, the activation of this pre-TCR signaling cascade is mediated through Lck. These findings directly link pre-TCR complex formation with specific downstream signaling components in vivo.

Key words: thymus • thymocyte differentiation • cellular signaling • fetal thymic organ culture • pre-TCR

During the maturation of T lymphocytes within the thymus, precursor lymphocytes undergo two key selection steps that ensure that T cells express a functional TCR–CD3 complex on their cell surfaces that can recognize peptide antigens in the context of self-MHC (1). The earliest thymocytes, which migrate to the thymus from the adult bone marrow or fetal liver, lack expression of CD4 and CD8 and are thus termed CD4⁻CD8⁻ double-negative (DN)[1] cells (2). The DN stage of development can be further subdivided by the ordered surface expression of CD117 (c-kit) and CD25 as follows: CD117⁺CD25⁻, CD117⁺CD25⁺, CD117^{lo/−}CD25⁺, and CD117⁻CD25⁻ (2, 3).

The first developmental checkpoint in T cell differentiation, which occurs at the CD117^{lo/−}CD25⁺ stage, assures that only thymocytes with a productively rearranged TCR β chain proceed to the next developmental stage. This critical regulatory checkpoint is known as β-selection (4–6). The TCR β chain covalently associates with the nonpolymorphic pre-Tα (pTα) chain, which together with CD3 molecules forms the pre-TCR complex (7, 8). Expression of the pre-TCR triggers a differentiation program that includes inhibition of further V(D)J recombination at the

TCR-β locus (allelic exclusion), rescue from apoptosis, exponential cellular proliferation, downregulation of CD25 expression, CD4 and CD8 surface expression, and initiation of TCR-α gene rearrangement (9–14). Notably, differentiation toward the CD4⁺CD8⁺ double-positive (DP) stage of development occurs through an immature single-positive (SP) stage (5, 6, 15, 16). The second regulatory checkpoint in T cell development occurs at the DP stage when thymocytes undergo positive and negative selection, resulting in the generation of mature CD4⁺CD8⁻ and CD4⁻CD8⁺ SP thymocytes (1).

The signaling pathways that mediate β-selection events are not well defined. However, the study of T cell development in mice lacking key signaling molecules has provided important clues regarding the possible signal transduction pathways essential for β-selection. The pre-TCR complex seems to function together with CD3 molecules to transduce differentiation and proliferation signals (16–23). Downstream signaling events appear to be dependent on the nonreceptor tyrosine kinases Lck/Fyn and ZAP-70/Syk (15, 24–28), as well as the adapter proteins SLP-76 (SH2 domain–containing leukocyte protein, 76 kD) and LAT (linker for activation of T cells) (29–31). Indeed, genetic deficiencies for pTα, TCR-β, CD3ε, CD3γ, Lck, SLP-76, and LAT all arrest differentiation at the CD117^{lo/−}CD25⁺ stage, at the onset of β-selection (16, 20, 24, 29–33).

Thymocytes are also arrested at the CD117⁻CD25⁺ stage of development in recombination activating gene 1 or 2–deficient (RAG⁻/⁻) and SCID mice due to an inability

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[1]*Abbreviations used in this paper:* DLR, DNA loading ratio; DN, double-negative; DP, double-positive; ERK, extracellular signal–regulated kinase; FTOC, fetal thymic organ culture; LAT, linker for activation of T cells; MAPK, mitogen-activated protein kinase; RAG, recombination activating gene; RLUs, relative light units; SLP-76, SH2 domain–containing leukocyte protein, 76 kD; SP, single-positive.

to initiate or complete rearrangement of their antigen receptors, respectively (34–38). In particular, the introduction of productively rearranged TCR-β transgenes into RAG$^{-/-}$ and SCID mice allows further differentiation of DN cells, indicating that a functional TCR β chain is sufficient for progression to the DP stage in these mice (39, 40). However, RAG$^{-/-}$ mice containing constitutively active Lck (F505), Ras (Ha-Ras^{V12}), and serine/threonine kinase c-Raf-1 (Raf-CAAX) transgenes allow for cellular proliferation and generation of DP cells by experimentally bypassing pre-TCR–dependent β-selection processes (41–43). These data support the involvement of Lck, Ras, and Raf in β-selection and suggest a signaling mechanism similar to that observed during T cell activation. Signaling through the mature α/β TCR is thought to involve the following events after TCR engagement: Lck-mediated phosphorylation of the CD3 ζ chain; recruitment of ZAP-70/Syk to the receptor complex; ZAP-70/Syk-mediated phosphorylation of downstream substrates, including LAT, SLP-76, and phospholipase C γ1; linkage of the TCR–CD3 complex to pathways leading to Ca^{2+} mobilization and Ras activation through phosphorylated LAT and SLP-76; and activation of the Ras/Raf/mitogen-activated protein kinase (MAPK)-1 (MEK1)/extracellular signal–regulated kinase (ERK; p44/42 MAPK) cascade (44). Notably, results from transgenic mice carrying dominant negative forms of Ras (Ha-Ras^{N17}; reference 45), Raf (46), and MEK1 (47) did not affect β-selection events. Thus, studies supporting the involvement of the Ras/Raf/MEK/ERK cascade during β-selection are conflicting. Furthermore, a direct link between pre-TCR expression by developing thymocytes and the activation of downstream signaling pathways has not been demonstrated.

In this report, we show that ERK-1/2 is phosphorylated and activated upon engagement of the pre-TCR expressed on a SCID mouse–derived pre-T cell line, indicating that the ERK signaling cascade is initiated by the pre-TCR. To study specific biochemical signaling events that ensue upon formation of the pre-TCR complex, we developed a novel experimental system that allows for the introduction of reporter plasmids into thymic lobes. This system enables signals generated by the pre-TCR to be assayed in real time within a biologically relevant setting, i.e., during thymocyte differentiation within an intact thymus. Using this system, we demonstrate that the ERK signaling cascade is activated in developing thymocytes as a physiological result of pre-TCR complex formation in vivo.

Materials and Methods

Animals. RAG-2$^{-/-}$ mice were bred and maintained in our animal facility (34). Timed-pregnant RAG-2$^{-/-}$ mice were used on day 14 of gestation.

Cell Lines. The SL-12β.12 cell line is a pre-T cell line derived from a spontaneous SCID mouse–derived thymoma that stably expresses functionally rearranged TCR β chain at the cell surface with endogenous pTα to form a pre-TCR (48, 49). The cell line was maintained in complete media (HG-DME media containing 10% FBS, 1% penicillin/streptomycin, 1% glutamine, 1% sodium pyruvate, 1% Hepes, 0.1% 2-ME, and 0.1% gentamicin [GIBCO BRL] supplemented with 0.5 mg/ml geneticin [GIBCO BRL]). Cells were kept in a humidified atmosphere of 5% CO$_2$ at 37°C.

SDS-PAGE and Western Blot Analysis. SL-12β.12 cells (5 × 10^6) were incubated at 4°C with 10 μg/ml biotinylated anti–TCR-β mAb (H57-597) for 30 min in DMEM supplemented with 0.1% BSA. Cells were then washed, resuspended in 100 μl DMEM/0.1% BSA containing 25 μg/ml avidin (Sigma Chemical Co.), and incubated at 37°C. Stimulation was stopped by the addition of 3 ml ice cold PBS followed by lysis in buffer containing 50 mM Tris, pH 7.4 (Boehringer Mannheim), 0.5% Triton X-100 (Boehringer Mannheim), 150 mM NaCl (ICN Biomedicals Inc.), 1 mM EDTA (BDH Chemicals Ltd.), 1 mM sodium orthovanadate (ICN Biomedicals Inc.), and Complete Inhibitor Cocktail (Boehringer Mannheim). Control cells (unstimulated) were treated similarly; however, avidin was not added before lysis. Lysates were resolved on 10% SDS-PAGE under reducing conditions and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Amersham Pharmacia Biotech). Phosphorylated ERK-1 (p44 MAPK) and ERK-2 (p42 MAPK) and total ERK-2 proteins were detected using phospho-p44/22 MAPK (Thr202/Tyr204) mAb (New England Biolabs Inc.) and total p42 MAPK antibody (New England Biolabs Inc.), followed by horseradish peroxidase–conjugated goat anti–rabbit IgG (Santa Cruz Biotechnology). Blots were revealed using Supersignal West Pico chemiluminescent substrate (Pierce Chemical Co.).

Transfection of SL-12β.12 Cells. All electroporations were carried out using a BTX Electro Cell Manipulator 600 apparatus. SL-12.β12 cells were electroporated at 450 μF, 186 Ω, 300 V, attaining a time constant of ∼30 ms. 3 × 10^6 cells were transfected with desired plasmid DNA (up to 40 μg), as indicated in the figure legends. Each sample was transfected with PathDetect reporter plasmids (Stratagene, Inc.; as indicated in figure legends) and 1 μg plasmid encoding β-galactosidase (pCMV-β-gal). The addition of a fixed amount of β-galactosidase plasmid allowed for the control of transfection efficiency during the experiment. The β-galactosidase activity was used to index the luciferase signal detected, as we were able to assay for luciferase and β-galactosidase activity within the same sample (50). In brief, cells were washed in electroporation media (RPMI 1640 containing 20% FCS) and resuspended at 1.2 × 10^7 cells/ml (250 μl per transfection). DNA and cells were combined in 4-mm sterile cuvettes (Bio-Rad Labs.) and incubated on ice for 10 min. The cells were electroporated with the conditions noted above and incubated on ice for a further 10 min. Transfected cells were put into fresh complete media and incubated for 24 h at 37°C, with or without the addition of exogenous stimuli, as indicated in the figure legends. The cells were then lysed and analyzed for luciferase and β-galactosidase activity.

Gene Gun Transfection and Fetal Thymic Organ Culture. Fetal thymic lobes from timed-pregnant RAG-2$^{-/-}$ mice at day 14 of gestation were placed on nucleopore filters resting on Gelfoam rafts (Upjohn) soaked in fetal thymic organ culture (FTOC) media (complete media supplemented with a further 5% FCS; five to six lobes per filter) (51). The thymic lobes were incubated at 37°C for 6 h in a humidified incubator with 5% CO$_2$ to allow them to adhere to the filters. After this time, the filters were briefly removed from the Gelfoam rafts, and thymi were immediately transfected by gene gun bombardment with a Helios gene gun set at 200 psi (Bio-Rad Labs.), with the desired DNA bound to gold particles (1 μg gold) (as indicated in the figure legends; one cartridge per set of thymic lobes). The amount of DNA loaded per microgram of microcarriers

is referred to as the DNA loading ratio (DLR; see figure legends). The filters carrying the transfected thymic lobes were then placed back on the Gelfoam rafts and incubated at 37°C for the indicated time (see figure legends). For short-term biochemical readout assays, the thymic lobes were incubated for a further 16–20 h, and then a single-cell suspension was prepared and the cells were lysed and assayed for luciferase and β-galactosidase activities as described below. For long-term developmental progression studies, the transfected thymic lobes were incubated for 7 d, with the FTOC media being changed once during the incubation. After this time, a single-cell suspension of the lobes was prepared, and the thymocytes were analyzed by flow cytometry as described below.

Flow Cytometry. FITC-, PE–, and APC-conjugated anti–mouse CD4, CD25, and CD8 were used for flow cytometric analysis (purchased from PharMingen). Staining of cells was carried out as described previously (51). In brief, thymic cell suspensions were washed in FACS buffer (HBSS containing 1% BSA and 0.1% sodium azide) and incubated with antibodies (1:300 dilution) for 30 min on ice. The cells were then washed in FACS buffer before analysis. Stained cells were analyzed with a FACS-Calibur™ flow cytometer (Becton Dickinson). Analysis was performed using CELLQuest software (Becton Dickinson); the data were live gated by size and lack of propidium iodide uptake.

Luciferase and β-galactosidase Assay. Cells transfected with the PathDetect reporter plasmids (Stratagene Inc.) were assayed for luciferase and β-galactosidase activities using the Dual-Light reporter gene assay system (Tropix; Perkin Elmer-Applied Biosystems) as previously described (50). In brief, the cells were lysed in lysis buffer (40 mM tricine, pH 7.8, 50 mM NaCl, 2 mM EDTA, 1 mM $MgSO_4$, 5 mM dithiothreitol, and 1% Triton X-100). Supernatant (25 μl) was combined with an equal volume of luciferase reaction buffer (30 mM tricine, pH 7.8, 3 mM ATP, 15 mM $MgSO_4$, 1 mM coenzyme A, and 10 mM dithiothreitol), and after addition of 1 mM luciferin, the sample was immediately assayed for luciferase activity with a Lumat LB 9507 Luminometer (Fisher Scientific Co.). To assay for β-galactosidase activity, Galacton-Plus (substrate for β-galactosidase; Tropix) was added to each tube after the luciferase assay was completed. The tubes were incubated for 30–60 min at room temperature, and then Accelerator-II (Tropix) was added to each tube and the samples were immediately assayed for β-galactosidase activity, measured as light emission with the Lumat LB 9507 Luminometer. Results represent the average luciferase activity indexed for β-galactosidase activity.

Results

Pre-TCR Complex Engagement Induces ERK-1/2 Phosphorylation. To aid in deciphering the signaling pathways that lead to the differentiative and proliferative events of β-selection, the phosphorylation status of ERK-1/2 was investigated by performing Western blot analyses using an anti–phospho-ERK-1/2 mAb. A SCID mouse–derived pre-T cell line, SL-12.β12, preincubated with biotinylated anti–TCR-β (H57-597) mAb, was stimulated for 1–30 min upon cross-linking with avidin. Engagement of the pre-TCR complex resulted in a rapid and transient phosphorylation of ERK-1/2 (Fig. 1 A). Phosphorylation was observed as early as 1 min but declined almost to background levels 5 min after stimulation; subsequently, a secondary, weaker level of phosphorylation was evident between 10 and 30 min (Fig. 1 A). To control for protein loading, the total amount
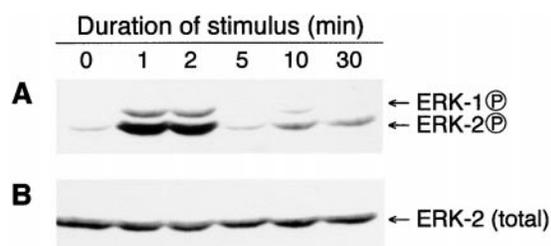

Figure 1. ERK-1/2 is phosphorylated upon cross-linking of the pre-TCR in the SL-12β.12 pre-T cell line. Lysates from SL-12β.12 cells stimulated with 10 μg/ml biotinylated anti–TCR-β mAb H57-597 and cross-linked with avidin (lanes 1, 2, 5, 10, and 30) or unstimulated cells (lane 0) were prepared and resolved on a 10% SDS-PAGE under reducing conditions and subsequently immunoblotted with specific antibodies against (A) phospho-ERK-1/2 and (B) ERK-2.

of ERK-2 was assessed by Western blot with an anti–ERK-2 antibody (Fig. 1 B).

To study whether the decline in phosphorylation of ERK-1/2 at 5 min was specific to pre-TCR activation, we performed a similar experiment with variants of the SL12 cell line expressing comparable levels of TCR β chain with either a TCR α chain (mature TCR) or a pTα chain (pre-TCR), as determined by flow cytometry (data not shown). The TCR β chain on both cell lines was cross-linked for 1–30 min, and the phosphorylation status of ERK-1/2 was determined. In both cases, there was an attenuation of ERK-1/2 phosphorylation levels at 5 min, indicating that this phosphorylation pattern is characteristic of the SL-12 cell line (data not shown). These results indicate that engagement of the pre-TCR on SL-12β.12 cells leads to the phosphorylation of ERK-1/2, demonstrating the activation of the Ras/Raf/MEK/ERK signaling cascade.

Reporter Plasmid Detection of ERK-1/2 Activation after Pre-TCR Engagement. We sought to determine if the phosphorylation of ERK-1/2 observed upon cross-linking of the pre-TCR (Fig. 1 A) leads to their activation. To this end, we employed a reporter plasmid system (described in Materials and Methods) that allows detection of ERK-1/2 activation within the cell. This system utilizes two plasmids: a fusion activator plasmid (pFA-Elk), which encodes for the transactivation domain of the ERK substrate, Elk-1 (residues 307–427), fused with the DNA-binding domain of GAL4; and a luciferase reporter plasmid (pFR-Luc), which encodes for the luciferase gene under the control of five GAL4 binding elements. Hence, in transfected cells, phosphorylation of the Elk-1 fusion protein can be read in the form of luciferase activity. Accordingly, we transfected SL-12β.12 cells with pFR-Luc, either alone or together with pFA-Elk. The transfected cells were then stimulated with immobilized anti-TCR-β mAb or with PMA and ionomycin.

Cells transfected with pFR-Luc alone displayed background luciferase activity (31 ± 18 relative light units [RLUs]). However, luciferase activity increased almost 850-fold above background levels in cells transfected with pFR-Luc and pFA-Elk (26,221 ± 1,119 RLUs; Fig. 2). This elevation in luciferase activity may reflect low-level
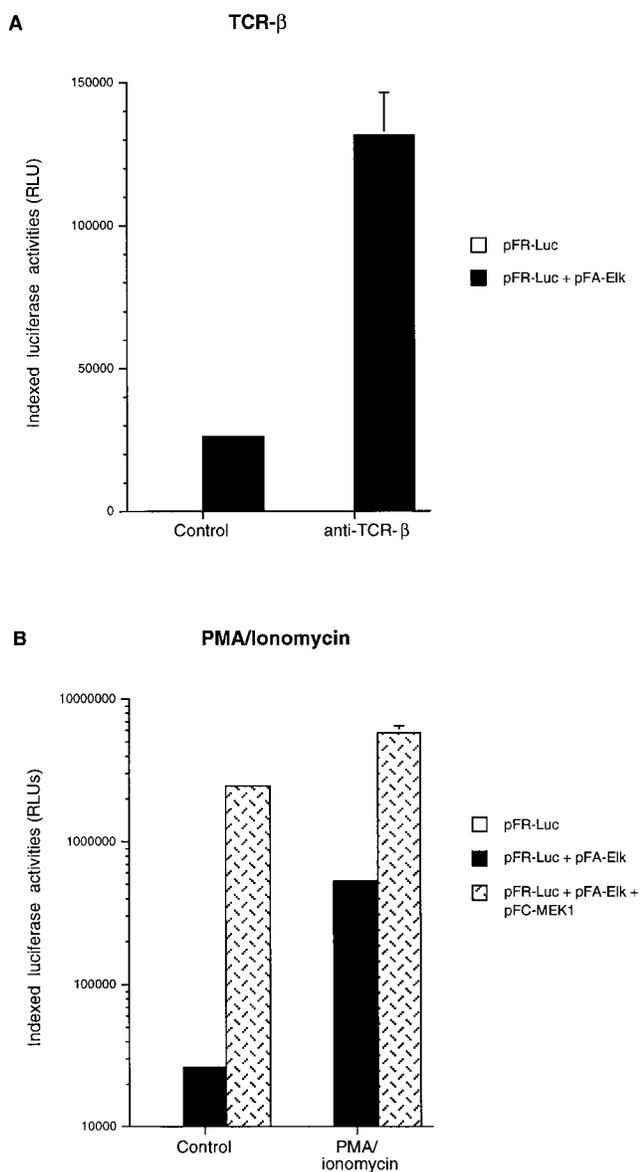
Figure 2. Activation of the ERK signaling cascade upon stimulation of SL-12β.12 cells with anti–TCR-β mAb (H57-597; A) or PMA/ionomycin (B). SL-12β.12 cells were transfected with reporter plasmids as indicated in the figure: pFR-Luc (10 μg), pFA-Elk (20 μg), and pFC-MEK1 (20 μg). All cells were transfected with a control β-galactosidase–encoding plasmid (1 μg). The transfected cells were incubated overnight at 37°C and then stimulated for 8 h with (A) immobilized anti–TCR-β or (B) phorbol ester, PMA, and the calcium ionophore, ionomycin (10 ng/ml of each). Cells were then lysed, and the lysates were assayed for luciferase and β-galactosidase activity. The experiment shown is representative of results carried out three times. Each experiment was carried out in triplicate, and the luciferase activities shown are indexed against β-galactosidase activity measured within each same sample.

constitutive ERK activity within SL-12β.12 cells (Fig. 1 A), as it was blocked by the addition of the MEK1 inhibitor, PD98059 (data not shown). Importantly, anti–TCR-β mAb stimulation of pFR-Luc/pFA-Elk–transfected SL-12β.12 cells resulted in a fivefold increase in luciferase activity compared with unstimulated cells (Fig. 2 A). This elevation

in luciferase activity was abrogated by the addition of PD98059 (129% inhibition). These data indicate that engagement of the pre-TCR complex results in the activation of ERK and subsequent phosphorylation of the ERK-1/2 substrate, Elk-1 (Fig. 2 A).

Activation of pFR-Luc/pFA-Elk–transfected cells by the addition of PMA/ionomycin induced a 20-fold stimulation in luciferase activity over unstimulated cells (Fig. 2 B); again, addition of PD98059 curtailed this activity (data not shown). As a control to demonstrate that ERK-1/2 activation can be efficiently detected with these reporter plasmids, pFR-Luc/pFA-Elk–transfected cells were also transfected with a plasmid encoding a constitutively active MEK1, pFC-MEK1. This serine/threonine kinase is responsible for the phosphorylation and activation of ERK-1/2. These cells showed maximal luciferase activity irrespective of PMA/ionomycin treatment (Fig. 2 B), indicating that this reporter system provides a highly sensitive method for detecting ERK activation.

Taken together, these results confirm that the ERK signaling cascade is activated upon engagement of the pre-TCR, as shown by the phosphorylation of ERK-1/2 (Fig. 1 A) and the phosphorylation/activation of the ERK-1/2 substrate, Elk-1 (Fig. 2 A), in the SL-12β.12 cell line.

Generation of DP Thymocytes in RAG-2$^{-/-}$ FTOCs Transfected with TCR-β and Active p56^{lck} Genes. An important application of the above reporter plasmid system would be to detect specific pre-TCR–mediated signals as they occur within an intact thymus. Therefore, an accelerated DNA/particle bombardment delivery system (gene gun) was adapted to transfect thymocytes in FTOC (52). This approach allows transfected cells to respond to normal microenvironmental stimuli encountered during differentiation within the thymic milieu (52, 53). We have previously used this method to study transcriptional regulation events in the developing fetal thymus (52).

The preservation of structural and functional integrity of gene gun–transfected thymi was tested by allowing FTOCs to complete a program of β-selection. Thus, RAG-2$^{-/-}$ mouse–derived FTOCs were gene gun transfected with control DNA and cultured for 7 d, either alone (Fig. 3 A, Control) or in the presence of anti-CD3 mAb (Fig. 3 A, anti-CD3). Transfected FTOCs were then analyzed by flow cytometry for evidence of differentiation to the DP stage (17–19). This analysis revealed that the thymocytes from anti-CD3–treated RAG-2$^{-/-}$ gene gun–transfected FTOCs progressed to the DP stage, as indicated by upregulation of CD8/CD4 (Fig. 3 A, anti-CD3). This differentiative event involved the loss of CD25 cell surface expression and proceeded through a CD8$^+$ immature SP stage (Fig. 3 A, anti-CD3). These results indicated that thymocytes and stromal elements within gene gun–transfected thymic lobes remain viable.

To determine the long-term developmental capacity of transfected thymocytes, RAG-2$^{-/-}$ thymic lobes were gene gun transfected with plasmids encoding either constitutively active Lck (Lck[F505]) or a productively rearranged TCR β chain. It has been previously shown in RAG$^{-/-}$
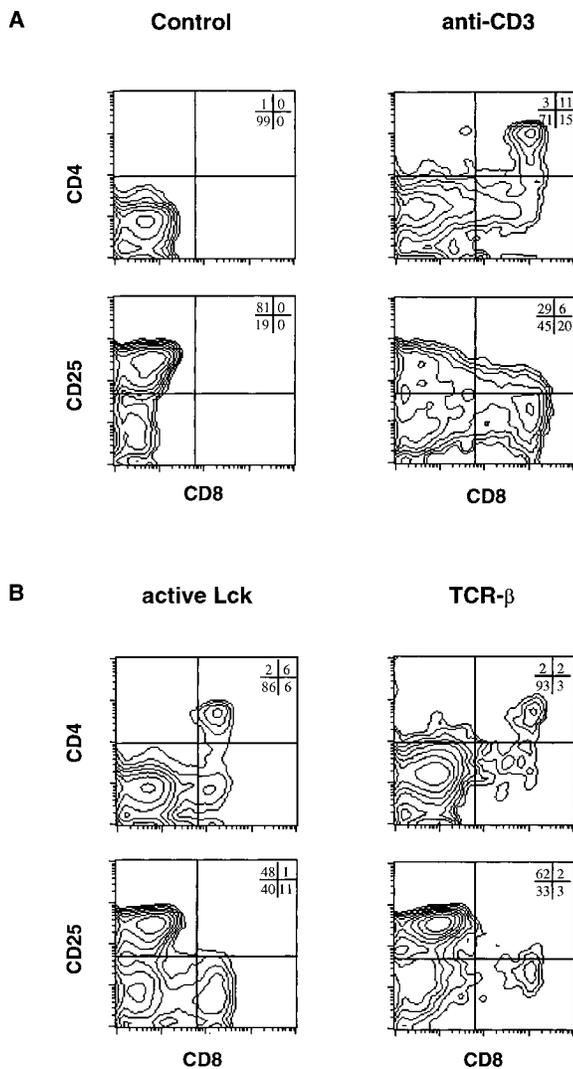
A

Control   anti-CD3

B

active Lck   TCR-β

Figure 3. Generation of CD4+CD8+ DP cells in RAG-2−/− FTOCs gene gun transfected with plasmid encoding a constitutively active Lck or a functional TCR β chain. Intact fetal thymic lobes removed from timed-pregnant RAG-2−/− mice (day 14 of gestation) were used as targets for DNA-covered gold particles as described in Materials and Methods. Fetal thymi were gene gun transfected with (A) control DNA and incubated in the absence (Control) or presence (anti-CD3) of 10 μg/ml anti-CD3 mAb (145-2C11) or (B) plasmid encoding a constitutively active Lck (DLR, 750 ng) or plasmid encoding a functionally rearranged TCR β chain (DLR, 250 ng). The transfected FTOCs were cultured for 7 d. After this time, a single-cell suspension was prepared and thymocytes were analyzed for surface expression of CD25, CD8, and CD4 by flow cytometry.

mice that introduction of transgenes encoding a kinase-active Lck or a functionally rearranged TCR β chain induced thymocyte proliferation and differentiation toward the DP stage of development (39, 41). Indeed, these events were also observed in gene gun–transfected RAG-2−/− FTOCs (Fig. 3 B). Flow cytometric analysis of Lck(F505)- and TCR-β–transfected FTOCs after 7 d revealed that thymocytes differentiated from the DN stage to the DP stage, as evidenced by upregulation of CD4 and CD8 surface expression and downregulation of CD25 surface expression (Fig. 3 B). The apparent difference in the levels of

CD8 surface expression in active Lck-transfected FTOCs was due to the fact that the experiment shown was analyzed separately and a different set of fluorochrome-labeled antibodies was used. These phenotypic changes are consistent with differentiation events that occur after completion of β-selection (Fig. 3 B; reference 5, 10–13).

Our finding that gene gun transfection of RAG-2−/− FTOCs with a functional TCR β chain resulted in the normal developmental progression of DN thymocytes indicated that a functional pre-TCR complex was generated (39). These data imply that after gene gun transfection, the formation of the pre-TCR results in signaling events that occur during normal β-selection in vivo.

ERK Signaling Cascade Is Activated upon Formation of the Pre-TCR in the Thymus. Because the cellular signaling processes that underlie β-selection are not well defined, we combined gene gun–mediated transfection of FTOCs with the reporter plasmid system. In this way, the intracellular signaling cascades that occur after pre-TCR formation can be monitored in a biological time frame and within a relevant in vivo setting. We hypothesized that gene gun–mediated transfection of RAG-2−/− mouse–derived FTOCs with a plasmid encoding a functionally rearranged TCR β chain, together with reporter plasmids, would allow us to capture de novo β-selection signaling events that occur after the generation of the pre-TCR.

Our results show that compared with FTOCs transfected with pFR-Luc alone (Fig. 4), there was a slight elevation in luciferase activity in FTOCs transfected with pFR-Luc/pFA-Elk (6 ± 1.8 vs. 59 ± 17 RLUs; Fig. 4). This level of luciferase activity may represent endogenous ERK-1/2 activity present in transfected FTOCs.

To directly test our hypothesis, RAG-2−/− FTOCs were gene gun transfected with reporter plasmids (pFR-Luc/pFA-Elk) together with a plasmid encoding a functional TCR β chain. Our analysis revealed an almost sixfold increase in luciferase activity in FTOCs transfected with a TCR-β plasmid as compared with FTOCs transfected with the reporter plasmids alone (333 ± 56 RLUs vs. 59 ± 17; Fig. 4, TCR-β). A substantial luciferase activity was also observed in FTOCs transfected with a constitutively active MEK1 plasmid together with the reporter plasmids (9,919 ± 1,933 RLUs; Fig. 4, MEK1). The level of stimulation observed in MEK1-transfected FTOCs demonstrates the sensitive nature of this assay.

Several reports have suggested that the Ras/Raf/MEK/ERK signaling cascade is involved at the β-selection stage (13, 42, 43, 54). However, our data provides the first direct evidence for coupling of the pre-TCR complex to the ERK signaling cascade in vivo.

Activation of the ERK Cascade Requires Lck. Lck is thought to play an essential role as a mediator of β-selection events (15, 17, 24, 41, 55). Therefore, we investigated whether Lck could activate the ERK signaling cascade in FTOCs. To this end, FTOCs were gene gun transfected with a kinase-active Lck together with the reporter plasmids. Fig. 5 shows that a sixfold increase in luciferase activity was observed in FTOCs transfected with constitutively active Lck
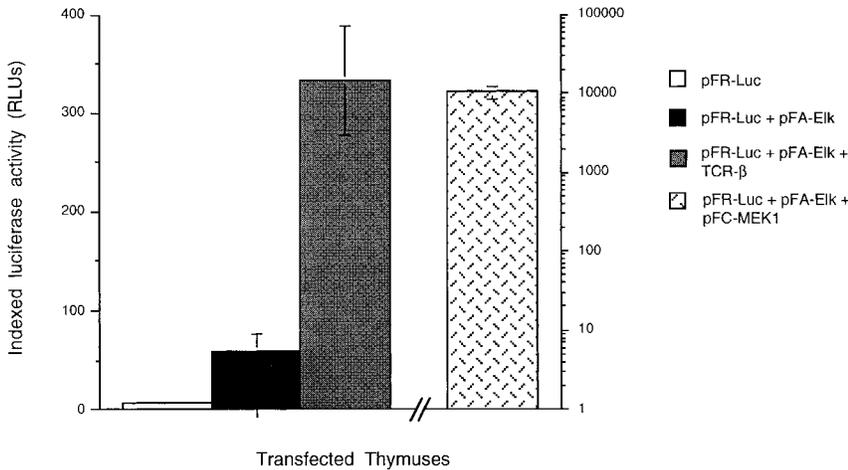
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(387 ± 75 RLUs; Fig. 5). This level of luciferase activity is comparable to the stimulation observed in TCR-β–transfected FTOCs (Fig. 5, TCR-β).

To address whether the observed ERK activation after the pre-TCR complex formation was dependent on Lck activity, we took advantage of a plasmid encoding a dominant negative Lck (Lck[R273]). This mutant form of Lck has been shown to cause a dramatic block in T cell development at the DN stage (15). Fig. 5 shows that transfection of RAG-2$^{-/-}$ FTOCs with the reporter plasmids together with plasmids encoding a TCR β chain and a kinase-negative Lck led to an attenuation of luciferase activity, as compared with FTOCs transfected with TCR-β alone (84 ± 56 vs. 333 ± 56 RLUs; Fig. 5).

These results indicate that expression of the pre-TCR complex by developing thymocytes activates the ERK signaling cascade and that this activation is directly dependent on Lck during thymocyte differentiation within the thymus.

Discussion

In this study, we demonstrate that formation and/or engagement of the pre-TCR complex results in the activation of the ERK signaling cascade. We have shown this in two distinct systems: (a) in a SCID mouse–derived pre-T cell line, SL-12β.12 (48), in which engagement of the pre-TCR leads to phosphorylation and activation of ERK-1/2; and (b) within the thymus, where formation of the pre-TCR in developing thymocytes induces ERK activation and phosphorylation of the downstream substrate, Elk-1. Furthermore, our findings confirm the central role of the nonreceptor tyrosine kinase Lck in β-selection by demonstrating that ERK activation is strictly dependent on Lck function in vivo.

The ability to introduce reporter plasmids into developing thymocytes in vivo provides an important diagnostic tool for the detection of signaling events during T cell development in the thymus (Figs. 3 and 4). The significance of this method is highlighted by the fact that, in the absence of the thymic microenvironment (i.e., cell suspension), thymocytes fail to respond to normal developmental

cues and undergo rapid programmed cell death (56, 57). Indeed, transfection of thymocytes from RAG$^{-/-}$ mice with reporter plasmids revealed that cells in suspension fail to respond when cotransfected with a plasmid encoding a TCR β chain (data not shown). Thus, thymocytes in cell suspension appear to be unresponsive to pre-TCR–mediated signals, possibly due to the lack of stromal elements to assist in this signaling. These results are consistent with previous data showing that anti-CD3 mAb–mediated differentiation of RAG$^{-/-}$ thymocytes to the DP stage in FTOC does not occur when these thymocytes are treated in cell suspension (58).
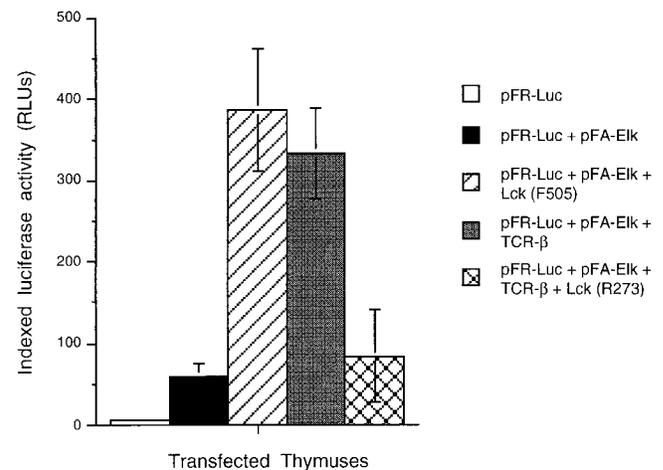
The notion that the Ras/Raf/MEK/ERK signaling cascade plays a central role during β-selection is supported by data obtained from mice bearing targeted genetic deficiencies of key signaling molecules such as Lck/Fyn, ZAP-70/Syk, LAT, or SLP-76 (24–26, 28–31). These mice displayed a block in T cell development at the DN stage, suggesting that these molecules are essential for differentiation to the DP stage of development (24–26, 28–31). However, several contradicting reports using transgenic mice carrying either constitutively active or dominant negative members of these signaling molecules have left the involvement of this pathway unresolved. Studies introducing constitutively active Lck, Ras, and Raf into RAG$^{-/-}$ mice also indicate that the Ras/Raf pathway is crucial at this stage of development (41–43). In contrast, studies using transgenic mice carrying dominant negative forms of Ras (Ha-Ras^{N17}) (45), Raf (46), and MEK-1 (47) failed to support a role for these signaling molecules during β-selection. In this regard, it is important to note that mice carrying a dominant negative Lck transgene displayed varying degrees of DN to DP thymocyte development arrest directly correlating with the levels of transgene expression (15). Thus, the conflicting results obtained using dominant negative molecules may reflect their ability to be sufficiently expressed at the appropriate stage of development. In addition, studies using retroviral infection of thymocytes with dominant negative MEK-1 showed a perturbation in the transition of thymocytes from the DN to DP stage, suggesting that the inhibitory effectiveness of dominant negative molecules appears to be dependent on the experimental system used (54).

Although several studies have indirectly supported the importance of the Ras/Raf/MEK/ERK-mediated signals downstream of the pre-TCR, in our system, the fact that the detection of ERK activation is predicated by the expression of the pre-TCR within individually transfected developing thymocytes directly links the pre-TCR to the activation of these kinases. Thus, this study provides the first evidence for the pre-TCR being responsible for the generation of specific downstream signals within the thymus (Fig. 4) and shows that Lck acts proximally to the nascent pre-TCR complex, leading to the subsequent activation of ERK (Fig. 5).

The transcription factor Elk-1 has been shown to be phosphorylated by ERK-1/2, although more recently it has been demonstrated that JNK (c-Jun NH$_2$-terminal kinase) also has the ability to phosphorylate and activate Elk-1 (59–61). However, mouse models with gene-targeted disruptions of key signaling components of the JNK signaling pathway (JNK2 and stress-activated protein kinase kinase 1) did not reveal any abnormalities in β-selection events (62–64). In this regard, our data with the SCID mouse–derived pre-T cell line SL-12β.12 show that engagement of the pre-TCR results in ERK phosphorylation and activation of the ERK substrate, Elk-1 (Figs. 1 and 2), and that this can be blocked by addition of the MEK1 inhibitor, PD98059. Together, these data indicate that, in this setting, Elk-1 is specifically activated by ERK.

Pre-TCR signals can be experimentally bypassed by introducing constitutively active mutants of certain signaling components, inducing β-selection–mediated events. Interestingly, the introduction of an activated form of Ras (Ha-Ras^{V12}) or Raf-1 (Raf-CAAX), although allowing the differentiation of RAG$^{-/-}$ thymocytes to the DP stage, failed to establish allelic exclusion at the TCR-β locus when introduced into wild-type mice (43, 65). This indicates that although certain β-selection outcomes are mediated by the Ras/Raf pathway, distinct pre-TCR–derived signals upstream of Raf are responsible for the control of allelic exclusion. Therefore, it is interesting to note that RAG$^{-/-}$ FTOCs retrovirally infected with constitutively active MEK1 do not differentiate to the DP stage of development (54). This finding suggests that Raf may activate additional signaling pathways enabling developing thymocytes to undergo full differentiation to the DP stage. Therefore, the intrathymic signal detection system developed here will allow for detailed studies of distal signaling events activated by the pre-TCR, facilitating the elucidation of potential signaling branchpoints.

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