Induction of Interleukin 2-Responsiveness in Thymocytes of the Transgenic Mice Carrying Ick-Transgene

Yoichi Moroi*,1, Yasuhiro Koga1, Kazuhiko Nakamura1, Masumi Ohtsu2, Genki Kimura2, and Kikuo Nomoto1

Departments of 1Immunology and 2Virology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Fukuoka 812, Japan

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Abstract: The role of Ick gene in T cell proliferation and differentiation was investigated with transgenic mice carrying human Ick cDNA whose expression was regulated by the promoter of mouse H-2Kb and the enhancer element of mouse IgH. RNase protection assay revealed that the Ick transgene was expressed in the thymus and spleen, whereas immunoblot analysis demonstrated that amounts of p56Ick in freshly isolated lymphoid organs were almost equal between transgenic mice and negative littermates. Cell-surface marker analyses of the thymocytes and peripheral lymphocytes revealed no remarkable difference between both groups. Notable finding is that the thymocytes from transgenic mice showed a significant proliferative response to the stimulation with IL-2, but not the thymocytes from negative littermates. Further analysis revealed that CD4+8- single positive thymocytes proliferated in response to IL-2. While surface expression levels of IL-2Rα and IL-2Rβ of these CD4+8- thymocytes from transgenic and control mice were almost equal before stimulation with IL-2, the expression of IL-2Rβ was induced only in transgenic thymocytes after stimulation with IL-2. Immunoblot analysis demonstrated that the expression of p56Ick of transgenic thymocytes was not down-regulated at 4 hr after stimulation with IL-2, whereas p56Ick of control ones were not detectable any more at 4 hr after stimulation with IL-2. Moreover, in vitro kinase assay substantiated such unchanged expression of p56Ick in the thymocytes from transgenic mice: the kinase activities of p56Ick did not decrease in thymocytes from transgenic mice after stimulation with IL-2, while kinase activities of control ones were significantly down-regulated by stimulation of IL-2. These results suggested that a significant proliferative response found in the thymocytes from Ick-transgenic mice after the stimulation with IL-2 was caused by a constitutive expression of p56Ick in these thymocytes even after the stimulation. Our findings, therefore, support a possibility that p56Ick may play a role in the IL-2R-mediated signaling system in CD4+8- thymocytes.

Key words: Transgenic mice, p56Ick, Interleukin 2 response, Thymocytes

The Ick gene encodes a membrane-bound protein tyrosine kinase (PTK) that is expressed in cells of lymphoid lineage, predominantly in T cells (19, 24, 51). The physical association of p56Ick, a main protein product of Ick, with the cytoplasmic tails of CD4 and CD8 T cell surface molecules, and phosphorylation of p56Ick by the cross-linking of CD4 antigens, suggest a specific role for p56Ick in signal transduction pathways mediating T cell differentiation and activation (34, 35, 43, 48, 49). Recently it has been demonstrated that the activation of PTK precedes the sequence of phosphatidylinositol (PI) breakdown when T cell is activated through the TcR-CD3 complex (5, 15, 18). The experiments with inhibitors of PTK such as genistein and herbimycin A have demonstrated that the inhibition of PTK activity by such agents results in failure of TcR to activate PI turnover (16, 47). On these bases it was concluded that the

*Address correspondence to Dr. Yoichi Moroi, Department of Immunology, Medical Institute of Bioregulation, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka, Fukuoka 812, Japan.

Abbreviations: FITC, fluorescein isothiocyanate; IgH, immunoglobulin heavy chain; IL-2, interleukin 2; IL-2R, IL-2 receptor; PE, phycoerythrin; PHA, phytoagglutinin; PI, phosphatidylinositol; PTK, protein tyrosine kinase; TcR, T cell receptor.
TcR must be coupled either directly or indirectly with nonreceptor PTK molecules to transmit the signal further and induce the turnover of PI pathway. The candidates for such nonreceptor PTKs are considered to be p56^ck and another member of nonreceptor PTK family, p59^fyn, which are expressed abundantly in T cells and associated physically with TcR-CD3 complex (17, 39, 41).

While a crucial role of p56^ck in TcR-mediated activation has been argued so far, a novel role of p56^ck as a signal-transducing molecule working downstream of IL-2R has also been proposed recently. Firstly, the increase in tyrosine phosphorylation of several protein species coupled with proliferative response was found in IL-2-dependent T cell lines by administration of IL-2 (30, 36, 37). Secondly, the addition of IL-2 to IL-2-dependent NK cell line or T cell clones stimulated the PTK activity of p56^ck specifically (6, 14). Thirdly it was demonstrated that IL-2R/β molecule was tyrosine-phosphorylated by stimulation with IL-2 (3, 8, 26, 42), and formed complex with p56^ck, which also phosphorylated IL-2R/β in vitro (11), supporting the participation of p56^ck as a critical signal-transducing molecule in the IL-2/IL-2R signal transduction system.

In the present study, we produced the transgenic mice that carried lck gene under the transcriptional control of H-2K promoter and IgH enhancer element. Using the lymphocytes from these transgenic mice, in which the expression of lck-transgene was regulated by these promoter and enhancer element in a manner different from that of lck gene of endogenous origin, the proliferative response of these lymphocytes to IL-2 was examined in order to investigate the role of p56^ck in IL-2R-mediated signal transduction. We found in this study that the CD4^+8^- thymocytes exhibited a marked proliferative response to IL-2, whereas the same subpopulation of thymocytes from transgene-negative littermates showed no detectable proliferative response to IL-2.

**Materials and Methods**

**Transgenic mice.** A plasmid vector named pHSE3' (31), a gift from Dr. H. Pircher, was used for transgene of lck in the present study. Briefly, pHSE3' consists of H-2Kb promoter located just upstream the cloning site, a genomic fragment of human β globin gene including exon 2 and 3, poly A signal, immunoglobulin heavy chain (IgH) enhancer element and vector sequence for cloning and amplification in the bacteria. A full-length human lck cDNA, YT 16 (3), was inserted into the SalI cloning site of pHSE3' (27) and the resultant construct was named pHSE3'-lck. For microinjection, 7.8 kbp DNA fragment consisting of chimeric gene was excised by digestion with XhoI and its structure is shown in this figure. A 3.5 kbp PstI fragment corresponds to the 3.5 kbp band detected by lck cDNA probe in Southern blot analysis of genomic DNA from lck-transgenic mice as shown in Fig. 2.

**Fig. 1. Structure of lck transgene for microinjection.** A human lck cDNA, YT 16 (3), was inserted into the SalI cloning site of pHSE3' (27) and the resultant construct was named pHSE3'-lck. For microinjection, 7.8 kbp DNA fragment consisting of chimeric gene was excised by digestion with XhoI and its structure is shown in this figure. A 3.5 kbp PstI fragment corresponds to the 3.5 kbp band detected by lck cDNA probe in Southern blot analysis of genomic DNA from lck-transgenic mice as shown in Fig. 2.

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**DNA and RNA extractions.** For the isolation of tail DNA, approximately 3 cm of tails of mice at 4 weeks of age were cut off and then incubated overnight at 37°C with gentle shaking in 1.8 ml of 0.05 M Tris-HCl [pH 8.0], 0.1 M NaCl, 20 mM EDTA, 1% SDS and 150 μg/ml proteinase K. DNA was isolated from the resulting homogenate by phenol extraction and ethanol precipitation. Total RNA was prepared by the guanidine isothiocyanate extraction and purified on CsCl gradient.

**DNA and RNA analyses.** For Southern blot analysis, 20 μg of total genomic tail DNA was digested with PstI, separated on 0.8% agarose gel and transferred to Gene Screen Plus (NEW, Boston, Mass., U.S.A.). The filter was hybridized with YT 16 cDNA probe labeled with 32P by random priming according to the method described elsewhere (7). For Northern blot analysis, 10 μg of
total RNA were glyoxylated, electrophoresed through 0.8% agarose gel and then transferred to a Gene Screen Plus. The filter was then hybridized with \(^{32}\)P-labeled probes of human lek cDNA and murine genomic \(\beta\)-actin cDNA as reported elsewhere (45).

**RNase protection assay.** RNase protection assay was performed as described by Nakamura et al (28). Briefly, an EcoRI-BglII fragment of YT 16, which corresponds to the 5'-UT region of human lek transcript, was cloned into pSPT 18. Using this constructed plasmid, \(^{32}\)P-labeled antisense RNA probe (362bp in length) for human lek mRNA was synthesized. This \(^{32}\)P-labeled antisense RNA probe was hybridized with 30 \(\mu\)g of total RNA extracted from the organs of mice in 30 \(\mu\)l of hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA and 40 mM PIPES [pH 6.7]). After hybridization, RNase was added to the reaction mixture and incubated at 34°C for 30 min. Following the treatment of samples with proteinase K and SDS, the precipitated RNA from the samples was loaded and electrophoresed on 6% polyacrylamide/8M urea gel. Then the gel was exposed to an X-ray film at -70°C.

**Immunoblot analysis.** Immunoblot analysis was performed according to the method described by Moroi et al (27). Briefly, cells were suspended in lysis buffer (1% Triton X, 300 mM NaCl, 50 mM Tris-HCl [pH 7.6], 1 mM sodium orthovanadate, 2.5 mM EDTA, 10 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml leupeptins and 2 mM phenylmethylsulfonylfluoride) and lysed by sonication. Cell lysates containing 100 \(\mu\)g protein were denatured in 1% SDS solution and electrophoresed on polyacrylamide gel. After transfer of proteins to a nitrocellulose membrane filter (Schleicher & Schuell, Dassel, Germany), the filter was treated with 50 \(\mu\)g/ml mouse anti-Lck monoclonal antibody, MOL 171 (27), and peroxidase-conjugated anti-mouse IgG (Tago Inc., Burlingame, Calif., U.S.A.) for visualization. For detection of Thy 1 antigens, rabbit anti-Thy 1 serum (Cedarlane, Hornby, Canada), horseradish peroxidase-conjugated anti-rabbit Ig and enhanced-chemiluminescence system (Amersham, Buckinghamshire, U.K.) were used.

**Cell preparation and purification.** A single cell suspension from the thymus or spleen was obtained by squeezing the organs with a pair of frosted slide glasses. To enrich T cells, spleen cell suspension was passed through the nylon wool column (12). Thymocytes were separated according to the panning method described by Wysocki and Sato (53). Briefly, thymocytes suspended in PBS at 4 \(\times\) 10^6/ml were poured onto a mAb-coated plastic petri dish (diameter 90 mm, Iwaki, Tokyo). After incubation for 90 min at 4°C, nonadherent cells were removed and then the bound cells were recovered by flashing. For preparation of CD4+/CD4− and CD8+/CD8− thymocytes by panning method, GK 1.5 (anti-CD4 mAb) and anti-mouse Lyt 2.2 (anti-CD8 mAb; 41-3.48, Meiji Institute of Health Science, Tokyo) were used, respectively.

**Flow cytometry analysis.** Analyses of the expression levels of surface antigens on thymocytes were performed using FACSscan (Becton Dickinson, Mountain View, Calif., U.S.A.). Cells were stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE)- and biotin-conjugated antibodies followed by incubation with streptavidin-PE (Becton Dickinson). Scatter was used to exclude dead cells, and fluorescence intensity was displayed on a logarithmic scale. The antibodies used in this study were FITC-conjugated anti-CD4 mAb (Becton Dickinson), PE-conjugated anti-CD8 mAb (Tago Inc.), biotin-conjugated anti-IL-2R\(\alpha\) mAb (7D 4; 29) and biotin-conjugated anti-IL-2R\(\beta\) mAb (TM-\(\beta\), generously provided by Dr. T. Tanaka; 46). In some analyses, analysis gates were set on CD4 positive cells, and the percentage of each IL-2R\(\alpha\) or \(\beta\) positive cells was calculated.

**Cell culture and stimulation.** Cells were suspended at 5 \(\times\) 10^6/ml in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin G and 100 \(\mu\)g/ml streptomycin, and incubated for 48 hr in the 96-well round-bottomed microtiter plate (0.1 ml/well) at 37°C in 5% CO_2. Six hours before harvesting cells by a cell harvester, 1 \(\mu\)Ci of \([3\text{H}]\)thymidine was added to each well for assay of cell proliferative activity. To stimulate the cells, human rIL-2 at 2 nM or at various concentration (TGP-3; Takeda Pharmaceutical Co., Osaka, Japan), murine rIL-4 at 100 U/ml (Genzyme Co., Boston, Mass., U.S.A) or PHA at the dilution of 50 times (Wellcome, Dartford, England) was added at the start of culture. For stimulation with immobilized antibody, H57-597 (anti-TcR \(\alpha\)-chain mAb; 21) or 145-2C11 (anti-CD3 mAb; 22), antibodies were diluted in PBS at 20 \(\mu\)g/ml and added to the 96-well plate (30 \(\mu\)l/well). Plates were incubated at 4°C overnight and washed 3 times with PBS and then used for the culture of the responding cells.

**Immunoprecipitation and in vitro kinase assay.** Immunoprecipitation and in vitro kinase assay were performed as described elsewhere (38, 50).
Cells (2 × 10⁶) were lysed with 1% NP40 lysis buffer (1% NP40, 50 mM Tris-HCl[pH 8.0] and 2 mM EDTA), and these lysates were precleared using rabbit anti-mouse Ig (Zymed, San Francisco, Calif., U.S.A.) and protein A Sepharose (Pharmacia, Uppsala, Sweden). These supernatants were incubated for 1 hr at 4°C with 20 μg/ml of mouse anti-Lck mAb, MOL171, then 15 μg/ml of anti-mouse Ig was added prior to incubation with protein A Sepharose. Protein A-antibody-antigen complexes were collected and washed four times with NET gel buffer (50 mM Tris-HCl[pH 7.5], 150 mM NaCl, 0.1% NP40, 1 mM EDTA[pH 8.0], 0.25% gelatin and 0.02% sodium azide). These complexes were incubated for 10 min at 30°C with reaction buffer (40 mM PIPES[pH 7.1], 10 mM MnCl₂ and 5 μCi of [γ-³²P]-ATP), then SDS-gel loading buffer (100 mM Tris-Cl[pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue and 20% glycerol) was added to stop the reaction and to denature the proteins in the samples. Samples were electrophoresed on polyacrylamide gel, then this gel was dried and was exposed to an X-ray film at −70°C.

Results

Transgenic Mice

Three independent lck transgene positive founder mice were obtained by microinjection of the lck-construct into (C57BL/6 × CBA/N) F₂ embryos. Figure 2 shows the detection of transgene integrated in tail DNA an 3.5 kbp band in two founder mice (F₂8 and F₂9), and no 3.5 kbp bands in DNA of two negative littermates (F₃₀ and F₃₁). In this filter, F₂⁹ was estimated to be integrated with approximately 7 copies of transgene by the densitometrical comparison with the control 3.5 kbp DNA fragment loaded in the parallel lane, which is excised from pHSE3'-lck by digestion with PstI (see Fig. 1). It was considered that the larger and smaller bands than 3.5 kbp detected in all mice were derived from endogenous lck gene. F₂⁹ (male mouse) was then crossed with normal female C57BL/6 mice and their offspring were used for further analysis after the confirmation of transgene integration. Expression of lck mRNA in the thymus and spleen was detected in transgene positive mice and their negative littermates by Northern blot analysis using human lck cDNA as probe (data not shown). The amounts of lck mRNA in the thymus and spleen were, however, almost equal between transgenic mice and negative littermates. No lck message was detected in other organs such as the brain, lung, liver or kidney (data not shown).

Detection of lck Transgene mRNA

The human and murine lck mRNA show high sequence homology with each other and exhibit almost the same message size of 2.2 kbp (19, 24, 51). Therefore it is difficult to discriminate the lck message of either transgene or endogenous origin by Northern blot analysis. To confirm the expression of lck transgene in the transgenic mice at its transcriptional level, RNase protection assay was performed with 362 by antisense RNA probe which was synthesized using human lck cDNA, YT 16 (Fig. 3). In transgenic mice (T), a 325 by fragment was detected in the thymus and less markedly in the spleen, whereas no such protected lck mRNA fragment was detected in the thymus or the spleen of negative littermates (N). A 238 by fragment detected in Jurkat cell line represented the other type of human mRNA transcribed from the distal lck promoter (28), there-
fore, no 238 bp band was detected in transgenic mouse because YT 16 of our transgene is not derived from the proximal promoter (19). This result demonstrated the functional expression of Ick transgene incorporated in the transgenic mice. Then the amount of p56Lck, a main protein product of Ick, was examined by immunoblot analysis using anti-Lck mAb, MOL171 (Fig. 4). As the total level of Ick mRNA was equal between the thymus of transgenic mice and that of negative littermates, the amount of p56Lck accumulated in thymus (Thy) was also almost equal in transgenic mice (T) and of negative littermates (N). Either in lymph nodes (LN), or spleen (Spl), no significant difference in the amount of p56Lck was observed. Moreover, no difference was found in kinase activities of p56Lck in thymocytes at resting state from both groups (Fig. 10, 0 hr). These results implied that the thymocytes from transgenic mice had no significant increase in the accumulation of p56Lck compared with their negative littermates at least in their freshly isolated preparation, which was considered as resting state in the cell activation cycle.

**Phenotypical Analysis of Transgenic Mice**

Cell-surface marker analysis of transgenic mice and negative littermates was performed by using FACScan. The expressions of CD4 and CD8 of thymocytes from transgenic mice and negative littermates were at almost equal levels, and the ratios of subpopulations of thymocytes, such as double negative, double positive and single positive cells, were also identical between both groups (data not shown). The ratios of high, low or negative expressions of CD3 or TcR of thymocytes are also equal between both groups (data not shown). Moreover, no difference of the ratio of T cell/B cell in splenocytes or lymph node cells was found between both groups, and no tumor in the thymus or in other organs developed in transgenic mice for 30 months' observation after birth (data not shown).

**Proliferative Response of Thymocytes to IL-2**

The proliferative responses of thymocytes to various stimulants were examined in transgenic mice (shaded bars) and their negative littermates (open bars) at 8 weeks after birth (Fig. 5a). When isolated thymocytes (5 × 10⁵/well) were set in the culture for 48 hr, a marked proliferative response of thymocytes to IL-2 added at 2 nM was found in transgenic mice (Fig. 5a, indicated by star). On the other hand, no such significant response of thymocytes from transgenic
mice was observed by the stimulation with IL-4, PHA, immobilized anti-TcR αβ or anti-CD3 mAb. A several-fold increase of proliferative response to IL-2 was also found in the thymocytes from transgenic mice when age-matched C57BL/6 or CBA/N mice were used as controls instead of negative littermates to exclude the possibility of strain-related difference in the response to IL-2 (data not shown). This augmented responsiveness of transgenic thymocytes after the stimulation with IL-2 was detected in every experiment, while the degree of augmentation was varying ranging 2 to 5 times. When the dose of IL-2 was reduced, the proliferative response of these thymocytes could...
not be detected any more at less than 0.2 nM (Fig. 5b). Previous reports (23, 52) implicated that the intermediate affinity IL-2Rs participate mainly in those IL-2-mediated proliferative responses of the thymocytes from transgenic mice. The degree of IL-2-mediated proliferative response of transgenic thymocytes was approximately compatible with that of IL-2-mediated response of splenic T cells from transgenic mice or control mice, both of which showed almost equal level of proliferative response to IL-2 (Fig. 5a). Then the kinetics of proliferative response of thymocytes to IL-2 were examined in the culture with fewer cell density (1 × 10^5/well) to keep the medium condition well up to 3 days (Fig. 6). Two days’ culture showed the highest response of thymocytes from transgenic mice (closed circles). Again a significant response was found only in the thymocytes from transgenic mice in all the one-, two- and three-day cultures.

Thymocyte Subsets Responsible for the Proliferative Response to IL-2

To determine the thymocyte subset exhibiting a significant proliferative response to IL-2 in transgenic mice, thymocytes were separated by panning method into CD8^-/CD8^+ or CD4^-/CD4^+ using anti-Lyt 2.2 (41-3.48) or anti-L3T4 (GK1.5), respectively. The efficiency of separation was monitored by two-color FACS analysis using 53.6.7 for CD8 antigen and GK 1.5 or anti-rat IgG for CD4 antigen. CD8^- subset (Fig. 7a) or CD4^- subset (data not shown), both of them collected as nonadherent cells from antibody-coated dishes, contained few CD8^+ or CD4^+ cells, respectively. Then each of thymocyte subpopulation derived...
from transgenic or control mice was examined on its proliferative response to the stimulation with IL-2 at fewer cell density (1 x 10^5/well) (Fig. 8). When thymocytes were separated into CD8^- and CD8^+ subpopulations, a significant proliferative response of transgenic mice (shaded bars), higher than the response of negative littermates (open bars), was found in CD8^- subset (the bar indicated by star) consisting of CD4^+ and CD4^-8^- thymocytes, but not found in CD8^+ subset consisting of CD4^+8^+ and CD4^-8^- thymocytes.

Moreover, after the separation of thymocytes into CD4^+ subsets consisting of CD4^-8^- and CD4^+8^+ cells, a response of transgenic group as significantly higher than that of control group (the bar indicated by star). Taken together, it was considered that the CD4^+8^- thymocytes of transgenic mice, which are included in both of the IL-2-responding CD8^- and CD4^+ subsets, are the cell population responsible for a proliferative response to stimulation with IL-2.

**Characterization of IL-2-Responsive Thymocyte Subset**

The ratios of CD4^+8^- cells as well as other cell subsets in the whole thymocytes were almost equal between transgenic mice and negative littermates (see "Results"). Therefore it seemed unlikely that the significant proliferative response by whole thymocytes from transgenic mice with IL-2 (Fig. 5) was caused by the increase in the ratio of CD4^+8^- cells in transgenic mice. To further characterize the IL-2-responsive thymocytes of transgenic mice bearing CD4^+8^- phenotype, the expression levels of surface IL-2Rα and IL-2Rβ on CD8^- thymocytes were examined by FACS analysis using anti-CD4 mAb and anti-IL-2Rα mAb/anti-IL-2Rβ mAb (Fig. 7). Figure 7a shows almost complete exclusion of CD8^+ cells in these CD8^- subsets obtained by panning method using anti-CD8 mAb. When CD4^+ cells in those CD8^- subsets (=CD4^+8^- cells) were gated for examination, the ratios of IL-2Rα-positive cells before the culture were only 4%, being almost equal both in transgenic (panels at left side) and negative littermate (panels at right side) groups (Fig. 7b). The ratios of IL-2Rβ-positive cells were also equal between both groups before stimulation (Fig. 7c). Incubation of those CD8^- subset with IL-2 for 42 hr, that is just before the addition of [3H]-thymidine in the cell proliferative response assay (see Fig.5), increased the ratios of IL-2Rβ-positive cells up to 15% in CD4^+8^- population in transgenic groups, but not in control ones (Fig. 7d). Although there was no significant difference in the positive ratios of IL-2Rα- and IL-2Rβ-expressing thymocytes in these groups of mice before culture, a moderate-increased expression of IL-2Rβ in this subset of transgenic thymocytes after stimulation may explain the augmented proliferative response of these thymocytes from transgenic group.

Then the level of p56^ck in CD4^+8^- thymocytes after stimulation with IL-2 was, subsequently, examined by immunoblot analysis (Fig. 9). Before stimulation, the amount of p56^ck accumulated in thymocytes was almost equal between transgenic mice (T) and negative littermates (N). However after the stimulation with IL-2, the amount of p56^ck in negative littermate group decreased at 1 hr and became almost undetectable at 4 hr whereas that of p56^ck in transgenic group was still significantly detectable during these periods of time. On the other hand, the amount of Thy 1 antigens, which were examined as the internal control of the protein amount loaded in the gel, from transgenic and control thymocytes were almost equal at 4 hr after stimulation of IL-2 (Fig. 9). Moreover, in vitro kinase assay, which detects the autophosphorylation of p56^ck itself and the phosphorylation of an exogenous PTK substrate, enolase, revealed that the kinase activities by
p56\(^{\text{Lck}}\) of transgenic thymocytes (T) increased transiently at 1 hr and were still enough even 4 hr after the stimulation with IL-2, whereas the activities of control ones (N) reduced progressively during this period (Fig. 10, a and b). It was reported that IL-2 stimulation induced the alteration in the mobility of p56\(^{\text{Lck}}\) to p60\(^{\text{Lck}}\) (14). Although MOL 171 used here was able to detect p60\(^{\text{Lck}}\) (27), it did not detect p60\(^{\text{Lck}}\) in these thymocytes from both group after stimulation with IL-2. These results proposed a potential role of p56\(^{\text{Lck}}\) in IL-2/IL-2R-mediated proliferative response such that the maintenance of significant amount of p56\(^{\text{Lck}}\) and the preservation of sufficient level of kinase activity of p56\(^{\text{Lck}}\) even after activation in CD4\(^{+}\)8\(^{-}\) cells of transgenic mice renders them highly responsive to the stimulation with IL-2.

Fig. 9. Immunoblot analysis of p56\(^{\text{Lck}}\) after stimulation with IL-2. CD8\(^{-}\) thymocytes were separated by panning method from whole thymocytes of lck-transgenic mice (T) and negative littersmates (N). These CD8\(^{-}\) thymocytes were suspended at 3 \times 10^6/ml in RPMI 1640 medium with 10% FCS and cultured with 2 nM of rIL-2 at 37°C. At 0 hr (lane 1), 1 hr (lane 2) and 4 hr (lane 3) after the start of culture, cells were collected and lysed for examination of accumulated p56\(^{\text{Lck}}\) in the cells by immunoblot analysis using MOL 171. For detection of Thy 1 antigens, rabbit anti-Thy 1 serum, peroxidase-conjugated anti-rabbit Ig were used.

Fig. 10. Immunoprecipitation and in vitro kinase assay of p56\(^{\text{Lck}}\) in thymocytes from transgenic mice. a) 2 \times 10^6 of CD8\(^{-}\) thymocytes from transgenic mice (T) or negative littersmates (N) was cultured with 2 nM of IL-2 for indicated times (0, 1 or 4 hr) as in Fig. 10. Cells were lysed by 1% NP40 buffer and immunoprecipitated with MOL 171 and anti-mouse Ig. These immunocomplexes were incubated for 10 min in reaction buffer containing exogenous substrate, enolase, and 32P-ATP then electrophoresed. The positions of p56\(^{\text{Lck}}\) and enolase were indicated. b) The kinase activities of p56\(^{\text{Lck}}\) after stimulation with IL-2 performed in Fig. 10a was measured by densitometry analyses. The relative levels of phosphorylation of p56\(^{\text{Lck}}\) (upper panel) and enolase (lower panel) in thymocytes from transgenic (solid line) or control mice (dotted lines) were calculated and plotted considering the level of phosphorylation p56\(^{\text{Lck}}\) or enolase in transgenic or control thymocytes at 0 hr as 1.00.
Discussion

Extracellular stimuli such as antigen and cross-linking of TcR with antibodies induce the activation of resting T cells. T cell activation results in the expression of IL-2R and the secretion of IL-2, and then IL-2/IL-2R interactions trigger the cell proliferation. So far, several of the growth factor receptors are revealed to possess PTK domains, and the tyrosine phosphorylation of receptor molecules and/or intervening molecules linked to these receptors has been considered to be involved in the pathway of signal transduction as one of the earliest detectable events after ligand binding (54).

IL-2R consists of two distinct subunits, IL-2Rα(p55) and IL-2Rβ(p75). The IL-2Rα molecule has no ability to mediate growth signals, whereas IL-2Rβ has a sufficient size of intracytoplasmic domain and is thought to be involved in transducing signals (10, 20). Recently the phosphorylation of IL-2Rβ molecule on tyrosine residues by stimulation with IL-2 was reported in IL-2-dependent T or NK cells (3, 8, 26, 42). Also the finding that p56lck is activated by stimulation with IL-2 in IL-2-dependent T cells (6, 14) suggested the possibility that tyrosine phosphorylation of IL-2Rβ molecule by IL-2 is mediated through p56lck. A scheme as to the role of p56lck in IL-2R-mediated signaling was further demonstrated by Hatakeyama et al. (11) such that IL-2Rβ forms a stable complex with p56lck in the cells. Moreover, as the result of interaction between them, IL-2Rβ becomes phosphorylated on its tyrosine residues by p56lck. These results strongly suggest that IL-2R does not work as a functional growth receptor without the association with p56lck.

In the present study using lck-transgenic mice, we found that CD4+8- thymocytes of these mice gain the ability to respond and proliferate to the stimulation with IL-2 to the extent comparable to the response of peripheral mature T cells stimulated with IL-2. While the expression levels of surface IL-2Rα and IL-2Rβ were almost equal both in such CD4+8- thymocyte subsets of transgenic mice and negative littermates before the simulation with IL-2, the stimulation with IL-2 induced the expression of IL-2Rβ only in transgenic thymocytes. When the amount and kinase activity of p56lck accumulated in this thymocyte subset was examined, on the other hand, a stable expression and its efficient kinase activity of p56lck without decrease was found in the transgenic mice after the stimulation with IL-2, contrasting to the negative littermates in which a marked reduction in the expression of p56lck occurred after the stimulation. Therefore it was thought that this constitutive expression of p56lck in CD4+8- cell of transgenic mice even after the stimulation with IL-2 makes their IL-2R complex sufficiently competent to receive the signal of IL-2-ligands and elicits the effective proliferative response to the thymocytes.

Induction of IL-2Rβ on transgenic thymocytes by IL-2 may be mediated through the augmented expression of p56lck and be concerned with efficient proliferative response by these thymocytes. The property of stable expression of lck in these thymocytes is considered to be exerted by IgH enhancer element incorporated in the construct pHSE3' used in the present study, because in the previous study by Pircher et al. (31) the pHSE3' exhibited a high level of expression of the integrated gene in the T cells carrying this construct. The expression of lck gene is down-regulated in normal T cells when the cells are activated (25), which has also been shown in the present study in the thymocytes from negative littermates after they were stimulated with IL-2. While the role of lck down-regulation in T cell activation remains to be elucidated, it may play a role in the maintenance of functional quiescence in normal thymocytes to immunological stimulation such as IL-2, because the aberrant expression of lck in the thymocytes from our transgenic mice broke unresponsiveness of their thymocytes to IL-2.

The expression of lck-transgene in our transgenic mice was not so high in thymocytes and splenocytes. On the other hand, the expression of lck in thymocytes from transgenic mice after stimulation with IL-2 was not down-regulated so much, and Fig. 9 showed that the expression level of lck at 4 hr after the stimulation with IL-2 was approximately 60% of that at 0 hr. It may be considered that in transgenic thymocytes this 60% of the total level of lck-expression is derived from transgene because the transgene is regulated by H-2K promoter which is considered not down-regulated when host cell is activated (31). Therefore it is estimated that the thymocytes from transgenic mice contain p56lck consisting of 60% from transgene lck and 40% from endogenous lck at resting state.

The thymus is the major site of T cell development and repertoire selection. During these processes, T cells were segregated into two subsets that express either CD4 or CD8 molecules and are referred to as “single” positive thymocytes bearing
the phenotype of peripheral T cells (9, 44). Although it was thought that such thymocytes had completed their maturation and were functionally competent, recent analysis of these thymocytes has challenged this view. As for CD4+8- thymocytes, it has been reported that no proliferative response was elicited by triggering TcR-CD3 complex due to the defect of secreting IL-2 (32). Moreover, it has been reported that no proliferative response was challenged this view. As for CD4+8- thymocytes, it has been reported that no proliferative response was elicited by triggering TcR-CD3 complex due to the defect of secreting IL-2 (32). Moreover, Ramsdell et al (33) recently demonstrated that the majority of CD4+8- thymocytes, which bear HSA+, Qa-2- phenotype, are functionally immature such that this thymocyte subset is nonresponsive to stimulation with anti-TcR-CD3 complex antibody even in the presence of various lymphokines including IL-2 or syngeneic APC. It seems therefore that stable expression of p56\textsuperscript{ck} in CD4+8- thymocytes renders their IL-2Rs competent for responding and proliferating to the stimulation with IL-2 without preceding triggering of T cell activation. The amount of IL-2 secreted by thymocytes from our transgenic mice, on the other hand, was negligible even after the stimulation with immobilized anti-CD3 or TcR \( \alpha \beta \) antibodies (data not shown).

According to the report using \textit{le}k-transgenic mice by Abraham et al (1, 2), a moderate, even only a twofold, increase of the p56\textsuperscript{ck} in thymocytes of transgenic mice substantially disrupts the appearance of CD4+8- thymocytes in these mice due to the maturation arrest. Moreover, extensively high expression of p56\textsuperscript{ck} in transgenic mice revealed tumor formation in the thymus. In these mice, the expression of \textit{le}k transgene is constitutive and its level is considerably higher than our transgenic mice especially at resting state without any stimulation. No increase in the constitutive level of p56\textsuperscript{ck} found in our \textit{le}k-transgenic mice will explain the absence of tumorigenesis and phenotypical change of thymocyte population in our mice.

Recent study of IL-2-deficient mice by gene targeting revealed that IL-2 was not required by T cell differentiation (40). Although CD4+8- thymocytes from our \textit{le}k-transgenic mice showed augmented proliferative response after the stimulation with IL-2 \textit{in vitro}, cell surface analyses demonstrated no abnormality in T cell maturation. Furthermore, \textit{in vitro}, IL-2-responsiveness of CD4+8-, CD4+8+ and CD4+8- thymocytes from our transgenic mice was not affected by the integration of our transgene. Our findings, therefore, suggested that IL-2 had no effect on the maturation of CD4+8- thymocytes \textit{in vivo}.

An interesting finding common to Abraham’s and our transgenic mice is that the effect of \textit{le}k transgene was exerted preferentially on CD4+8- thymocytes but not on CD4-8+ thymocytes, such that both the IL-2-responsiveness in our study and the maturation arrest in their study are found only in CD4+8- thymocytes. In this context, it may be important to note that CD8\( \beta \) chain interacts less well with p56\textsuperscript{ck} than do CD4 molecules, and that the CD8\( \beta \) chain fails to associate with p56\textsuperscript{ck} entirely (48). The relevance of this association of p56\textsuperscript{ck} with CD4/CD8 to a putative role of p56\textsuperscript{ck} as a signal-transducing molecule associated with IL-2R needs to be elucidated further.

References


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