p56<sup>lck</sup>, a member of the src family of non-receptor protein tyrosine kinases (PTKs), is expressed predominantly in T-lymphocytes. Association of p56<sup>lck</sup> with CD4 and CD8 T-cell receptor (TcR) accessory molecules suggests that p56<sup>lck</sup> may play a specialized role in antigen-induced T-cell activation. CD4 and CD8 molecules are known to stabilize the interaction between TcR and the major histocompatibility complex during T-cell activation. To examine the role of p56<sup>lck</sup> in the dynamics of the CD4 molecule, p56<sup>lck</sup>-expressing transfectant cell clones were prepared by the transfection of an lck-gene plasmid containing an inducible promoter into a human monocytoid cell line. When these transfectant cells were stimulated with phorbol ester, p56<sup>lck</sup> expression was selectively and markedly retarded, as compared to p56<sup>lck</sup>-negative control cell lines. When cell-surface CD4 and intracellular CD4 were selectively precipitated after stimulation, the intracellular CD4 molecules were dissociated from p56<sup>lck</sup> whereas the surface-retained CD4 molecules were still associated with p56<sup>lck</sup>. Moreover, the dissociation of p56<sup>lck</sup> from CD4 appeared to occur prior to the PMA-induced internalization of CD4. These data indicate that p56<sup>lck</sup> regulates the PMA-induced internalization of CD4 possibly via its association with CD4. Treatment with genistein, a PTK inhibitor, revealed that the PTK activity of p56<sup>lck</sup> might not be involved in this regulatory effect of p56<sup>lck</sup> on CD4 internalization.

Introduction

The lck gene is a member of the src-related family of genes that encode a class of closely related, membrane-bound, non-receptor protein tyrosine kinases (PTKs), including src, yes, fgr, fyn, lck, hek and lyn [1]. The lck gene product, p56<sup>lck</sup>, is normally expressed predominantly in T-lymphocytes [2,3]. Physical association of p56<sup>lck</sup> with the intracytoplasmic domains of CD4 and CD8 T-cell surface antigens [4,5] and the phosphorylation of p56<sup>lck</sup> following antibody-mediated cross-linking of these surface antigens have been reported so far [6]. The CD4 and CD8 antigens function as accessory molecules in concert with the T-cell receptor (TcR)-CD3 complex in the major histocompatibility complex (MHC)-restricted antigen-mediated T-cell activation. In one way, these accessory molecules are shown to act as stabilizers of the physical interaction among the T-lymphocyte, the antigen and the antigen-presenting cell through the interaction with MHC [7–9]. On the other hand, the inhibition of T-cell activation by antibody-mediated cross-linking of CD4 surface antigens in the absence of accessory cells has raised the possibility that CD4 molecules transduce a signal independent of TcR-CD3 molecules in the activation of T-cells [10–12]. Moreover, it is reported that the cross-linking of the CD4 receptor induces a rapid phosphorylation of the ζ-subunit of TcR-CD3 complex on its tyrosine residue [6]. These results have strongly suggested that p56<sup>lck</sup> may act as an intervening molecule through its PTK activity in the pathway of CD4- and CD8-mediated signal transduction. Activation of protein kinase C (PKC) has been shown to represent a potentially critical early event in T-lymphocyte response to antigen recognition. Actually, physiological T-lymphocyte activation can be mimicked

Key words: CD4; PMA; Tyrosine kinase; Protein tyrosine kinase; Receptor internalization
by a variety of agents such as the combination of phorbol esters (e.g., phorbol 12-myristate 13-acetate (PMA), a potent PKC stimulus) and mitogenic ligands. PMA is known to cause the aggregation and the subsequent internalization of CD4 molecules [13,14], which may, at least partially, represent the aggregation of CD4 molecules during the activation of T-helper cells by antigens [15]. Moreover, p56Lck is rapidly downstream its promoter sequence and the constructedotics.

In the present study, we examined the role of p56Lck in the dynamics of CD4 via its interaction with CD4.

**Materials and Methods**

**Cells**. A human monocytic cell line, U937 clone2, expressing surface CD4 but no Lck gene at its transcriptional level [21], was cultured in RPMI-1640 medium with 10% fetal calf serum (FCS) and antibiotics.

**Construction and transfection of the p56Lck-expressing plasmid**. An expression vector, pSMT [22], was constructed by inserting the promoter portion of human metathionin (IgA gene) [23] into pVS2neo which has the gene resistant to the antibiotic G418. A full-length human cDNA, YT16 [24], was put into the pSMT downstream its promoter sequence and the constructed plasmid was designated pSMTick. The plasmid pSMTick was then transfected by electroporation method [25] into U937 clone2 cell line. U2Mlck-4 and U2Mlck-6, cell lines stably integrated with pSMTick and U2Ml, a control cell line integrated with pSMT, were cloned after selection with 1 mg/ml of G418 and maintained in RPMI-1640 medium with 10% FCS in the presence of G418.

**Northern blot analysis**. Total cellular RNA was extracted by guanidine-HCI method [26] from U2Mlck-4 cells that had been cultured in the presence of 20 µg of C6C3, for the given hours. 10 µg of RNA from each cell sample was electrophoresed, blotted onto a nitrocellulose filter and hybridized with the YT16 probe. Densitometric analysis was performed to quantify the detected RNA levels.

**Anti-bodies and reagents**. A monoclonal antibody (mAb), OKT4 (anti-CD4) was purchased from Ortho (Raritan, NJ). An anti-p56Lck mAb, MOL, 171, was produced by our group [27]. PMA was purchased from Sigma (St. Louis, MO) and was dissolved in dimethyl-sulfoxide at 500 µg/ml as a stock solution. N-hydroxy-succinimidy-LC-biotin (NHS-LC-biotin) was purchased from Pierce (Rockford, IL). Genistein, a PTK inhibitor, was obtained from Extrasynthese (Genay, France).

**Cell surface biotinylation**. Cell surface biotinylation was performed as described elsewhere [28]. Briefly, cells were washed once in ice-cold phosphate-buffered saline (PBS) and suspended in freshly prepared 0.5 mg/ml NHS-LC-biotin in PBS at 10⁶ cells/ml. After labeling for 30 min at 4°C with constant gentle agitation, cells were centrifuged and washed twice in 0.2 M glycine in PBS. Cell viability was checked after labeling using Trypan blue; usually >95% of the cells excluded the dye.

**Immunoprecipitation and immunoblot analysis**. For immunoprecipitation of CD4, cells were lysed in 1% Nonidet P-40 (NP-40), 50 mM Tris-HCl (pH 8.0), 2 mM ethylenediaminetetraacetic acid, supplemented with 10 µg/ml aprotinin, 10 µg/ml of leupeptin, and 2 mM phenylmethylsulfonyl fluoride. Cell lysates from 5·10⁶ cells were incubated with 10 µg of OKT4 for 1 h at 4°C. Immune complexes were precipitated with protein A-Sepharose beads and then blotted onto a nitrocellulose filter. Immuno-precipitation of p56Lck was performed likewise with MOL, 171, an anti-p56Lck mAb [27]. For the differential immunoprecipitation of surface CD4 and internalized CD4, 5·10⁶ cells were lysed with 10 µg of OKT4 for 30 min at 4°C. Then cells were lysed and the surface CD4 molecules, which were bound to OKT4 antibody, were collected with protein A-Sepharose beads. The residual supernatants of the cell lysates, from which surface CD4 molecules had been removed, were further incubated with 10 µg of OKT4 for the immunoprecipitation of internalized CD4. Immune complexes were collected with protein A-Sepharose beads. Each protein A-Sepharose beads-bound immune complex was washed, boiled in Laemmli sample buffer, resolved on 10% SDS-PAGE and then blotted onto a nitrocellulose filter.

**For the visualization of precipitated p56Lck, filters were incubated with MOL 171 or biotinylated MOL 171 and then treated with peroxidase-conjugated goat anti-mouse Ig antibodies, or peroxidase-conjugated avidin. For the visualization of biotinylated CD4, filters were directly incubated with peroxidase-conjugated avidin. Development of the blot was performed as described elsewhere [29]. For the comparison of the visualized protein bands, densitometric analysis was performed on the developed membranes**.

**Immunofluorescence analysis**. Aliquots of 1·10⁶ cells were incubated with CD4 mAb, OKT4, and then treated with peroxidase-conjugated goat anti-mouse Ig antibodies, or peroxidase-conjugated avidin. Development of the blot was performed as described elsewhere [29]. For the comparison of the visualized protein bands, densitometric analysis was performed on the developed membranes.
were washed in ice-cold PBS, resuspended in 100 μl of PBS containing 0.1% bovine serum albumin and 0.1% sodium azide and incubated for 30 min on ice with OKT4. After washing, the expression level of surface CD4 was analyzed by fluorescence-activated cell sorter (FACS; FACScan, Becton Dickinson, San Jose, CA).

Assessment of tyrosine phosphorylation. The effect of genistein, a PTK inhibitor, on the CD4-cross-linking-induced tyrosine phosphorylation was assessed by visualization of alkali-resistant phosphoproteins [30]. Briefly, U2Mlk-4 cells (5 × 10⁶) that had been treated with CdCl₂ for 20 h were washed and suspended in phosphate-free RPMI medium supplemented with dialyzed FCS at 1×10⁵ cells/ml. After adding 1 mg/ml of [γ³²P]orthophosphate (10 μCi/ml in aqueous solution, Amer sham, UK), incubation was continued for 4 h in the presence of CdCl₂ at 20 μM. During the last 45 min of the incubation, aliquots of 1×10⁶ cells were mixed with 80 or 120 μg of digitonin. Then cells were incubated with a saturating concentration of OKT4 to induce CD4-mediated activation of p56LK [6] for 30 min on ice, washed and were mixed with rabbit anti-mouse Ig antibodies (RAM) for 5 min at 37°C. Cells were lysed with RIPA buffer (1% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), supplemented with 10 mM Na₃PO₄, 10 mM NaF, 2 mM Na₂VO₄, and protease inhibitors). Cell lysates were resolved in 10% SDS-PAGE, and the gel was treated in 1 M NaOH for 2 h at 50°C to visualize alkali-resistant phosphoproteins.

Results

Expression of the lck gene in the transfected cells
The expression of the lck gene was examined in U2Mlk-4 cells without or with addition of CdCl₂, as an inducer for the murine leukemia virus promoter (Fig. 1a). A marginal level of lck mRNA (0.22 kbp) was detected without addition of the inducer (0 h) in U2Mlk-4 cells (Fig. 1a). Northern blot analysis of the lck gene in the transfected cells (Fig. 1b) also showed the expression level of the lck gene in the transfectant cells (open circles) that did not express the lck gene in the control cell line (closed circles). The expression level of the lck gene in the transfectant cells was also slightly higher than on U2Mlk-2 clone 2 (data not shown). The expression of the lck gene in U2Mlk-6 cells, which induced lck expression of the lck gene in the transfectant cells, was also slightly higher in the U2Mlk-6 clone cells without CdCl₂ treatment than in U2Mlk-2 clone 2 and the level became much higher after induction of p56LK with CdCl₂ (data not shown).

Effect of p56LK on the expression of surface CD4
On U2Mlk-4 cells, the expression level of surface CD4 antigens without CdCl₂ treatment was slightly higher than that on U2Mlk-1 cells (Fig. 2b compared to a) or U937 clone 2 (data not shown). When p56LK was induced with CdCl₂, in U2Mlk-4 cells, the level of surface CD4 expression was slightly increased at 24 h after induction (Fig. 2a). The expression level of surface CD4 on U2Mlk-6 cells without CdCl₂ treatment was also slightly higher than on U2Mlk-2 clone 2 and the level become much higher after induction of p56LK with CdCl₂ (data not shown).

Effect of p56LK on PMA-induced CD4 internalization
PMA is known to cause rapid down-modulation of surface CD4 molecules of human T-cells, though its mechanisms have not yet been elucidated [13,14]. To examine the effect of p56LK on the internalization of surface CD4 induced by PMA, U2Mlk-4, U2Mlk-6, U2Mlk-2 clone 2, all of which had been treated with CdCl₂, for 24 h and U937 clone 2 (U2Mlk-6 clone 2) cells were incubated in RPMI medium at 37°C for 1×10⁵/ml of cell density. PMA was added to each cell suspension at a final concentration of 25 nM/ml and aliquots of 1×10⁶ cells were removed after the indicated times (abscissa in the figure), washed with ice-cold PBS, and analyzed for surface CD4 expression with fluorescence isothiocyanate-conjugated OKT4 mAb (ordinate in the figure). As shown in Fig. 3a, the expression level of surface CD4 molecules via the two cysteine residues in its cytoplasmic domain [30,31] was not altered by the treatment of PMA (data not shown). Results are shown as the mean ± S.E. of triplicate experiments.

To examine whether the effect of p56LK on the internalization of surface molecules is specific to CD4, the expression levels of CD4 antigens on each cell line were examined (Fig. 3b). The expression levels of CD4 antigens were decreased in U2Mlk-4 cells (open circles) and U2Mlk-2 cells (closed circles), which were treated with PMA stimulation on each cell line was examined (Fig. 3b). The expression levels of CD4 antigens were decreased in U2Mlk-4 cells (open circles) and U937 clone 2 (U2Mlk-6 clone 2) cells (closed circles), which were treated with PMA stimulation on each cell line was examined (Fig. 3b).

The expression level of surface CD4 antigen was also slightly higher than on U2Mlk-1 cells, while the rate of the internalization of CD4 on U2Mlk-6 cells without CdCl₂ treatment was also slower than in U2Mlk-2 clone 2 and U937 clone 2 cells (Fig. 3b). Therefore, it was suggested that p56LK is specifically concerned with the down-modulation of CD4.

Dissociation of p56LK from CD4 prior to the internalization of CD4
Aside from inducing CD4 internalization, PMA has been reported to cause the dissociation of p56LK from CD4 [19]. p56LK associated with the cytoplasmic tail of CD4 molecules via its two cysteine residues in its amino-terminal domain [32,33], is linked to the plasma membrane via its myristylated amino-terminal glycine residue [34]. It is, therefore, of interest whether this association of p56LK with CD4 molecules is related to its internalization in the intact cell population. As shown in Fig. 3a, the expression level of surface CD4 molecules via the two cysteine residues in its cytoplasmic domain [30,31] was not altered by the treatment of PMA (data not shown). Results are shown as the mean ± S.E. of triplicate experiments.

To further confirm that p56LK is involved in the retardation of CD4 internalization observed in p56LK-expressing cell lines, U2Mlk-4 cells that had been treated with CdCl₂ for 24 h were incubated with 25 ng/ml of PMA at 37°C for 24 h. After PMA stimulation, the expression level of surface CD4 antigens on each cell line was examined (Fig. 3b). The expression levels of CD4 antigens without CdCl₂ treatment were stimulated with PMA stimulation on each cell line was examined (Fig. 3b). The expression levels of CD4 antigens without CdCl₂ treatment were stimulated with PMA stimulation on each cell line was examined (Fig. 3b). The expression levels of CD4 antigens without CdCl₂ treatment showed a slower rate of the CD4 internalization induced by PMA than p56LK-expressing cell lines. As shown in Fig. 3a, the expression level of surface CD4 molecules via the two cysteine residues in its cytoplasmic domain [30,31] was not altered by the treatment of PMA (data not shown). Results are shown as the mean ± S.E. of triplicate experiments.
30 min after stimulation, reflecting the decrease of total precipitable CD4 (Fig. 4b) due presumably to the lysosomal degradation of internalized CD4 molecules [35]. There was a faint amount of CD4 in the cytoplasm (Fig. 4a, 0 min 'total') even before PMA stimulation, due presumably to the subtle internalization of CD4 during the cell-surface biotinylation and the incubation with OKT4, note that protein A-Sepharose beads added after removal of surface CD4 (Fig. 4c) complexes collected no biotinylated CD4 (Fig. 4c, 'residual'), thereby indicating that all cell-surface CD4 molecules were collected with the first round of treatment with OKT4 and protein A-Sepharose beads. Then p56Ick bound either to cell-surface CD4 or to internal CD4 was selectively precipitated with the same procedure (Fig. 4d). First, the amount of p56Ick associated with surface CD4 after PMA stimulation was evaluated. Before stimulation, surface CD4 is associated with a substantial amount of p56Ick (Fig. 4d, 0 min 'surface'). 5 min after stimulation, the amount of p56Ick associated with surface CD4 strikingly decreased and no detectable p56Ick was coprecipitated with CD4 molecules 15 min after the stimulation, though, during this period, surface CD4 was precipitated at a detectable level (see Fig. 4a, 'surface'). Total amount of p56Ick in U2Mlck-4 cells was not altered after PMA stimulation (Fig. 4c), indicating that the decrease in the amount of p56Ick associated with surface CD4 was not the result of the reduction of total p56Ick. Next, when internal CD4 molecules were precipitated after PMA stimulation, no detectable amount of p56Ick was coprecipitated with CD4 throughout the time course (Fig. 4d, 'internal'). Hence, it was demonstrated that the internalized CD4 molecules were dissociated from p56Ick, even at the earlier periods after PMA stimulation (as early as 5 min after stimulation), at no time surface-exposed CD4 molecules were still associated with p56Ick. And virtually, the internalized CD4 molecules were dissociated from p56Ick before the stimulation (Fig. 4d, 0 min 'internal'). Densitometric comparison of the rate of disappearance of the surface CD4 (Fig. 4a, 'surface') with that of the dissociation of p56Ick from surface CD4 (Fig. 4d, 'surface') after PMA stimulation indicated that the rate of the dissociation was significantly faster than that of surface CD4 down-modulation. Thus, these data indicate that the dissociation of p56Ick from CD4 in response to PMA precedes the PMA-induced internalization of CD4 and also that cell-surface CD4 molecules associated with p56Ick are able to internalize only after their dissociation from p56Ick.

Effect of genistein on PMA-induced CD4 internalization on U2Mlck-4 cells
To examine the role of PTK activity of p56Ick on the p56Ick-mediated regulation of CD4 internalization, an anti-OKT4, a PTK selective inhibitor [36], was used when...
cells were stimulated with PMA for the induction of CdC4 internalization (Fig. 2). U2Mick-4 cells that had been treated with CdC4, for the induction of p56
superscript 1ck (closed symbols) were treated with open circles, 80 ng/ml PMA at 37°C for 5 min, and the expression was assayed for the induction of CdC4 internalization, which is comparable to U2Mick-6 cells transfected with CdC4, treated with CdC4 and U2Mick cells [31].

What is the molecular basis of the regulatory effect of p56
superscript 1ck on the association of CdC4 with its ligand, and the mechanism of CdC4 internalization? The association of CdC4 with its ligand is dependent on the presence of p56
superscript 1ck, and the activity of p56
superscript 1ck is stably associated with CdC4 via cysteine residues and may be involved in the effect of p56
superscript 1ck on the regulation of CdC4 internalization.

In this study, a panel of transfectant cell clones, U2Mick-4 and U2Mick-6 (CD4-p56
superscript 1ck-) and U2Mick (CD4-p56
superscript 1ck+), together with parental U937 clone2 (CD4-p56
superscript 1ck+) were transfected with the expression plasmid for p56
superscript 1ck, and the expression of p56
superscript 1ck in the transfected cells was confirmed with the visualization of phosphotyrosine that demonstrated the decreased in the amount of phosphotyrosine and also the disappearance of autophosphorylated p56
superscript 1ck induced by CdC4 crosslinking in U2Mick-4 cells treated with CdC4 (Fig. 5b, lane 3; 80 μM genistein and lane 4, 120 μM genistein, compared with lane 2, without genistein). These results indicated that the PTK activity of p56
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Discussion

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