A lymphocyte-specific protein tyrosine kinase, p56~, regulates the PMA-induced internalization of CD4
A lymphocyte-specific protein tyrosine kinase, p56<sub>1ck</sub>, regulates the PMA-induced internalization of CD4

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p56<sub>1ck</sub>, a member of the src-family of non-receptor protein tyrosine kinases (PTKs), is expressed predominantly in T-lymphocytes. Association of p56<sub>1ck</sub> with CD4 and CD8 T-cell receptor (TcR) accessory molecules suggests that p56<sub>1ck</sub> 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<sub>1ck</sub> in the dynamics of the CD4 molecule, p56<sub>1ck</sub>-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, CD4 internalization on these p56<sub>1ck</sub>expressing cell lines was selectively and markedly retarded, as compared to p56<sub>1ck</sub>-negative control cell lines. When cell-surface CD4 and intracellular CD4 were selectively precipitated after stimulation, the intracellular CD4 molecules were dissociated from p56<sub>1ck</sub> whereas the surface-retained CD4 molecules were still associated with p56<sub>1ck</sub>. Moreover, the dissociation of p56<sub>1ck</sub> from CD4 appeared to occur prior to the PMA-induced internalization of CD4. These data indicate that p56<sub>1ck</sub> 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<sub>1ck</sub> might not be involved in this regulatory effect of p56<sub>1ck</sub> 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, hck and lyn [1]. The lck gene product, p56<sub>1ck</sub>, is normally expressed predominantly in T-lymphocytes [2,3]. Physical association of p56<sub>1ck</sub> with the intracytoplasmic domains of CD4 and CD8 T-cell surface antigens [4,5] and the phosphorylation of p56<sub>1ck</sub> 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 surface antigens 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<sub>1ck</sub> 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...
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 dimethylsulfoxide at 500 μg/ml as a stock solution. N-hydroxysuccinimidyl-2-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 10^7 cells/ml. After labeling for 30 min at 4°C with constant gentle agitation, cells were collected by centrifugation 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 5·10^6 cells were incubated with 10 μg of OKT4 for 1 h at 4°C. Immune complexes were precipitated with protein A-Sepharose beads (Pharmacia, Uppsala, Sweden), washed 2 times with immunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% NP-40, 0.25% gelatin and 0.02% sodium azide) and then with 10 mM Tris-HCl in 1% NP-40. Samples were then solubilized in Laemmli sample buffer, resolved on 10% SDS-PAGE and then blotted onto a nitrocellulose filter. Immunoprecipitation of p56Lck was performed likewise with MOL 171, an anti-p56Lck mAb [27].

For the differential immunoprecipitation of surface CD4 and internalized CD4, 5·10^6 cells were collected 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 method [25] into U937 clone2 cell line. U2Mick-4 and U2Mick-6, cell lines stably integrated with pSMtick and U2M1, a control cell line integrated with pSM1Tc, were cloned after selection with 1 μg/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-HCl method [26] from U2Mick-4 cells that had been cultured in the presence of 20 μM of CdCl_2 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.

Materials and Methods

Cells. A human monocytic cell line, U937 clone2, expressing surface CD4 but no p56Lck 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, pSM [22], was constructed by inserting the promoter portion of human metallothionein IIA gene [23] into pSV2neo which has the gene resistant to the antibiotic G418. A full-length human cDNA, YT16 [24], was put into the pSM downstream its promoter sequence and the constructed plasmid was designated pSMtick. The plasmid pSMtick was then transfected by electroporation.

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 IgG 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 1·10^5 cells were collected with RAM and protein A-Sepharose beads, washed and resolved in 10% SDS-PAGE and then blotted onto a nitrocellulose filter. The filter was treated with peroxidase-conjugated RAM for visualization of the precipitated proteins. (c) Association of p56Lck with surface CD4. U2Mick-4 cells that had been cultured in the presence of CdCl_2 for 24 h were incubated with surface-matched mouse Ig (clone 'Con1'). Immune complexes were collected with RAM and protein A-Sepharose beads, washed and resolved in 10% SDS-PAGE and then blotted onto a nitrocellulose filter. The filter was treated with peroxidase-conjugated RAM for visualization of the precipitated proteins.
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 jasmonic acid, a PTX inhibitor, on the CD4-transfected cells was evaluated with TCA-precipitable radioactivity. The expression level of the transfected CD4 antigen was analyzed by FACScan.

Results

Expression of the cck gene in the transfected cells

The expression of the cck gene was examined in U2Mick-4 cells without or with addition of CcIk, as an inducer for the mps2-deoxycholate promoter (Fig. 1a). A marginal level of cck transcription at 2.2 kb was detected without addition of the inducer (0 h) in U2Mick-4 cells (Fig. 1a).

Northern blot analysis. Addition of deoxycholate, a buffer (1 % Na4P2O7, 10 mM Tris-HCl (pH 7.5), supplemented with 10 mM Na2PO4, 10 mM NaF, 2 mM Na4VO4, and protease inhibitors. Cell lysates were resolved in 10% SDS-PAGE and the gel was treated in 1 M NaOH for 2 h at 59°C to visualize alkali-resistant phosphoproteins.

Effect of p56ck on the expression of surface CD4

On U2Mick-4 cells, the expression level of surface CD4 antigens without CcIk treatment was slightly higher than that on U2M1 cells (Fig. 2a, indicated as open circles) and the level become much higher after induction of p56ck (data not shown).

The effect of p56ck on the expression of surface CD4

The expression levels of surface CD4 antigens on each cell line are shown in Fig. 2b. The expression levels reached their nadirs (data not shown). The expression level of surface CD4 antigen was slightly increased at 24 h after induction (Fig. 2c). The expression level of surface CD4 reached its nadir at 24 h (indicated as closed circles) and before and after CcIk treatment (Fig. 2a). When the expression levels reached their nadirs (data not shown).

To further confirm that p56ck is involved in the retardation of CD4 internalization observed in p56ck-expressing cells, U2Mick-4 cells that had been treated with CcIk for 24 h to induce the expression of p56ck maximally and U2Mick-4 cells without CcIk treatment were stimulated with PMA and the internalization of CD4 on each cell line was examined (Fig. 3b). The expression levels of CD4 on both U2Mick-4 cells (open circles) showed a much faster rate of the internalization of CD4 than that with CcIk treatment (closed circles). The rate of the internalization of CD4 on U2Mick-4 cells without CcIk treatment was comparable to that of U2M1 or U937 clone2 cells (Fig. 3a). The result was therefore, that the CD4 internalization of dependent on p56ck expressed in the cells.

To examine whether the effect of p56ck on the internalization of surface molecules is specific to CD4, the expression levels of CD11b, CD18 and CD45 on U2Mick-4 cells were examined before and after induction of p56ck and before and after PMA stimulation on U937-treated cells. As expected, no alteration of the expression of these molecules was observed either by the induction of p56ck or by the PMA stimulation (data not shown).

During the last 5 min on ice with CdCl2 for 24 h, the expression level of surface CD4 in the treated U2M1 cell line decreased significantly, indicating that p56ck expressing cell
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 "before") 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-OKT4 complexes collected no biotinylated CD4 (Fig. 4c, "residual"), thereby indicating that all the cell-surface CD4 molecules were collected with the first round of treatment with OKT4 and protein A-Sepharose beads. Then p56\(^{ck}\) bound either to cell-surface CD4 or to internal CD4 was selectively precipitated with the same procedure (Fig. 4d). First, the amount of p56\(^{ck}\) associated with surface CD4 after PMA stimulation was evaluated. Before stimulation, surface CD4 is associated with a substantial amount of p56\(^{ck}\) (Fig. 4d, 0 min "surface"). 5 min after stimulation, the amount of p56\(^{ck}\) associated with surface CD4 remarkably decreased and no detectable p56\(^{ck}\) 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 p56\(^{ck}\) in U2Mlck-4 cells was not altered after PMA stimulation (Fig. 4c), indicating that the decrease in the amount of p56\(^{ck}\) associated with surface CD4 was not the result of the reduction of total p56\(^{ck}\). Next, when internal CD4 molecules were precipitated after PMA stimulation, no detectable amount of p56\(^{ck}\) was coprecipitated with CD4 throughout the time course (Fig. 4d, "internal"). Hence, it was demonstrated that the internalized CD4 molecules were dissociated from p56\(^{ck}\), even at the earlier periods after PMA stimulation (as early as 5 min after stimulation), as well as time surface-precipitated CD4 molecules were still associated with p56\(^{ck}\). And virtually, the internalized CD4 molecules were dissociated from p56\(^{ck}\) 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 p56\(^{ck}\) 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 p56\(^{ck}\) from CD4 in response to PMA precedes the PMA-induced internalization of CD4 and also that cell-surface CD4 molecules associated with p56\(^{ck}\) are able to internalize only after their dissociation from p56\(^{ck}\).

**Effect of genistein on PMA-induced CD4 internalization on U2Mlck-4 cells**

To examine the role of PTK activity of p56\(^{ck}\) on the p56\(^{ck}\)-mediated regulation of CD4 internalization, genistein, a PTK selective inhibitor [36], was used when...
may affect this process, thereby raising the expression level of CD4. Support to the hypothesis that p56\(^{ck}\) on the cell surface is required for the dissociation of CD4 from the cell comes from the fact that the expression level of other cell-surface molecules (CD45, CD11b and CD14) remained unaltered when p56\(^{ck}\) was not phosphorylated by PMA in control C\(\text{C}^2\) cells treated with genistein (Fig. 3b). The amount of CD4 was still precipitated at the same time. This is in agreement with the observation that the dissociation of CD4 from the CD4 molecule is also unknown. It is, however, indicated that PMA may not directly initiate the dissociation but promote or facilitate the mechanism of the dissociation, since the internalization of CD4 is already dissociated from CD4 by the stimulation with PMA. Each of these effects is suggested that the association of p56\(^{ck}\) with CD4 is important in understanding the role of p56\(^{ck}\) in the internalization of CD4 on human peripheral blood mononuclear cells, though the internalization of CD4 was induced by CD4-crosslinking via covalent bonds, such as by PMA. Due to the high homology of the amino-acid sequence of p56\(^{ck}\) with the other p56\(^{ck}\) expressed in various other tissues, it is suggested that the PTK activity of p56\(^{ck}\) might be involved in the regulatory effect of p56\(^{ck}\) on the internalization of CD4. From this point of view, we examined the association of p56\(^{ck}\) with CD4 molecules before PMA stimulation with the selective incubation of cell surface and internalized CD4, it was demonstrated that the internalization of CD4 was dissociated from p56\(^{ck}\), even in the earlier period after stimulation (e.g., 5 min after stimulation, Fig. 4d, "internal"). It was noteworthy that the intracellular CD4 stimulation, due presumably to the "instantaneous" internalization during the procedure, was also dissociated from p56\(^{ck}\) indicating that the dissociation was not triggered directly by PMA stimulation. On the contrary, cell-surface CD4 before stimulation is associated with a significant amount of p56\(^{ck}\) (Fig. 4d, 0 in surface). 5 minutes after stimulation, however, the amount of p56\(^{ck}\) that coprecipitated with cell-surface CD4 was remarkably reduced when compared with the amount of precipitated CD4 at the same time. And no detectable amount of p56\(^{ck}\) was coprecipitated from CD4 after stimulation, and a CD4 15 min after stimulation, though a considerable amount of CD4 was still precipitated at the same time. This dissociation of p56\(^{ck}\) from CD4, which is shown to be dependent on the internalization induced by PMA stimulation, is a significantly slower rate than the PMA-induced dissociation of p56\(^{ck}\) from CD4 upon CD4 internalization. Hence, it is proposed that the association of p56\(^{ck}\) plays a regulatory role in the internalization of CD4. The surface expression of CD4 on U2Mick-4 and U2Mick-6 cells was slightly higher than that on U2Mick-1 clone 2 and the level was raised after treatment with genistein to a comparable level to that of U2Mick-1 clone 1, which shows a lower expression of p56\(^{ck}\). As shown in this study, p56\(^{ck}\) plays a regulatory role in the internalization of CD4. 5. p56\(^{ck}\) may perturb the cytoplasmic portion of CD4 to interact with cellular elements for internalization, such as the cytoskeleton or coated pits. The mechanism of the dissociation of p56\(^{ck}\) from CD4 molecule is also unknown. It is, however, indicated that PMA may not directly initiate the dissociation but promote or facilitate the mechanism of the dissociation, since the internalization of CD4 is already dissociated from p56\(^{ck}\).

In the PTK activity of p56\(^{ck}\) involved in the effect of p56\(^{ck}\) on the internalization of CD4 (Table 1), the ability of p56\(^{ck}\) to endogenous phosphatase has been reported to be reduced in response to PMA stimulation (28, 29), therefore, the effect was confirmed with the visualization of phosphotyrosine that demonstrated the decrease in the amount of phosphotyrosine and also the decrease in the amount of phosphoprotein that decreased the amount of phosphotyrosine and also the decrease in the amount of phosphosite that decreased the amount of phosphotyrosine.

The surface expression of CD4 was induced by cross-linking (closed squares) and confirmed with the visualization of phosphotyrosine that demonstrated the decrease in the amount of phosphotyrosine that decreased the amount of phosphotyrosine and also the decrease in the amount of phosphosite that decreased the amount of phosphotyrosine.

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