A lymphocyte-specific protein tyrosine kinase, p56^, regulates the PMA-induced internalization of CD4

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A lymphocyte-specific protein tyrosine kinase, p56^{lck}, regulates the PMA-induced internalization of CD4

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p56^{lck}, a member of the *src* family of non-receptor protein tyrosine kinases (PTKs), is expressed predominantly in T-lymphocytes. Association of p56^{lck} with CD4 and CD8 T-cell receptor (TcR) accessory molecules suggests that p56^{lck} 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^{lck} in the dynamics of the CD4 molecule, p56^{lck}-expressing transfectant cell clones were prepared by the transfection of an *lck*-gene plasmid containing an inducible promoter into a CD4^{+}*lck*$^{-}$ human monocytoid cell line. When these transfectant cells were stimulated with phorbol ester, CD4 internalization on these p56^{lck}-expressing cell lines was selectively and markedly retarded, as compared to p56^{lck}-negative control cell lines. When cell-surface CD4 and intracellular CD4 were selectively precipitated after stimulation, the intracellular CD4 molecules were dissociated from p56^{lck} whereas the surface-retained CD4 molecules were still associated with p56^{lck}. Moreover, the dissociation of p56^{lck} from CD4 appeared to occur prior to the PMA-induced internalization of CD4. These data indicate that p56^{lck} 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^{lck} might not be involved in this regulatory effect of p56^{lck} 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^{lck}, is normally expressed predominantly in T-lymphocytes [2,3]. Physical association of p56^{lck} with the intracytoplasmic domains of CD4 and CD8 T-cell surface antigens [4,5] and the phosphorylation of p56^{lck} 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^{lck} 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

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Abbreviations: PTK, protein tyrosine kinase; TcR, T-cell receptor; MHC, major histocompatibility complex; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; FCS, fetal calf serum; mAb, monoclonal antibody; PBS, phosphate-buffered saline; NP-40, Nonidet P-40; FACS, fluorescence-activated cell sorter; RAM, rabbit anti-mouse Ig antibodies.

by a variety of agents such as the combination of phorbol esters (e.g., phorbol 12-myristate 13-acetate (PMA), a potent PKC stimulator) and mitogenic lectins. 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 antigen [15,16], though the significance of the aggregation and the internalization of CD4 molecules has not been elucidated yet. The recognition, however, by the T-cell receptor of the antigen-MHC complex is not often sufficient for maximal response of MHC class-II-restricted T-helper cells and the binding of CD4 to MHC class-II molecules on the surface of antigen presenting cells augments or potentiates the activation of responding cells [17,18]. Some of this effect can be ascribed to increased adhesion provided by the binding of CD4 to MHC class-II molecules. Thus, it is potentially possible that the internalization of CD4 molecules plays an important role in the activation of T-cells. PMA is also known to cause the dissociation of $p56^{lck}$ from CD4 molecules [19]. Moreover, $p56^{lck}$ is rapidly converted to a product with slower gel-mobility (approx. 60 kDa) in response to PMA, with a slight decrease in the ability to undergo autophosphorylation [20]. It is, therefore, of great interest whether $p56^{lck}$ affects the kinetics of CD4 molecules during the internalization after PMA stimulation.

In the present study, we examined the role of $p56^{lck}$ in the dynamics of CD4 in response to PMA stimulation by preparing a panel of $CD4^+p56^{lck+}$ and $CD4^+p56^{lck-}$ transfectant cell clones. We demonstrated that the rate of the PMA-induced internalization of CD4 was markedly slower in $p56^{lck}$-expressing cell lines than in non-$p56^{lck}$-expressing cell lines. Moreover, the dissociation of $p56^{lck}$ from CD4 molecules preceded the internalization of CD4. From these observations, it is indicated that $p56^{lck}$ plays a regulatory role in the dynamics of CD4 via its interaction with CD4.

Materials and Methods

Cells. A human monocytoid 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 $p56^{lck}$-expressing plasmid. An expression vector, pSMT [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 *lck* cDNA, YT16 [24], was put into the pSMT downstream its promoter sequence and the constructed plasmid was designated pSMTlck. The plasmid pSMTlck was then transfected by electroporation method [25] into U937 clone2 cell line. U2Mlck-4 and U2Mlck-6, cell lines stably integrated with pSMTlck and U2M1, 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-HCl method [26] from U2Mlck-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.

Antibodies and reagents. A monoclonal antibody (mAb), OKT4 (anti-CD4) was purchased from Ortho (Raritan, NJ). An anti-$p56^{lck}$ 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-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 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 $5 \cdot 10^6$ 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 once 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 $p56^{lck}$ was performed likewise with MOL 171, an anti-$p56^{lck}$ mAb [27].

For the differential immunoprecipitation of surface CD4 and internalized CD4, $5 \cdot 10^6$ cells were incubated 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 $p56^{lck}$, 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 $1 \cdot 10^6$ cells

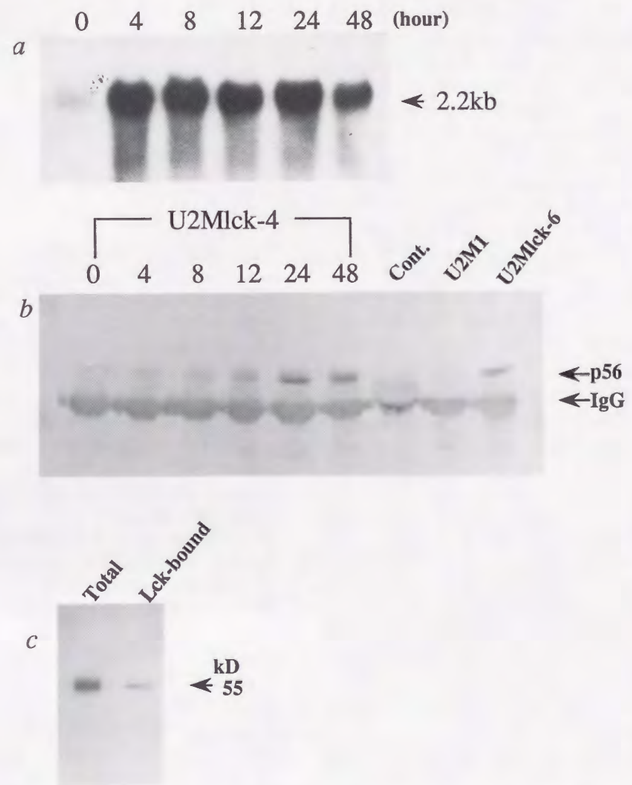

Fig. 1. Expression of the *lck* gene in U2Mlck-4 cells. (a) Northern blot analysis. Total RNA was extracted from U2Mlck-4 cells cultured for 0, 4, 8, 12, 24 and 48 h as indicated at the top of each lane in the presence of 20 μM of $CdCl_2$. RNA (10 μg per lane) was electrophoresed, transferred onto a nitrocellulose filter and hybridized with YT16 as probe. (b) Immunoprecipitation and immunoblot analysis of $p56^{lck}$. Cell lysates were obtained from U2Mlck-4 cells after treatment with 20 μM of $CdCl_2$ for 0, 4, 8, 12, 24, 48 h as indicated on the top of each lane and from U2M1 and U2Mlck-6 cells that had been treated with $CdCl_2$ for 24 h. Cell lysates were incubated with MOL 171 for 1 h at 4°C. As control, lysates of U2Mlck-4 that had been treated with $CdCl_2$ for 24 h were incubated with subclass-matched mouse Ig (lane 'Cont.'). Immunocomplexes were collected with RAM and protein A-Sepharose beads, washed and resolved in 10% SDS-PAGE and blotted onto a nitrocellulose filter. The filter was treated with peroxidase-conjugated RAM for visualization of the precipitated proteins. (c) Association of $p56^{lck}$ with surface CD4. U2Mlck-4 cells that had been cultured in the presence of $CdCl_2$ for 24 h were treated with NHS-LC-biotin for the cell-surface biotinylation as described in Materials and Methods. Then cells were lysed and cell lysates from $5 \cdot 10^6$ cells were precipitated with OKT4 for the precipitation of total CD4 (Total) or with MOL 171 for the precipitation of $p56^{lck}$-bound CD4 (Lck-bound). Immunocomplexes were collected with RAM and protein A-Sepharose beads, resolved in 10% SDS-PAGE and blotted onto a nitrocellulose filter. The filter was treated with peroxidase-conjugated avidin for the visualization of the precipitated CD4.

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, U2Mlck-4 cells ($5 \cdot 10^6$) that had been treated with $CdCl_2$ for 20 h were washed and suspended in phosphate-free RPMI medium supplemented with dialyzed FCS at $1 \cdot 10^7$ cells/ml. After adding 1 mCi/ml of [^{32}P]orthophosphate (10 mCi/ml in aqueous solution, Amersham, UK), incubation was continued for 4 h in the presence of $CdCl_2$ at 20 μM. During the last 45 min of the incubation, aliquots of $1 \cdot 10^6$ cells were mixed with 80 or 120 μM of genistein. Then cells were incubated with a saturating concentration of OKT4 to induce CD4-mediated activation of p56^{lck} [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 $Na_4P_2O_7$, 10 mM NaF, 2 mM Na_5VO_4 and protease inhibitors. Cell lysates were resolved in 10% SDS-PAGE and the gel was treated in 1 M NaOH for 2 h at 55°C to visualize alkali-resistant phosphoproteins.

Results

Expression of the lck gene in the transfectant cells

The expression of the *lck* gene was examined in U2Mlck-4 cells without or with addition of $CdCl_2$ as an inducer for the metallothionein promoter (Fig. 1a). A marginal level of *lck* message at 2.2 kb was detected without addition of the inducer (0 h) in U2Mlck-4 cells by Northern blot analysis. Addition of 20 μM $CdCl_2$ induced an almost 20-fold increase of *lck* message after 4 h, continuing for more than 24 h.

Immunoblot analysis of the p56^{lck} precipitated with MOL 171, an anti-p56^{lck} mAb, also demonstrated a faint amount of p56^{lck} expressed in U2Mlck-4 cells at 0 h and an approx. 10-fold increase of it at 8–48 h with its peak at 24 h after induction (Fig. 1b). p56^{lck} was also expressed in $CdCl_2$-treated U2Mlck-6 cells, another cell line integrated with pSMTlck, but not in U2M1, a control cell line integrated with pSMT, nor in U937 clone2, a parental cell line (data not shown). To confirm the association of p56^{lck} with CD4 in U2Mlck-4, U2Mlck-4 cells that had been treated with $CdCl_2$ for 24 h were surface biotinylated and lysed. Total CD4 (Fig. 1c, Total) and p56^{lck}-bound CD4 (Lck-bound) were precipitated with OKT4 and MOL 171, respectively, and the densitometric analysis demonstrated that about 20% of the surface CD4 antigens were associated with the expressed p56^{lck}.

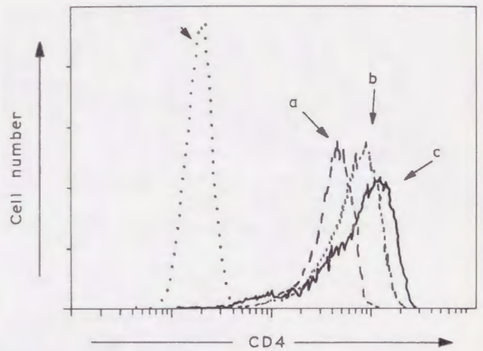

Fig. 2. Expression levels of surface CD4 on U2Mlck-4 and U2M1 cells. The expression level of surface CD4 antigen was analyzed by FACS with fluorescein isothiocyanate-conjugated OKT4 mAb in U2M1 (a), U2Mlck-4 cells before (b) and after (c) $CdCl_2$ treatment for 24 h. Arrowhead shows an autofluorescence level.

The effect of p56^{lck} on the expression of surface CD4

On U2Mlck-4 cells, the expression level of surface CD4 antigens without $CdCl_2$ treatment was slightly higher than that on U2M1 cells (Fig. 2b compared to a) or U937 clone2 (data not shown). When p56^{lck} was induced with $CdCl_2$ in U2Mlck-4 cells, the level of surface CD4 expression was slightly increased at 24 h after induction (Fig. 2c). The expression level of surface CD4 on U2Mlck-6 cells without $CdCl_2$ treatment was also slightly higher than on U2M1 or U937 clone2 and the level become much higher after induction of p56^{lck} with $CdCl_2$ (data not shown).

Effect of p56^{lck} 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 p56^{lck} on the internalization of surface CD4 induced by PMA, U2Mlck-4, U2Mlck-6, U2M1 cells, all of which had been treated with $CdCl_2$ for 24 h and the parental U937 clone2 cells were mixed with 25 ng/ml of PMA at 0 min at 37°C (Fig. 3a). The decrease of surface CD4 antigen was retarded in U2Mlck-4 cells (closed circles) and U2Mlck-6 cells (closed squares) compared with that in U2M1 cells (open circles) and U937 clone2 cells (open squares). The expression levels of CD4 antigens on each cell line were almost identical (about 15% of the initial expression) at 120 min after PMA stimulation, when the expression levels reached their nadirs (data not shown). It was, therefore, indicated that p56^{lck}-expressing cell lines examined here showed a slower rate of the CD4 internalization induced by PMA than p56^{lck}-non-expressing cell lines.

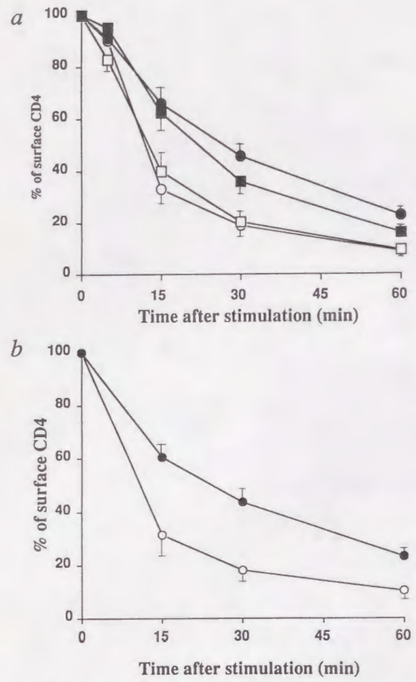

Fig. 3. Effect of p56^{lck} on the PMA-induced internalization of surface CD4. (a) Differences in the PMA-induced CD4 internalization in p56^{lck}-expressing and p56^{lck}-non-expressing cell lines. U2Mlck-4 (●), U2Mlck-6 (■), U2M1 (○), all of which had been treated with $CdCl_2$ for 24 h and U937 clone 2 (□) cells were suspended in RPMI medium at 37°C at $1 \cdot 10^7$/ml of cell density. PMA was added to each cell suspension at a final concentration of 25 ng/ml and aliquots of $1 \cdot 10^6$ cells were removed after the indicated times (abscissa in the figure), washed with ice-cold PBS and analyzed for surface CD4 expression with fluorescein isothiocyanate-conjugated OKT4. % of surface CD4 (ordinate in the figure) = 100×(mean fluorescence value [MFV] of the surface CD4 on sample cells - MFV of the unstained cells)/(MFV of the surface CD4 on stained cells at 0 min - MFV of the surface CD4 on unstained cells). (b) Differences in PMA-induced CD4 internalization in U2Mlck-4 cells before and after $CdCl_2$ treatment. U2Mlck-4 cells with (●) or without (○) pretreatment with $CdCl_2$ were resuspended in RPMI medium at $1 \cdot 10^7$/ml. Surface CD4 expression in each cell population after PMA stimulation was analyzed and % of surface CD4 (ordinate in the figure) was calculated.

To further confirm that p56^{lck} is involved in the retardation of CD4 internalization observed in p56^{lck}-expressing cell lines, U2Mlck-4 cells that had been treated with $CdCl_2$ for 24 h to induce the expression of p56^{lck} maximally and U2Mlck-4 cells without $CdCl_2$ treatment were stimulated with PMA and the internalization of CD4 on each cell line was examined (Fig. 3b). Without $CdCl_2$ treatment, U2Mlck-4 cells (open circles) showed a much faster rate of the internalization of CD4 than that with $CdCl_2$ treatment (closed circles). The rate of the internalization of CD4 on U2Mlck-4 cells without $CdCl_2$ treatment was comparable with that of U2M1 or U937 clone2 cells (Fig. 3a). Thus, it was shown that the retardation of PMA-induced CD4 internalization is dependent on p56^{lck} expressed in the cells.

To examine whether the effect of p56^{lck} on the internalization of surface molecules is specific to CD4, the expression levels of CD11b, CD18 and CD45 on U2Mlck-4 cells were examined before and after induction of p56^{lck} and before and after PMA stimulation on $CdCl_2$-treated cells. As expected, no alteration of the expression of these molecules was observed either by the induction of p56^{lck} or by the PMA stimulation (data not shown). Moreover, the rate of the mAb-induced internalization of CD4 molecules was also slower in U2Mlck-4 cells than in U2M1 cells, while the rate of the antibody-induced internalization of CD11b was identical between U2Mlck-4 and U2M1 cells [31]. Therefore, it was suggested that p56^{lck} is specifically concerned with the down-modulation of CD4.

Dissociation of p56^{lck} from CD4 prior to the internalization of CD4

Aside from inducing CD4 internalization, PMA has been reported to cause the dissociation of p56^{lck} from CD4 [19]. p56^{lck}, 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 p56^{lck} with CD4 molecules is related to the difference in the internalization of CD4 observed above. To address this issue, the association of p56^{lck} with the surface-retained CD4 or the internalized CD4 after PMA stimulation was investigated. First, U2Mlck-4 cells that had been treated with $CdCl_2$ were cell-surface biotinylated and were stimulated with PMA for 0, 5, 15 and 30 min. Then, cells were incubated with a saturating amount of OKT4 for 30 min, washed and solubilized. Cell-surface CD4 bound to OKT4 were collected with protein A-Sepharose beads and a gradual decrease in the amount of the surface CD4 was found after PMA stimulation (Fig. 4a, indicated as 'surface'), which paralleled the CD4 internalization on $CdCl_2$-treated U2Mlck-4 cells revealed by FACS analysis (Fig. 3a). Then, the remainder of CD4 molecules was precipitated with OKT4 from each cell lysate, out of which surface CD4 molecules had already been removed (Fig 4a, indicated as 'internal'). After PMA stimulation, the internal CD4 increased from 0 to 5 min and slightly to 15 min and then slightly decreased

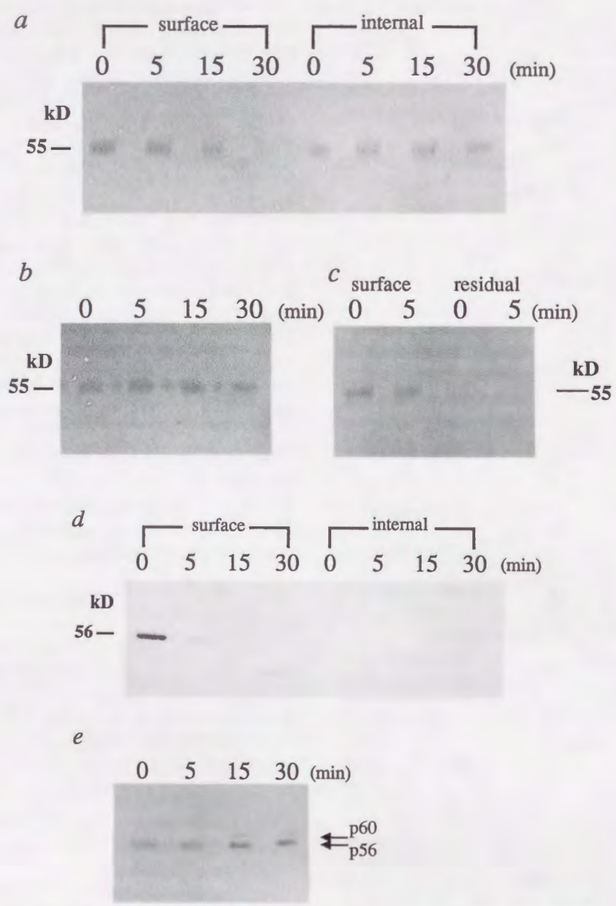

Fig. 4. Dissociation of p56^{lck} from CD4 after PMA stimulation. (a) Selective precipitation of surface CD4 and internalized CD4. U2Mlck-4 cells after $CdCl_2$ treatment were surface biotinylated as described in Materials and Methods. 0, 5, 15, and 30 min after PMA stimulation, cells were incubated with a saturating amount of OKT4 for 30 min and were solubilized. Surface-retained CD4 molecules (indicated as 'surface') bound to OKT4 were collected with protein A-Sepharose beads. The residual supernatants of cell lysates, from which surface CD4 molecules had been removed, were incubated with OKT4 to collect the residual internalized CD4 molecules ('internal'). After separation by SDS-PAGE, precipitated CD4 molecules were visualized by direct treatment of the blotted filter with peroxidase-conjugated avidin. The position of CD4 (55 kDa) is indicated. (b) Total precipitable CD4. U2Mlck-4 cells with $CdCl_2$ treatment were surface biotinylated and stimulated with PMA. After indicated minutes, cells were lysed, incubated with OKT4 followed by protein A-Sepharose beads. Precipitated immunocomplexes were separated on SDS-PAGE. (c) $CdCl_2$-treated U2Mlck-4 cells were surface biotinylated and stimulated with PMA as in Fig. 5a. Before and 5 min after stimulation, surface CD4 molecules ('surface') were removed. The rest of each cell lysate was treated with protein A-Sepharose beads for 1 h to collect the residual OKT4-bound, cell-surface CD4 molecules and were applied on SDS-PAGE ('residual'). (d) Selective precipitation of surface CD4-bound and internalized CD4-bound p56^{lck}. U2Mlck-4 cells after $CdCl_2$ treatment were stimulated with PMA as in Fig. 5a. 0, 5, 15 and 30 min after PMA stimulation, cell-surface CD4 and internalized CD4 molecules were selectively precipitated as in Fig. 5a. After separation by SDS-PAGE, co-precipitated p56^{lck}, either bound to surface CD4 ('surface') or internalized CD4 ('internal') was visualized by incubating the blotted filter with biotinylated MOL 171, followed by treatment with peroxidase-conjugated avidin. The position of p56^{lck} (56 kDa) is indicated. (e) Total precipitable p56^{lck}. U2Mlck-4 cells with $CdCl_2$ treatment were stimulated with PMA for the indicated times. Cells were lysed and incubated with MOL 171 for 1 h, then with RAM for additional 1 h. Immunocomplexes were collected with protein A-Sepharose beads, separated on SDS-PAGE and blotted onto a nitrocellulose filter. The filter was treated with biotinylated MOL 171 for the visualization of precipitated p56^{lck}. The positions of p56^{lck} (p56) and its immunoreactive form with diminished gel-mobility (p60) are indicated.

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 in 'internal') 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^{lck} bound either to cell-surface CD4 or to internal CD4 was selectively precipitated with the same procedure (Fig. 4d). First, the amount of p56^{lck} associated with surface CD4 after PMA stimulation was evaluated. Before stimulation, surface CD4 is associated with a substantial amount of p56^{lck} (Fig. 4d, 0 in 'surface'). 5 min after stimulation, the amount of p56^{lck} associated with surface CD4 remarkably decreased and no detectable p56^{lck} 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^{lck} in U2Mlck-4 cells was not altered after PMA stimulation (Fig. 4e), indicating that the decrease in the amount of p56^{lck} associated with surface CD4 was not the result of the reduction of total p56^{lck}. Next, when internal CD4 molecules were precipitated after PMA stimulation, no detectable amount of p56^{lck} was coprecipitated with CD4 throughout the time course (Fig. 4d, 'internal'). Hence, it was demonstrated that the internalized CD4 molecules were dissociated from p56^{lck}, even at the earlier periods after PMA stimulation (as early as 5 min after stimulation), at which time surface-retained CD4 molecules were still associated with p56^{lck}. And virtually, the internalized CD4 molecules were dissociated from p56^{lck} before the stimulation (Fig. 4d, 0 in 'internal'). Densitometric comparison of the rate of disappearance of the surface CD4 (Fig. 4a, 'surface') with that of the dissociation of p56^{lck} 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^{lck} from CD4 in response to PMA precedes the PMA-induced internalization of CD4 and also that cell-surface CD4 molecules associated with p56^{lck} are able to internalize only after their dissociation from p56^{lck}.

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

To examine the role of PTK activity of p56^{lck} on the p56^{lck}-mediated regulation of CD4 internalization, genistein, a PTK selective inhibitor [36], was used when

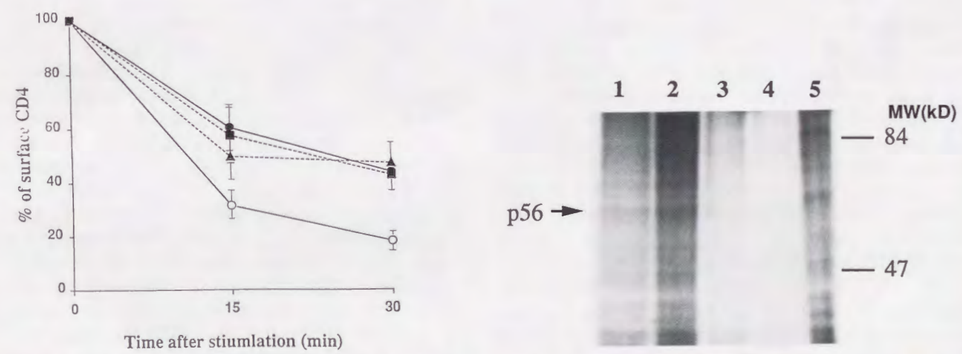

Fig. 5. Effect of genistein on the internalization of CD4. (a) CD4 internalization in genistein-treated cells. U2M1 cells (○) and U2Mlck-4 cells (closed symbols) were cultured for 24 h in the presence of $CdCl_2$. During the last 45 min of the culture, aliquots of U2Mlck-4 cells were treated with 0 (●), 80 (■) and 120 (▲) μg/ml of genistein. Then cells were washed, resuspended in RPMI medium at 37°C and were stimulated with PMA. Surface CD4 expression in each cell population after PMA stimulation was analyzed and % of surface CD4 (ordinate in the figure) was calculated as described in the legend of Fig. 3. (b) Inhibition of PTK activity by genistein. U2Mlck-4 cells, cultured in the presence of $CdCl_2$ were radiolabeled with [^{32}P]orthophosphate for 4 h in phosphate-free medium as described in Materials and Methods. The last 45 min of the culture, aliquots of $1 \cdot 10^6$ cells were treated with 0 (lanes 1, 2 and 5), 80 (lane 3) and 120 (lane 4) μM of genistein, respectively. For the induction of p56^{lck}-mediated tyrosine phosphorylation [6], cells were incubated with OKT4 at the saturating concentration (lanes 2–4) for 30 min. Then, cells were washed and mixed with RAM for 5 min at 37°C. A cell aliquot not treated with OKT4 was stimulated with PMA at 25 ng/ml for 5 min at 37°C (lane 5). Then, cells were lysed in phosphatase inhibitor-supplemented RIPA buffer, boiled in Laemmli sample buffer and resolved in 10% SDS-PAGE. The gel was treated with 1 M NaOH for the visualization of alkali-resistant phosphoproteins as described in Materials and Methods.

cells were stimulated with PMA for the induction of CD4-internalization (Fig. 5a). U2Mlck-4 cells that had been treated with $CdCl_2$ for the induction of $p56^{lck}$ (closed symbols) were treated with 0 (closed circles), 80 (closed squares) and 120 μM (closed triangles) genistein for 45 min before stimulation with PMA. Each of the three populations of $p56^{lck}$-expressing U2Mlck-4 treated with or without genistein showed similar rates of internalization of CD4, which were slower than that of U2M1 cells (open circles). The effective inhibition of PTK activity by genistein in this experiment was confirmed with the visualization of phosphotyrosine that demonstrated the decrease in the amount of phosphotyrosine and also the disappearance of autophosphorylated $p56^{lck}$ induced by CD4-crosslinking in U2Mlck-4 cells treated with genistein (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 $p56^{lck}$ was not involved in its effect on the internalization of CD4.

Discussion

In this study, a panel of transfectant cell clones, U2Mlck-4 and U2Mlck-6 ($CD4^+p56^{lck+}$) and U2M1 ($CD4^+p56^{lck-}$), together with parental U937 clone2 ($CD4^+p56^{lck-}$), were prepared to elucidate the effect of $p56^{lck}$ on the PMA-induced modulation of the CD4 molecule. When the internalization of CD4 in these cell clones was induced by PMA stimulation, a marked delay of the CD4 internalization was observed in U2Mlck-4 and U2Mlck-6 cells expressing $p56^{lck}$ compared with that in the control U2M1 cells or the parental U937 clone2 cells (Fig. 3a). To confirm that the delay in CD4 internalization in U2Mlck-4 and U2Mlck-6 cells is dependent on the presence of $p56^{lck}$ generated in cells, PMA-induced CD4 internalization was compared between $CdCl_2$-treated U2Mlck-4 cells, which express $p56^{lck}$ maximally and $CdCl_2$-non-treated U2Mlck-4 cells, which express very little $p56^{lck}$ (Fig. 3b). Cells not treated with $CdCl_2$ showed a much faster rate of CD4 internalization, which is comparable to U2M1 or U937 clone2, than cells treated with $CdCl_2$. This effect of $p56^{lck}$ was also observed when the internalization of surface CD4 was induced by cross-linking of CD4 antigens with anti-CD4 mAb and RAM [31]. These results suggest that $p56^{lck}$ plays a regulatory role in the internalization of CD4. The surface expression of CD4 on U2Mlck-4 and U2Mlck-6 cells was slightly higher than that on U2M1 and U937 clone2 cells and the level was raised after treatment with $CdCl_2$ to induce the expression of $p56^{lck}$ (Fig. 2). This observation may also be ascribed to the presence of $p56^{lck}$; as CD4 has been reported to be continuously internalized and recycled in the absence of stimulation [28], $p56^{lck}$ may affect this process, thereby raising the expression level of CD4. Support to the effect of $p56^{lck}$ on the kinetics of CD4 comes from the fact that the expression level of other cell-surface molecules (CD45, CD11b and CD18 were examined) was not affected by the expression of $p56^{lck}$ nor PMA treatment (data not shown) and the fact that the rate of antibody-induced internalization of CD11b was identical for $CdCl_2$-treated U2Mlck-4 and U2M1 cells [31].

What is the molecular basis of the regulatory effect of $p56^{lck}$ on the internalization of CD4? $p56^{lck}$ is myristylated at the amino-terminal end to bind to the cell membrane [34]. In T-lymphocytes, $p56^{lck}$ is stably but non-covalently associated with CD4 [5] via cysteine motifs in its amino-terminal domain [32, 33] and is dissociated from CD4 by the stimulation with PMA, which induces the CD4 modulation. It is, therefore, conceivable that the association of $p56^{lck}$ with CD4 may regulate the internalization of CD4. From this piont of view, we examined the association of $p56^{lck}$ with CD4 molecules after PMA stimulation. With the selective immunoprecipitation of cell surface and internalized CD4, it was demonstrated that the internalized CD4 was dissociated from $p56^{lck}$, even in the earlier period after stimulation (e.g., 5 min after stimulation, Fig. 4d, 'internal'). It was noteworthy that the intracellular CD4 before stimulation, due presumably to the spontaneous internalization during the procedure, was also dissociated from $p56^{lck}$ 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 $p56^{lck}$ (Fig. 4d, 0 in surface). 5 minutes after stimulation, however, the amount of $p56^{lck}$ 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 $p56^{lck}$ was coprecipitated with CD4 15 min after stimulation, though a considerable amount of CD4 was still precipitated at the same time. These data, taken together, demonstrate that the CD4 internalization induced by PMA stimulation occurs at a significantly slower rate than the PMA-induced dissociation of $p56^{lck}$ from surface CD4 and also that the dissociation precedes the internalization of surface CD4. Similar observation was reported by Juszczak et al. [37] that gp120 of HIV induced the dissociation of $p56^{lck}$ from CD4 followed by CD4 internalization. Hence, it is proposed that the association of $p56^{lck}$ may function to keep CD4 molecules on the cell surface and that the perturbation of the association may provide a trigger to initiate the internalization of CD4. It is unknown how the association of $p56^{lck}$ with CD4 restrains the internalization of CD4. The association of $p56^{lck}$ may perturb the cytoplasmic portion of CD4 to interact with cellular elements for internalization, such as the cytoskelton or coated pits. The mechanism of the dissociation of $p56^{lck}$ 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 internal CD4 before PMA stimulation is already dissociated from $p56^{lck}$.

Is the PTK activity of $p56^{lck}$ involved in the effect of $p56^{lck}$ on the internalization of CD4? The ability of $p56^{lck}$ to undergo autophosphorylation has been reported to be reduced in response to PMA stimulation [20]. We examined, therefore, the effect of genistein, a potent PTK inhibitor, on the PMA induced CD4 internalization. 80 to 120 μM genestein, concentrations at which the PTK activity in U2Mlck-4 cells was sufficiently inhibited (Fig. 5b), did not affect the rate of CD4 internalization on $p56^{lck}$-expressing U2Mlck-4 cells. Treatment of cells with such concentrations of genistein did neither affect the cell viability nor the surface expression of CD4 (data not shown). Hence, it is suggested that the PTK activity of $p56^{lck}$ may not be involved in the regulatory effect of $p56^{lck}$ on the internalization of CD4. Support to this idea may be provided by the report by Thuillier et al. [38] that genistein did not inhibit but facilitated the mAb-induced internalization of CD4 on human peripheral blood mononuclear cells, though the internalization of CD4 was induced in a different way (PMA-induced or mAb-induced). Due to the high homology of the amino-acid sequence of epidermal growth factor receptor (EGF receptor) and CD4 along with $p56^{lck}$ [35], however, the possibility is suggested that the PTK activity of $p56^{lck}$ might be involved in its regulatory effect on CD4 internalization, because the intrinsic PTK activity of EGF receptor is reported to enhance the ligand-induced internalization of EGF receptor by stabilizing receptor association with the apparatus for endocytosis [39]. Actually, the possibility cannot be excluded that a small amount of PTK activity remains after the genistein treatment, which is involved in the regulation of CD4 internalization, though the PTK activity appears to be completely inhibited by the treatment. Further investigation of the role of PTK activity of $p56^{lck}$ on the internalization of CD4 along with detailed knowledge of the mechanisms of CD4 endocytosis will be important in understanding the role of $p56^{lck}$ on CD4 internalization.

In conclusion, our data indicate that the PMA-induced internalization of CD4 in $p56^{lck}$-expressing cells are retarded as compared to the process in $p56^{lck}$-negative cells. This effect is probably induced by the association of $p56^{lck}$ with CD4 and it seems that the internalization of CD4 molecules must be preceded by the dissociation of $p56^{lck}$ from CD4. However, the PTK activity of $p56^{lck}$ appears not to be involved in this effect. This effect of $p56^{lck}$ to keep CD4 molecules on the cell surface might play a substantial role aside from or rather in concert with the role of $p56^{lck}$ as a signal-transducing molecule in Ag-induced cell activation.

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