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# Assembly of the CD8α/p56$^{lck}$ protein complex in stably expressing rat epithelial cells

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**Abstract We have previously characterized the biogenesis of the human CD8α protein expressed in rat epithelial cells. We now describe the biosynthesis, post-translational maturation and hetero-oligomeric assembly of the human CD8α/p56$^{lck}$ protein complex in stable transfectants obtained from the same cell line. There were no differences in the myristilation of p56$^{lck}$, or in the dimerization, *O*-glycosylation and transport to the plasma membrane of CD8α, between cells expressing either one or both proteins. In the doubly expressing cells, dimeric forms of CD8α established hetero-oligomeric complexes with p56$^{lck}$, as revealed by co-immunoprecipitation assays performed with anti-CD8α antibody. Moreover, p56$^{lck}$ bound in these hetero-oligomeric complexes was endowed with auto- and hetero-phosphorylating activity. The present study shows that: (1) the newly synthesized p56$^{lck}$ binds rapidly to CD8α and most of the p56$^{lck}$ is bound to CD8α at steady state; (2) CD8α/p56$^{lck}$ protein complexes are formed at internal membranes as well as at the plasma membrane; and (3) about 50% of complexed p56$^{lck}$ reaches the cell surface.** **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

*Key words:* p56$^{lck}$; CD8α; Protein expression; Interaction; Myristilation; Phosphorylation

## 1. Introduction

Human CD8 is a transmembrane glycoprotein uniquely expressed on the surface of T-lymphocytes, prevalently as an α/α homodimer, and to a lesser extent as an α/β heterodimer [1,2]. This protein exerts a dual function. Extracellularly, it is a T-cell co-receptor because its ectodomain binds MHC class I protein exposed on the surface of antigen-presenting cells [3–5]. Intracellularly, its cytosolic domain forms a stable complex with p56$^{lck}$ tyrosine kinase [6,7]; consequently the protein is involved in the signal transduction process determined by activation of the T-cell receptor [8,9].

p56$^{lck}$ belongs to the src-family of tyrosine kinases, it is expressed only in lymphocytes, and binds the cytosolic carboxy-terminal tail of CD4 and CD8 through the amino-terminal portion [10,11]. The amino-terminus of p56$^{lck}$ is myristilated and may also be palmitoylated [12–15], which favors the membrane association and the enzymatic activity of the protein. The binding between CD4/CD8 and p56$^{lck}$ is stable and is mediated by cysteine residues on both proteins [5,16,17]. The interaction between T-cells and antigen-presenting cells stimulates p56$^{lck}$ tyrosine kinase activity, which leads to auto- and hetero-phosphorylation [18–20]. In fact, most of the signalling due to T-cell receptor activation would be transmitted through the CD4/CD8–p56$^{lck}$ complex [21,22].

We previously described the biogenesis and post-translational maturation of CD8α stably expressed in the rat cell line FRT-U10 [23]. Other studies described the route of transport of p56$^{lck}$ to the plasma membrane and the kinetics of p56$^{lck}$ association mainly with the T-cell coreceptor CD4 [24,25]. To investigate the assembly and function of the CD8α/p56$^{lck}$ complex in the absence of the other proteins that form or interact with the T-cell receptor complex, we generated stable FRT clones in which CD8α and p56$^{lck}$ form stable and enzymatically active complexes. We found that newly synthesized p56$^{lck}$ binds rapidly to CD8α throughout the exocytic pathway and about 50% of complexed p56$^{lck}$ reaches the cell surface.

## 2. Materials and methods

### 2.1. Reagents

All culture reagents were supplied by Sigma (St. Louis, MO, USA). Solid chemicals and liquid reagents were obtained from E. Merck (Darmstadt, Germany), Farmitalia Carlo Erba (Milan, Italy), Serva Feinbiochemica (Heidelberg, Germany); SDS was purchased from BDH (Poole, UK). [$^{35}$S]Cysteine (specific activity 1000 Ci/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA, USA); [$^{3}$H]myristic acid (specific radioactivity 30 Ci/mmol) was from Amersham International, (Amersham, Bucks., UK). Mouse monoclonal CD8 (OKT8) was supplied by Ortho (Raritan NJ, USA). Mouse monoclonal Lck (3A5), rabbit polyclonal Lck (2102) and mouse monoclonal p-Tyr (PY20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein A–Sepharose was from Pharmacia (Uppsala, Sweden). The ECL kit (Amersham International) was used for the chemiluminescent detection of horseradish peroxidase (HRP).

### 2.2. Cell culture and transfection

Parental FRT cells and FRT-derived clones were cultured in Coon's modified Ham's F12 medium supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine in a 95% air, 5% $CO_2$ incubator. The pRc-lck expression vector carrying the murine lck cDNA under the control of the CMV promoter was co-transfected in FRT-U10 cells with a plasmid RSV-igro carrying the bacterial igro gene, in FRT cells with a plasmid RSV-neo carrying the bacterial neo gene [23,26]. Stable transformants were selected in the presence of 250 μg/ml igromicin-B or 600 μg/ml G-418, respectively. Positive clones were screened by immunofluorescence, propagated and frozen in liquid nitrogen. In all experiments, the cultured clones were used before the tenth passage after thawing.

### 2.3. Radioactive labeling and immunoprecipitation

The cells were allowed to grow to subconfluence. For [$^{35}$S]cysteine

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*Abbreviations:* FRT, Fisher rat thyroid; FCS, fetal calf serum; DTT, dithiothreitol; HRP, horseradish peroxidase; P-tyr, phosphotyrosine

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labeling, cells were first incubated for 1 h in labeling medium containing cysteine-free Dulbecco's modified Eagle's medium, supplemented with 1% FCS, and then pulse-labelled for various time with the same medium containing 100 μCi/ml [$^{35}$S]cysteine. Cells were chased for various times with labeling medium containing 10-fold excess cold cysteine and 10 μg/ml cycloheximide. Immunoprecipitation analyses are described elsewhere [27]. Immunoprecipitation of CD8 present at the plasma membrane was obtained by loading monoclonal anti-CD8 antibody on UL23 cell monolayers for 45 min at 4°C. The antibody was removed and the washed monolayer was then lysed and incubated with protein A–Sepharose as described previously [27]. Cells used for [$^{3}$H]myristic acid labeling were incubated for 24 h with Dulbecco's modified Eagle's medium supplemented with 1% FCS and labelled for 4 h in serum-free medium containing 200 μCi/ml [$^{3}$H]myristic acid.

*2.4. Immunofluorescence, SDS–PAGE and immunoblot analysis*

Indirect immunofluorescence analysis, preparation of cell extracts, SDS–PAGE analysis and fluorography were performed as previously described [27]. For immunoblot analysis, equal amounts of cell extracts (100 μg of protein/lane) were separated on 10% SDS–PAGE and transferred to nitrocellulose filters. Filters were saturated with 10% milk solution and incubated with antibody. Filters were washed several times and incubated for 1 h at room temperature with HRP-conjugated antibody; HRP activity was evaluated with chemiluminescent assays.

*2.5. In vitro kinase assay*

CD8α/p56$^{lck}$ complexes were immunoprecipitated with anti CD8 mouse monoclonal antibody OKT8. After extensive washes, the immunocomplexes were resuspended in 50 ml of kinase buffer (10 μCi of [γ-$^{32}$P]ATP, 50 μM of cold ATP, 100 μM Na-orthovanadate, 20 mM $MgCl_2$, 10 mM $MnCl_2$, 0.1% Triton X-100 and 20 mM HEPES pH 7.2) [28] and incubated for 10 min at room temperature. Incubation was stopped with 1 ml of ice-cold stopping buffer (20 mM HEPES pH 7.2, 0.1% Triton X-100, 100 mM Na-orthovanadate); the proteins were precipitated in 20% trichloroacetic acid and analyzed by SDS–PAGE and autoradiography.

## 3. Results

*3.1. Expression of CD8α and p56$^{lck}$ in FRT clones*

Parental FRT cells and the FRT-U10 clone, which expresses high levels of CD8α [26], were transfected (see Section 2) with the pRc-p56$^{lck}$ expression vector carrying the murine lck cDNA under the control of the CMV promoter. Two representative clones were selected, 'FL15' and 'UL23', that stably expressed p56$^{lck}$ alone and with CD8α, respectively.

Both proteins were associated mainly with cellular membranes, particularly the plasma membrane (Fig. 1A–D). As expected, CD8α was not required for the membrane association of p56$^{lck}$ [13,29]. Indeed, the distribution of p56$^{lck}$ was the same in FL15 and UL23 cells (Fig. 1E).

We next used Western immunoblotting assays to determine whether FL15 and UL23 cells express recombinant CD8α

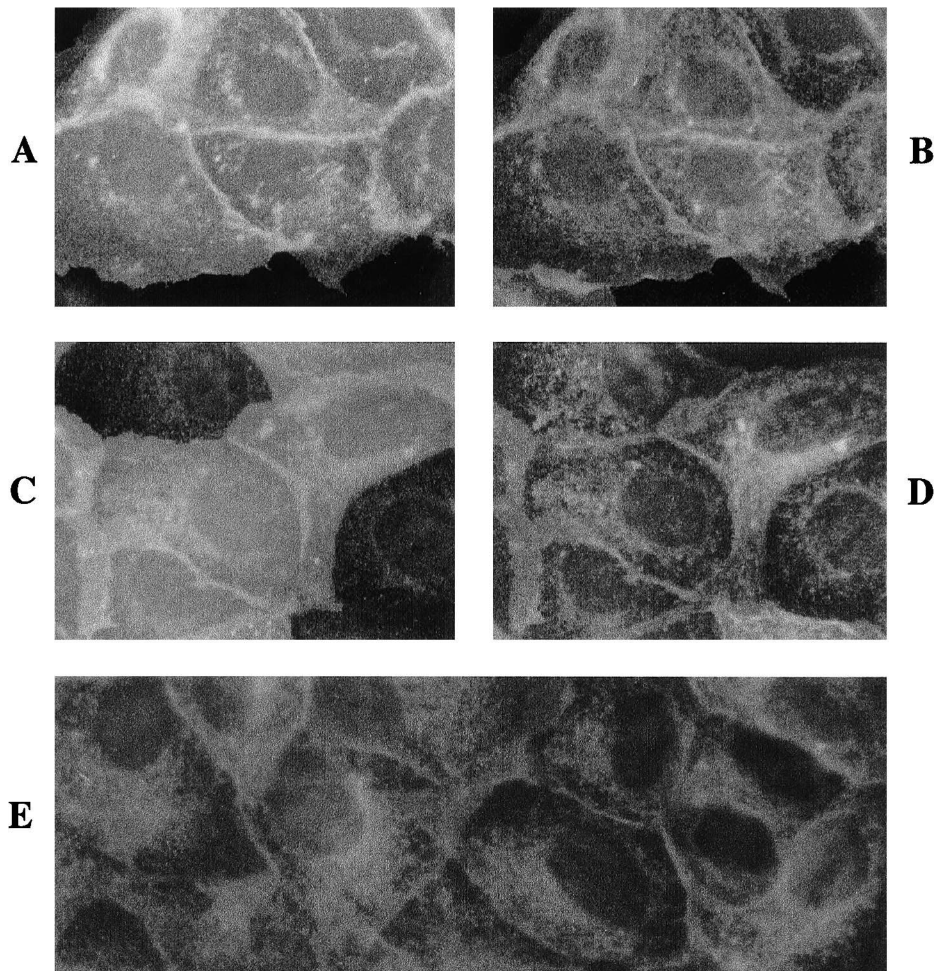


Fig. 1. Plasma membrane localization of CD8α and p56$^{lck}$ proteins in UL23 and FL15 cells. Cells were grown on glass coverslips and processed for immunofluorescence light microscopy as detailed in Section 2. (A–D) UL23 cells; (E) FL15 cells. (A,C) anti-CD8α monoclonal antibody; (B,D,E) anti-p56$^{lck}$ polyclonal antibody.

and/or p56$^{lck}$ of the predicted molecular weight; we used the U10 and Jurkat cell lines as control (Fig. 2A). p56$^{lck}$ occurred only in UL23 and FL15 cells (Fig. 2A, lanes 3 and 5). The apparent molecular weight of the murine p56$^{lck}$ expressed in FL15 cells was similar to that of the endogenous p56$^{lck}$ synthesized in Jurkat cells (Fig. 2A, lane 2); consequently, the FRT clones correctly expressed exogenous p56$^{lck}$. Conversely, CD8α was expressed only in UL23 and U10 cells (Fig. 2A, lanes 8 and 9). The two slower migrating bands (Fig. 2A, lanes 8 and 9) were probably due to residual CD8α homodimers. Similar results were obtained with immunoprecipitation assays of pulse-labelled cells (data not shown).

Finally, we investigated if like lymphocytes and U10 cells [26], UL23 cells expressed homodimeric forms of CD8α. SDS–PAGE analysis of immunoprecipitated products with or without reducing agents resulted in a band of about 55 kDa (the expected size for the CD8 α/α homodimer) only in the absence of reducing agents (Fig. 2B, lanes 3 and 4). Therefore, also in UL23 cells CD8α is assembled in homodimers stabilized by disulfide bonds. The pattern of CD8α maturation (*O*-glycosylation) was identical in UL23 and U10 cells (M.C. Pascale, unpublished results), indicating that the coexpression of p56$^{lck}$ does not modify the main parameters of CD8α biogenesis in FRT cells.

### 3.2. CD8α/p56$^{lck}$ complexes in UL23 cells

Next we examined the interaction of CD8α with p56$^{lck}$ in UL23 cells (Fig. 3). Cell lysates obtained from the UL23 clone were immunoprecipitated with anti-CD8α (lanes 1 and 4) or anti-p56$^{lck}$ (lane 2). The immunoprecipitated products (lanes 1 and 2) were separated by SDS–PAGE and analyzed by Western blot with anti-p56$^{lck}$ antibody together with total lysates (Fig. 3, lanes 1–3). The level of precipitation with p56$^{lck}$ protein was similar with anti-CD8α and anti-p56$^{lck}$ antibody (Fig. 3: lanes 1 and 2). This finding strongly suggested that at steady state, almost all p56$^{lck}$ protein was bound to CD8α. Conversely, after immunoprecipitation with anti-p56$^{lck}$, CD8 was not revealed by Western blot with anti-CD8 antibody (data not shown). Control experiments in which immunoprecipitation with anti-CD8 antibody was performed with lysates of mixed U10 and FL15 cells did not result in p56$^{lck}$ protein. This indicates that CD8α/p56$^{lck}$ complexes were not assembled after cell lysis (data not shown). Conversely, p56$^{lck}$ co-immunoprecipitated from metabolically labeled cells immunoprecipitated with anti-CD8 monoclonal antibody (Fig. 3, lane 4). Again, monoclonal anti p56$^{lck}$ antibody failed to retrieve CD8α in immunoprecipitation experiments on metabolically labeled cell lysates (data not shown).

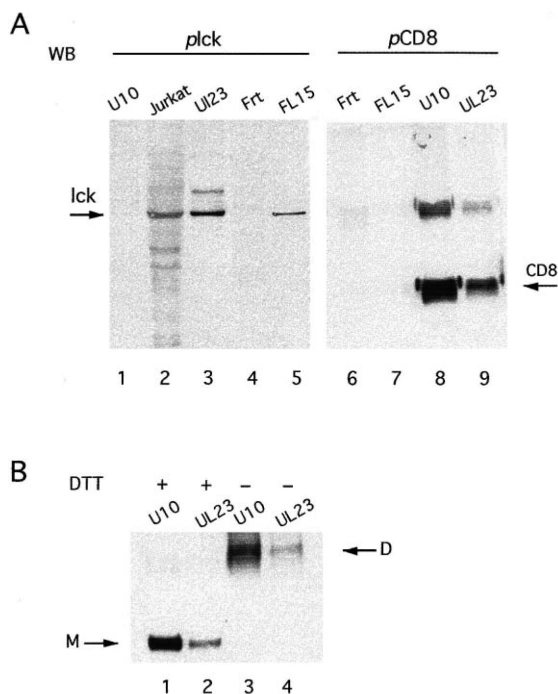


Fig. 2. Expression of CD8α and p56$^{lck}$ proteins in FRT-derived clones. (A) Aliquots of cell lysates were separated by SDS–PAGE and analyzed by Western blot (WB) with anti-p56$^{lck}$ (lanes 1–5) and anti-CD8α (lanes 6–9) polyclonal antibody. (B) Aliquots of cell lysates from U10 and UL23 cells were analyzed as in A with anti-CD8α polyclonal antibody with or without reducing agent (DTT). Arrows indicate p56$^{lck}$ (lck) and CD8α (CD8). D, dimeric CD8α; M, monomeric CD8α.

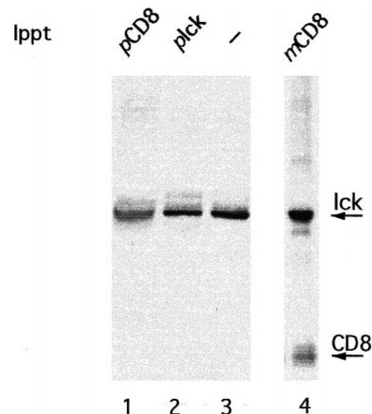


Fig. 3. Co-immunoprecipitation of CD8α/p56$^{lck}$ complexes assembled in UL23 cells. Aliquots of UL23 cell lysates were immunoprecipitated (lanes 1 and 2) and analyzed by SDS–PAGE followed by Western blot with anti-p56$^{lck}$ monoclonal antibody (lanes 1–3). Lane 1, immunoprecipitation (Iptt) with anti-CD8α (pCD8) polyclonal antibody; lane 2, immunoprecipitation with anti-p56$^{lck}$ polyclonal antibody; lane 3, total UL23 cell lysate; lane 4, cell lysates from UL23 cells metabolically labeled for 4 h and immunoprecipitated with anti-CD8α monoclonal antibody.

These results show that CD8α/p56$^{lck}$ complexes are efficiently assembled in UL23 cells.

To establish the timing and the site of assembly of CD8α/p56$^{lck}$ protein complexes UL23 cells were pulse-labelled with [$^{35}$S]cysteine, chased for different times, and processed for surface immunoprecipitation with anti-CD8α antibody followed by cell lysis and a second round of immunoprecipitation to reach intracellularly located CD8α. The total amount of complexes coimmunoprecipitated was similar during the pulse-chase (Fig. 4A,B). About 25% of the total complexed p56$^{lck}$ was present at the cell surface at the end of the pulse time (Fig. 4C). Conversely, newly synthesized CD8α appeared later; this confirms the specificity of the immunoprecipitation from the surface.

In conclusion, we found that newly synthesized p56$^{lck}$ binds rapidly to CD8α and about 50% of complexed p56$^{lck}$ reaches the cell surface.

Moreover, the time of conversion of the unglycosylated CD8α to the fully glycosylated form (half-time of about 15 min), the appearance of CD8α at the surface (half-time of

about 30–60 min) and the total amount present at steady state at the plasma membrane (75%) were consistent with previously reported values [23,30].

### 3.3. Myristilation and phosphorylation of p56$^{lck}$

p56$^{lck}$ is myristilated at its amino terminus in lymphocytes [12], thus we investigated if this modification occurs in UL23 and FL15 cells. Parallel cultures were pulse-labelled with [$^3$H]myristic acid for 4 h, the cell lysates immunoprecipitated with monoclonal antibodies anti-lck, and the immunoprecipitated products analyzed by SDS–PAGE. p56$^{lck}$ was indeed myristilated in both FL15 and UL23 cells (Fig. 5, lanes 1 and 2) and the covalent nature of the binding of $^3$H-myristic acid to the protein backbone was demonstrated by the resistance to 1 M hydroxylamine (data not shown).

Next we used a monoclonal anti-phosphotyrosine antibody to determine the phosphorylation status of p56$^{lck}$ synthesized in UL23 cells. Two assays were performed: Western immunoblotting on total lysates of FL15 (Fig. 5, lanes 3 and 5) and UL23 cells (Fig. 5, lanes 4 and 6) or immunoprecipitation followed by Western immunoblotting with polyclonal anti-lck antibody (Fig. 5, lanes 7 and 8). Both assays demonstrated that p56$^{lck}$ was tyrosine-phosphorylated in FL15 and UL23 cells. Differences in the phosphorylation levels of p56$^{lck}$ paralleled the expression levels of the protein in the diverse clones (compare Figs. 1 and 5).

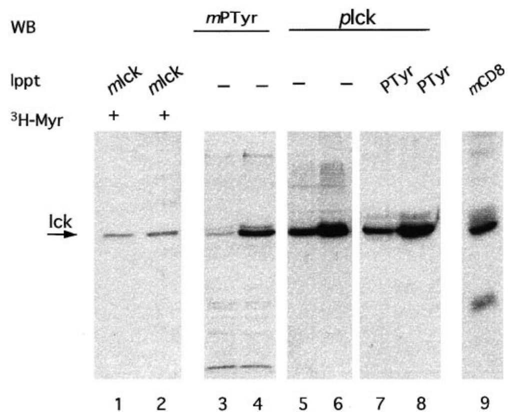


Fig. 5. Myristilation, tyrosine phosphorylation, and tyrosine-kinase activity of p56$^{lck}$ synthesized in UL23 and FL15 cells. Parallel cultures of UL23 (lanes 1) and FL15 cells (lane 2) were pulsed with [$^3$H]myristic acid for 4 h, lysed and immunoprecipitated with anti-p56$^{lck}$ monoclonal antibody and the immunoprecipitated products analyzed by SDS–PAGE and fluorography. Cell lysates from FL15 (lanes 3, 5, 7) and from UL23 cells (lanes 4, 6, 8) were separated by SDS–PAGE and analyzed by Western blot (WB) with anti-phosphotyrosine monoclonal antibody (lanes 3–4) or with anti-p56$^{lck}$ polyclonal antibody (lanes 5 and 6). Cell lysates from FL15 (lane 7) or from UL23 cells (lane 8) were immunoprecipitated with anti-phosphotyrosine antibody and revealed by Western blot with anti-lck antibody. Lane 9, UL23 cell lysate was incubated with anti-CD8α antibody and the immunoprecipitated products tested in a kinase assay supplemented by [γ-$^{32}$P]ATP, followed by SDS–PAGE analysis and autoradiography.

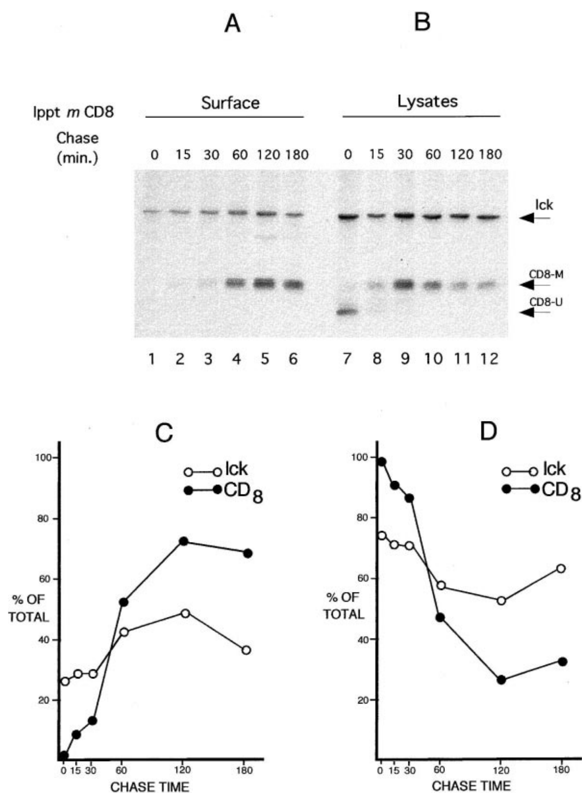


Fig. 4. Timing of complex formation by newly synthesized CD8α and p56$^{lck}$ proteins. UL23 cells were pulse-labelled with [$^{35}$S]cysteine for 15 min and chased for different times as indicated. The immunoprecipitated products were analyzed by SDS–PAGE and fluorography. (A) surface immunoprecipitation with anti-CD8α antibody. (B) Second round of immunoprecipitation of cell lysates with anti-CD8α antibody to reach intracellularly located CD8α/p56$^{lck}$ complexes. p56$^{lck}$ (lck) and CD8α (CD8) proteins are indicated by arrows. CD8-M, mature CD8α; CD8-U, unglycosylated precursor CD8α. Quantitative densitometry of autoradiograms A and B: (C) percent of total complexed p56$^{lck}$ (lck) and of total CD8α (CD8) at the cell surface at the chase times indicated; (D) percent of total complexed p56$^{lck}$ (lck) and of total CD8α (CD8) present intracellularly at the chase times indicated. Totals were calculated by adding surface values to intracellular values.

Finally, we tested CD8α-bound p56$^{lck}$ for tyrosine kinase activity. CD8α/p56$^{lck}$ complexes were immunoprecipitated with anti-CD8 antibody, and the immunoprecipitated products used for an in vitro kinase assay. The major labelled protein was p56$^{lck}$ (Fig. 5, lane 9), which confirms a significant autophosphorylating activity. However, a smaller protein, most likely the immunoglobulin light chain, was also labelled (Fig. 5, lane 9), indicating that p56$^{lck}$ bound to CD8α also phosphorylates exogenous substrates in vitro.

## 4. Discussion

We have characterized the expression and assembly of CD8α/p56$^{lck}$ protein complexes in stably transfected FRT cells. CD8α and p56$^{lck}$ proteins synthesized together in UL23 cells are indistinguishable from those individually synthesized in U10 and FL15 cells, respectively, and in lymphocytes. This observation is supported by the results of the analysis of folding, dimerization, *O*-glycosylation and kinetics of transport to the plasma membrane of CD8α; and of the myristilation and hetero-phosphorylating activity of p56$^{lck}$ molecules assembled in complex with CD8α. Finally, the bond between the two proteins survived treatment with non-ionic detergent, as previously described [31].

We also described the dynamics of the formation of the complexes and the intracellular compartment where the interaction between CD8α and p56$^{lck}$ occurs in UL23 cells. The interaction of CD8α with p56$^{lck}$ and the consequent traffick-

ing of CD8α/p56$^{lck}$ complexes differ with respect to the assembly process of p56$^{lck}$ with CD4 in lymphocytes [24]. In UL23 cells, newly synthesized p56$^{lck}$ forms complexes with CD8α very rapidly and, most likely, p56$^{lck}$ binds CD8α directly at the plasma membrane. This means that p56$^{lck}$ interacts with pre-existing CD8α molecules present throughout the exocytic pathway. Indeed, p56$^{lck}$ is synthesized on free ribosomes in the cytosol, so it can rapidly reach any target membrane of the cell.

Conversely, newly synthesized p56$^{lck}$ binds CD4 in intracellular membranes within 10 min, and within 30–45 min all the complexes are transported to the plasma membrane [24]. Differently, we detected 25% of the total complexed newly synthesized p56$^{lck}$ bound to pre-existing CD8α molecules at the plasma membrane already at the end of the short pulse time. This finding strongly suggests that p56$^{lck}$ binds CD8α at the plasma membrane.

Surprisingly, we found that even after 2 and 3 h of chase, no more than about 50% of complexed p56$^{lck}$ was on the plasma membrane versus about 75% of total CD8α on the surface. This finding suggests that p56$^{lck}$ has preferential binding, and/or localization (complexed with CD8α) in intracellular membranes with respect to the plasma membrane.

This preferential localization could be explained by a reversible binding of p56$^{lck}$ to CD8α, which generates a retrograde flow of bound p56$^{lck}$ from the plasma membrane to counteract the anterograde flow of CD8α toward the plasma membrane. At steady state, most of the CD8α is in the mature form which is generated in the *trans*-Golgi network [23,30] Therefore, complexed p56$^{lck}$ would accumulate on the intracellular membranes of the late compartments of the exocytic pathway.

In conclusion, the model system described herein has revealed that p56$^{lck}$ binds rapidly to CD8α located at the plasma membrane as well as at intracellular membranes; and that 50% of p56$^{lck}$ complexed with CD8α is located on the plasma membrane. Further experiments are required to elucidate the differences in CD4 and CD8 binding to p56$^{lck}$, in the transport of their complexes to the cell surface and in their association with other components of the plasma membrane.

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