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The Hierarchical Importance of Components of The Peptide-loading Complex*

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Abstract TAP2, tapasin and calreticulin (CRT) are three crucial components of the peptide loading complex (PLC) in MHC class Ⅰ antigen processing and presentation. While the functions of the proteins within the complex have been progressively defined, the hierarchical importance of the components has not been clearly and quantitatively analyzed. To compare the efficiency of MHC class Ⅰ antigen processing in different cell lines, we established a novel suilysin-mediated antigen delivery method to quantitatively analyze antigen processing in a number of cell lines, including K41, the CRT-deficient line K42, the tapasin-deficient line 90a and the tapasin/TAP2-deficient cell line 91a. We ranked the importance of the components of MHC class Ⅰ antigen processing such as CRT, TAP and tapasin using OVA challenge as the antigen model. Surprisingly, CRT was shown to be the most important component of the PLC, even though TAP was speculated to be the most crucial protein involved in the pathway. This is the first report that ranks the components of the antigen-processing pathway. Further investigation is required for elucidation of the roles played by the individual proteins within the complex.

Key words MHC-Ⅰ, peptide loading complex (PLC), suilysin (SLY), quantitative analysis
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The peptide loading complex (PLC) of MHC-Ⅰ, consisting of the ATP-dependent TAP heterodimer (TAP1 and TAP2), tapasin, calreticulin (CRT) and oxidoreductase Erp57 [1–2], is essential for the classic MHC class Ⅰ antigen processing and presentation pathway. Short peptides (usually 8～10 a.a. in length) derived from proteins within the cell are generated in the cytosol and transported *via* the PLC to the lumen of endoplasmic reticulum (ER). These peptides bind to the nascent MHC-Ⅰ molecules to form a peptide/MHC-Ⅰ complex[3–4] and are subsequently transported to and presented on the cell surface, where they interact with T cells.

In the PLC, TAP2, in association with TAP1, is responsible for the transportation of peptides from cytosol to ER [5–7]. CRT specifically binds to the monoglycosylated N-linked glycan of MHC-Ⅰ to regulate MHC-Ⅰ folding [8–12]. Tapasin is a trans-membrane protein that binds to both TAP1 and a region of MHC-Ⅰ, thereby bridging them. Tapasin also assists the binding of peptides transported by TAP

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to MHC-Ⅰ proteins, forming stable peptide/MHC-Ⅰ complexes [12–13]. Although the functions of PLC components have been progressively well defined in past studies, the hierarchical importance of constituents of the PLC is still obscure, mainly due to the lack of methods that yield quantitative results of antigen processing efficiency[14]. Previous research has described that streptolysin O (SLO), a member of the cholesterol-dependent cytolysin (CDC) family, has the potential to perforate cell membranes and deliver intact proteins into the cytosol. However, the method itself is still far from flawless[14–15].

In this study, we developed a novel method using suilysin (SLY), a homologous protein of SLO and also a member of the CDC family, to introduce exogenous antigen directly into the cells to investigate the efficiency of antigen processing and presentation. By utilizing SLY to perforate cells, we successfully delivered intact ovalbumin (OVA) into the cytosol of K41 cells. The OVA epitope H-2K^b presented on the cell surface was detected and quantified using the B3Z ovalbumin presentation assay[16]. We further detected the efficiency of MHC-Ⅰ antigen processing and presentation by comparing K41 cells with the CRT-deficient cell line K42[8], the TAP2 and tapasin-silenced cell line 91a, and the tapasin-silenced cell line 90a.

We found that compared with the 90 and 91a cell lines, the K42 line displayed a lower efficiency of antigen processing and presentation, even though MHC-Ⅰ expression on the surface of K42 cells is not significantly different from that on 90a and 91a cells. These findings advanced our understanding of the divergent importance of PLC constituents, and demonstrated that the role of CRT in the PLC may require deeper understanding and reevaluation. In addition, our research developed a novel protein delivery method to further understand the role of MHC-Ⅰ in the PLC, which enriches the methodology available for current MHC-Ⅰ research, as well as other biomedical research areas.

1 Materials and methods

1.1 Cells

K41 is a mouse fibroblast cell line derived from a normal embryo, as described previously [8]. K42 fibroblasts were given to us by Dr. Michalak M, *et al*[11]. Both the TAP2- and tapasin-inhibited cell line 91a and tapasin-inhibited cell line 90a were derived from wild type K41 cells, and kindly given to us by Dr. Wu Ying, *et al*(Beijing, China)[17]. B3Z cell is a T-cell hybridoma that specifically recognizes the K^b-SIINFEKL complex, originally obtained from Dr. Shastri N, *et al*[18]. T2 cell is a human lymphoblast cell line kindly given by Dr. Wu Ying(Beijing, China). All the cells were cultured in RPMI 1640 supplemented with 10% FBS and L-glutamine (Gibco BRL, USA). Murine spleen cells were obtained as described by Fu *et al*[14].

1.2 Proteins and antibodies

Suilysin was expressed by the *E. coli* strain BL21 and affinity purified using Ni-columns. The plasmid construction was kindly given to us by Dr. George Fu Gao.25-D1.16, obtained originally from Dr. R. Germain, is a monoclonal antibody specific to the K^b-SIINFEKL complex [19]. Antibodies Y3 (against peptide-bound K^b [20]) were affinity purified using protein G columns (ImmunoPure, Pierce, USA). Rabbit anti-mouse TAP2 sera and rabbit anti-mouse tapasin sera were generated in rabbits immunized with *E. coli*-expressed inclusion bodies of mouse TAP2-GST fusion and tapasin-GST fusion proteins, respectively [17, 21]. The rabbit anti-calnexin (CNX) serum was generated as described previously [17]. Chicken ovalbumin (OVA) was purchased from Sigma (St. Louis, MO). FITC-tagged OVA was purchased from Epigen Biotech.

1.3 Western blotting

10^6 cells were washed twice by PBS, centrifuged at 1 500 r/min for 5 min, then resuspended in PBS containing 1% Triton X-100 and lysed on ice for 15 min. The lysate was separated by 12% SDS-PAGE, and the gel was blotted onto Hybond Extra-C membrane (Amersham, USA). The membrane was then washed once in PBS, blocked in PBS with 2% skimmed milk powder at room temperature for 1 h. After blocking, the membrane was incubated with primary antibodies in PBS containing 2% skimmed milk powder for 1 h, followed by anti-rabbit or anti-mouse antibody conjugated with HRP for another 1 h at room temperature, and washed three times with PBS-T (0.05% Tween). Finally, bands on the membrane were visualized using an enhanced chemiluminescence substrate (Pierce, USA). Software

Quantity One 4.6.2 (Bio-RAD) was used to analyze the scanned blot quantitatively.

1.4 Flow cytometry

For surface flow cytometry, 1×10^6 cells were labeled consecutively by primary antibody and FITC-conjugated IgG in PBS on ice for 30 min, for each antibody treatment. Cells were then washed twice with PBS and the level of green fluorescence was analyzed using FACS Calibur and FlowJo software. For the optimization of the suilysin permeabilization methodology, FITC-tagged intact OVA was added to cells permeabilized with suilysin, and incubated at 37 ℃ for 30 min. Peptide-loaded cells were washed twice with ice-cold growth medium and resuspended in 500 μl of ice-cold growth medium. 10 μg of propidium iodide (Sigma) was added and the cells were incubated for 10 min on ice. Samples were then analyzed using FACS Calibur and FlowJo software.

1.5 B3Z endogenous ovalbumin presentation assay

Cells were permeabilized for 30 min by suilysin at room temperature, with the exogenous protein added into the solution as described above. Cells were then washed twice with normal cell growth medium and resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS. Properly diluted target cells were added into wells of a 96-well plate, with the cell concentration of the first well being 1×10^5 cells/well and then double-diluted for the next five wells to reach a different Effector∶Target (E∶T) ratio. Next, 1×10^5 B3Z cells were added into each well and co-cultured 16 h in 37℃. Cells were washed twice with PBS (pH 7.4) and lysed by addition of 100 μl lacZ buffer containing 0.15 mmol/L chlorophenol red-β-D-galactopyranoside (CPRG) substrate (Calbiochem, La Jolla, CA), 0.125% NP40 (EMD Sciences, La Jolla, CA), 9 mmol/L $MgCl_2$ (Aldrich, USA) and 100 mmol/L 2-mercaptoethanol in PBS. After 3 h incubation at 37℃ 50 μl stop buffer (300 mmol/L glycine and 15 mmol/L EDTA in water) was added to each well, and the Lac Z expression level was quantified by the hydrolysis of CPRG. The A value was read on a 96-well plate reader (BioRad Model 3550) at 595 nm with 620 nm as the reference wavelength.

1.6 Statistical analysis and reproducibility

All experiments were repeated at least three times. Statistical analysis were performed by independent t-test using SPSS 21. $P < 0.05$ was considered significant compared with the control group.

2 Results

2.1 Delivery of intact proteins into the cytosol by SLY

Because information regarding the application of SLY for permeabilizing cells was unavailable in the literature, both the SLY concentration and duration of treatment suitable for cell permeabilization were unknown. Therefore, we optimized the conditions for SLY-mediated permeabilization, and tested the effect of different concentrations of SLY on cell permeabilization. Cells were stained with propidium iodide (PI) to label dead cells and the presence of FITC-labeled OVA in the cytoplasm was used as an indicator of successful permeabilization. Similar to streptolysin O[14], the rates of both successful delivery and cell death rose with increasing doses of SLY, but the rate of increase varied. As shown in Figure 1, application of less than 20 U of SLY / 0.1 million cells increased cell mortality slowly, from (4.87±1.51)% for no sly to (17.75±3.44)%, whereas the rate of successful delivery of FITC-labeled OVA increased rapidly, from (0.95 ±0.05)% to (44.31 ±3.10)%. However, a concentration higher than 20 U/0.1 million cells increased the death rate significantly, although the rate of permeabilization did not increase over that obtained with 20 U/0.1 million cells. When the concentration of SLY was increased to 40 U/0.1 million cells, 47% of the detected cells were PI-positive, indicating that they might die during the course of the treatment. When the concentration of SLY increased to 60 U/0.1 million cells, nearly 74% of the cells were dead, with approximately 18% of the cells alive and permeated. This result was much lower than the 33% of live/permeable cells observed at a dose of 40 U/0.1 million cells (Figure 1 b, c).

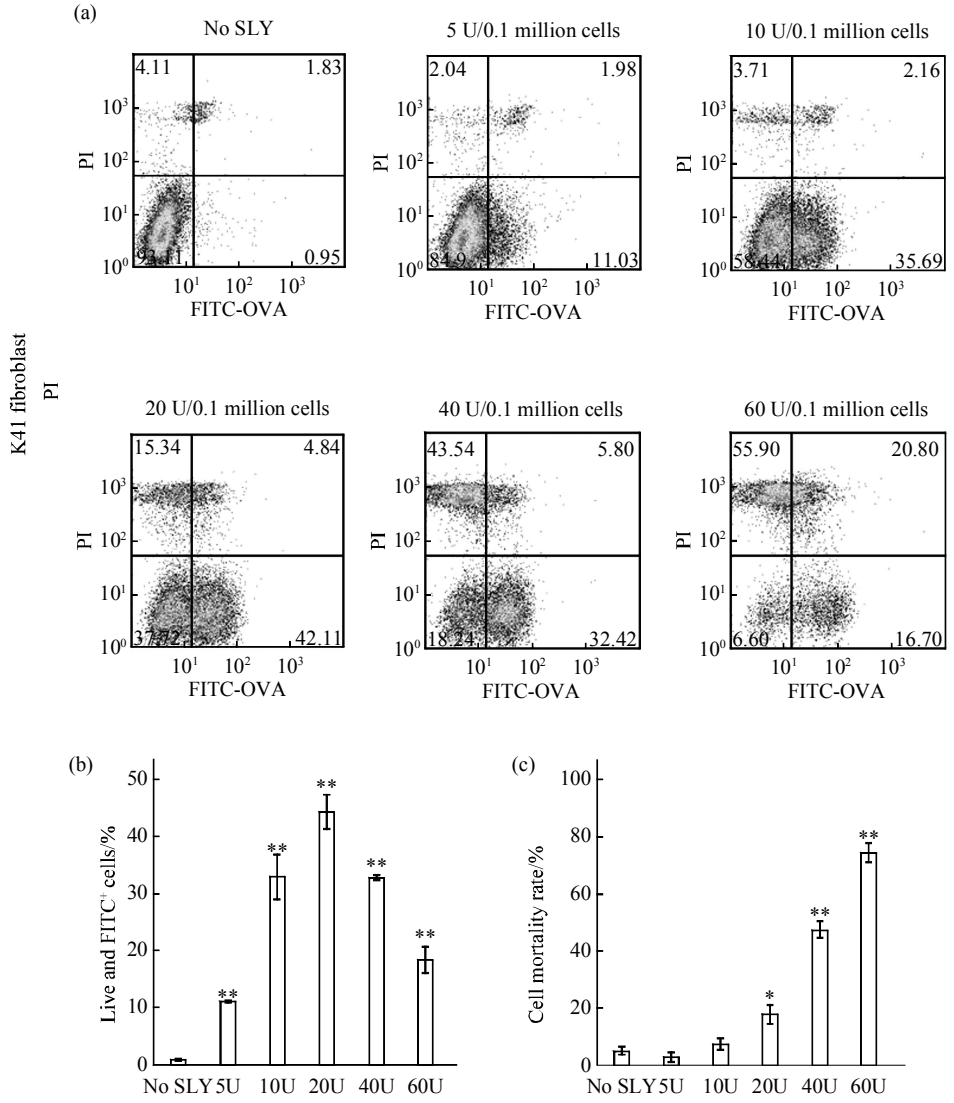

Fig. 1 Flow cytometry analysis of cell permeabilization with different suilysin concentrations

(a) Flow cytometry analysis of cell permeabilization with different concentrations of suilysin. K41 mouse fibroblasts were treated with different concentrations of suilysin under the same other conditions (30 min, 37℃). Cells were co-incubated with 100 mg/L fluorescein isothiocyanate (FITC)-tagged ovalbumin (OVA). Cells were then stained with PI after extensive washing. We performed flow cytometry for the presence of FITC and/or PI-labeled cells. (b, c) Percentage of (b) live cells with FITC-tagged OVA ($FITC^+$ PI^-) and (c) cell mortality (PI^+ cells) of flow cytometry results shown in Figure 1a, $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* the control group (no SLY). Data are representative of five independent experiments.

In addition, we optimized the treatment duration by evaluating the efficiency of protein delivery after different durations of SLY exposure. Although SLY treatment duration from 10 min to 60 min under a SLY concentration of 20U/0.1 million cells could all result in a successful delivery of FITC-OVA (Figure 2a and b), increasing the duration of treatment from 30 min to 60 min did not appreciably change the rates of both protein delivery and cell death, with the differences of death rate or delivery efficiency between 40 min group and 60 min group insignificant ($P > 0.05$). However, increasing the treatment duration from 10 min to 30 min increased the efficiency of delivery by more than 20%, changing from (17.88 ± 2.51)% to (44.73 ± 2.50)% (Figure 2b and c). By contrast, increasing the SLY treatment duration increased the proportion of PI positive cells proportionately. Therefore, considering the time economy and the rising death rate of the cells, we concluded that 30 min was the optimal duration of SLY treatment.

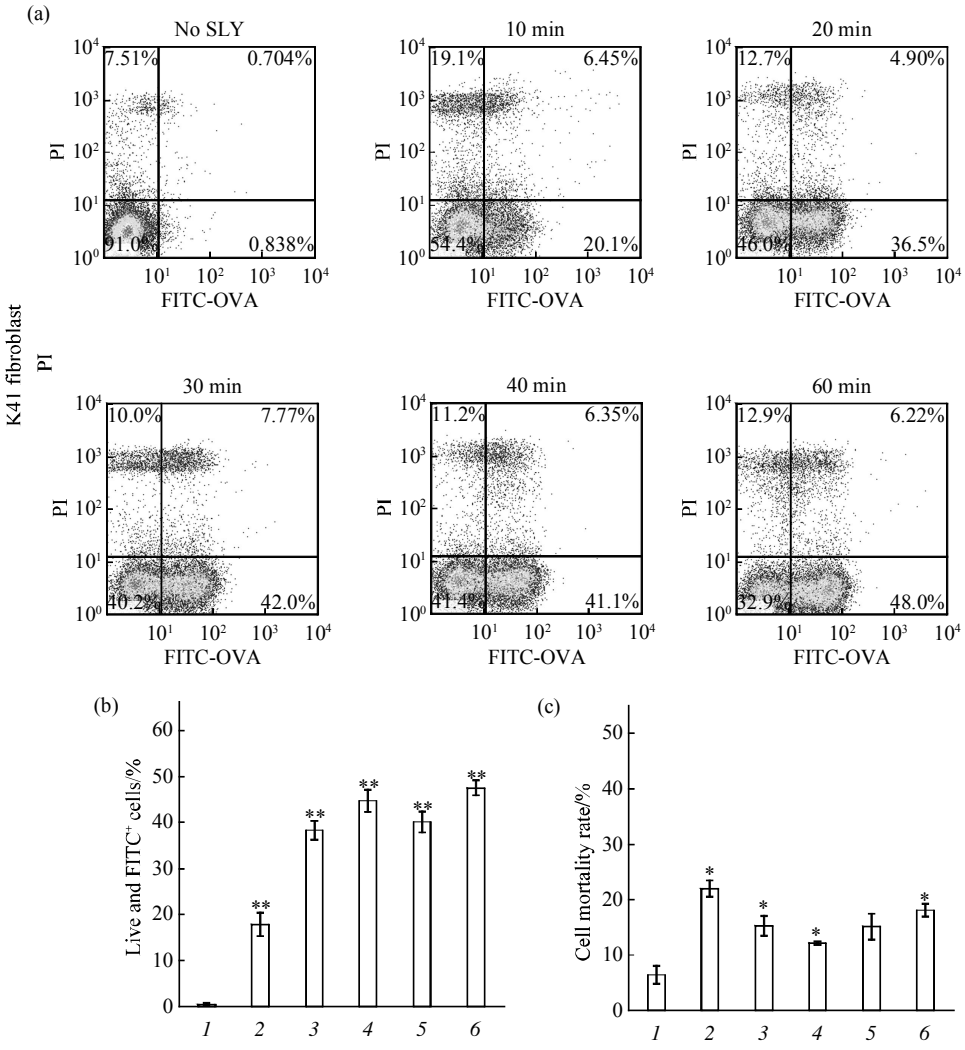

Fig. 2　Flow cytometry analysis of cell permeabilization treatments of different durations

(a)K41 mouse fibroblasts were treated with identical concentrations of suilysin (20 U/0.1 million cells) for different durations. Cells were co-incubated with 100 mg/L fluorescein (FITC)-tagged oval albumin (OVA). Cells were stained with propidium iodide (PI) after extensive washing. We performed flow cytometry for the presence of FITC and/or PI-labeled cells. (b. c) Percentage of (b) live cells with FITC-tagged OVA ($FITC^+$ PI^-) and (c) cell mortality (PI^+ cells) of flow cytometry results shown in Figure 2a, $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* the control group (no SLY). Data are representative of five independent experiments. *1*: No SLY; *2*: 10 min; *3*: 20 min; *4*: 30 min; *5*: 40 min; *6*: 60 min.

2.2　Intact proteins could be delivered into K41 cells by SLY and induced specific MHC-Ⅰ antigen processing

According to previous studies, exogenous proteins that delivered into the cytosol will enter the classical MHC-Ⅰ processing and presentation pathway. When exogenous protein is delivered into the cytosol of K41 cells, epitope is processed within the MHC-Ⅰ complex and be presented on the surface of the cells. Antigen processing efficiency can be measured by OVA antigen presentation [22]. If intact OVA delivered through SLY system can enter the MHC-Ⅰ antigen processing pathway, the dominant epitope SIINFEKL will be presented and a MHC class Ⅰ H-$2K^b$/SIINFEKL complex will be formed on the cell surface. Therefore, by co-incubating treated cells with B3Z cells that specifically recognize the K^b/SIINFEKL epitope, the efficiency of the antigen delivery system can be measured by assessing lac Z activity in the B3Z cells.

K41 cells were permeabilized with SLY for 30 min, and incubated with either BSA or OVA. Permeabilized cells were cultured for 4 h and the presentation of K^b-SIINFEKL complexes on the cell surface was detected by flow cytometry. As shown in Figure 3a, using the K^b-SIINFEKL-specific monoclonal

antibody 25-D1.16, a significant amount of K^b-SIINFEKL complex was detected on the surface of K41 cells at a level similar to that of K41-91a-OVA-hβ2m cells. In contrast, the K41 cells not treated with SLY, barely exhibited any formed K^b-SIINFEKL complexes, even though a same 5 g/L concentration of OVA was added. K41 treated with SLY and OVA exhibited significant higher level of Median Fluorescence Intensity (MFI) and percentage of FITC positive cells than K41 cells not treated with SLY, with $P < 0.05$ for MFI as well as percentage of FITC positive cells versus control group (Figure 3c, d). In the B3Z ovalbumin presentation assay, permeabilized K41 cells were co-incubated overnight with B3Z cells and the CPRG-dependent lac Z activity was quantified. As shown in Figure 3b, compared with the K41 cells co-incubated for 30 min with SLY and exogenous BSA, the OVA-exposed cells presented markedly higher level of B3Z lac Z activation, with $P < 0.05$ for all differences between K41-OVA versus K41-BSA under same E ∶ T ratio, indicating that K^b-SIINFEKL complexes were formed on the cell surface. As a result, we demonstrate that intact endogenous protein loaded by SLY could enter the K41 MHC-Ⅰ pathway and be presented by MHC-Ⅰ molecules.

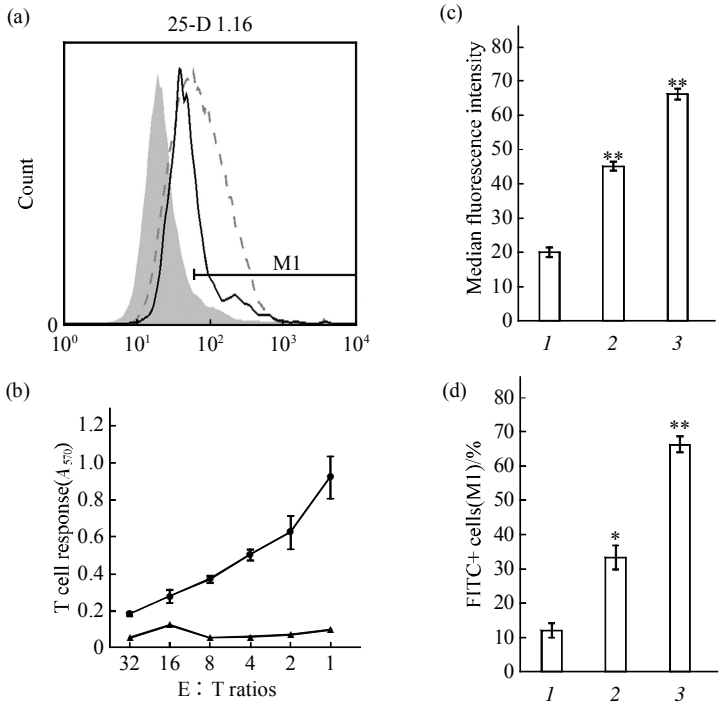

Fig. 3 Delivery of intact proteins into K41 cells by suilysin induces specific antigen processing

(a, c, d) Flow cytometry analysis of permeabilized K41 fibroblasts using 25-D 1.16 mAb. After perforating cells with SLY, the cells were cultured in standard medium at 37℃ for 4 h. Cells were then stained by 25-D1.16 mAb, followed by FITC-conjugated goat anti-mouse IgG Ab. The level of green fluorescence was analyzed by FACS (black line). K41-91a-OVA-hβ2m cell (mentioned above) were used as positive controls (grey dashed line). K41 cells not treated with SLY and stained with Abs were used as negative controls (filled area in each panel). The Median Fluorescence Intensity (MFI) and percentage of FITC positive cells are shown in (c) and (d), $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* the control group (no SLY). (b) SLY-treated K41 cells could induce specific activation of the B3Z T cell hybridoma. K41 cells were perforated using SLY with the presence of OVA/BSA as a negative control. Protein-containing cells were co-cultured with B3Z cells overnight at different effector/target ratios (E ∶ T ratios) to assess the processing of H-2K^b-$OVA_{257-264}$ complex. The B3Z activation level was measured by β-Gal activity using CPRG. Data shown is representative for 3 individual experiments, each one comprised of triplicate samples.●—●: K41-OVA; ▲—▲: K41-BSA. *1*: No SLY; *2*: K41-OVA; *3*: 91a-hβ2m.

2.3 K42 cells displayed lower efficiency of antigen processing than 91a and 90a cells

Once the K41, K42, 90a and 91a cells were permeabilized by suilysin, exogenous OVA enters the MHC-Ⅰ pathway, and the SIINFEKL peptide is presented on the cell surface by MHC molecules, the

antigen processing efficacy can be detected both by flow cytometry using the 25-D1.16 monoclonal antibody and the B3Z endogenous ovalbumin presentation assay. With divergent characteristics, the cell lines K41, K42, 90a and 91a displayed different levels of antigen processing efficiency, which could be interpreted as an indicator of the hierarchical importance of these PLC components (Figure 4a). By using the B3Z assay, we detected that K41, K42, 90a and 91a cells all successfully processed and presented the dominant epitope SIINKEFL, as the B3Z activation level by all of the cell lines was significantly higher than the negative control group (with all cell line group *vs*. negative group under same E∶T ratio, $P < 0.05$) , in which K41 cells were treated with SLY and non-relevant protein BSA. Furthermore, K41 and the PLC component-deficient cell lines displayed divergent B3Z activation efficiency under the same conditions. Compared with K41 cells, K42 cells exhibited a 60% loss of processing efficiency (E/T=1, K41 *vs*. K42, $P < 0.001$), and 91a cells exhibited a 40% (E/T=1, K41 *vs*. 91a, $P < 0.01$) loss. Under E∶T ratio equals to 1, The B3Z activation efficiency of 91a was (0.479±0.028), approximately 1.5 times of that in K42 (0.334±0.030), with $P < 0.01$ for the difference (Figure 4b).The tapasin silenced 90a cells exhibited a processing efficiency that was 75% of the K41 cells or 120% of 91a cells under E∶T ratio equaled to 1, and the difference under E∶T ratio = 2 or 4 insignificant, suggesting that the role of TAP2 in the PLC might not be as important as estimated (E/T =1, K41 *vs*. 90a, Figure 4b). Under all the E∶T ratio condition within the experiment, the K42 cells exhibited a significant lower B3Z activation level compared with 90a and 91a, indicating that the antigen processing and presentation efficiency of K42 is lower than its counterparts in which TAP2 or tapasin is inhibited (Figure 4b ～g). In the negative control group of SLY-permeabilized K41 cells pulsed with non-relevant BSA, no B3Z activation was observed. K41 cells pulsed with 5 g/L OVA but not permeabilized by SLY also did not activate B3Z cells, even under conditions in which E/T ratio equaled to 1 (data not shown).

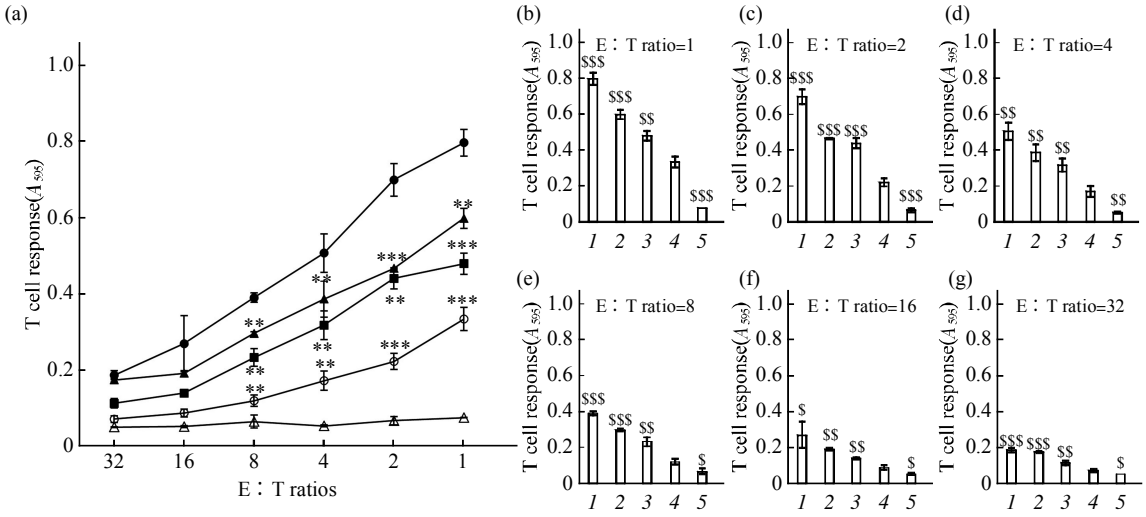

Fig. 4　Different levels of B3Z activation are induced in K41, 91a, 90a and K42 cells

(a) Cells were perforated using SLY in the presence of OVA. Treated cells were co-cultured with B3Z overnight at different effector/target ratios (E∶T ratios). The B3Z activation level was assessed as described above. K41 cells into which BSA was delivered served as a negative control. The means ± standard errors (SE) of triplicate samples are shown. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ *vs* the K41 group under same E∶T ratio. ●—●: K41-OVA; ▲—▲: 90a-OVA; ■—■: 91a-OVA; ○—○: K42-OVA; △—△: K41-BSA. (b～g) Statistic analysis of different levels of B3Z activation shown in Figure 4a. The B3Z activation levels for different cell line under the same E∶T ratio are shown in same graph. (b) E∶T ratio = 1, (c) E∶T ratio = 2, (d) E∶T ratio = 4, (e) E∶T ratio = 8, (f) E∶T ratio = 16, (g) E∶T ratio = 32. $^{\$\$\$}P < 0.001$, $^{\$\$}P < 0.01$, $^{\$}P < 0.05$ *vs* the K42 group under same E∶T ratio. Data shown is representative of 3 individual experiments, each one comprised of triplicate samples. *1*: K41-OVA; *2*: 90a-OVA; *3*: 91a-OVA; *4*: K42-OVA; *5*: K41-BSA.

Western blotting was used to confirm the deficiency of the above-mentioned PLC constituents in 90a and 91a cells. Tapasin expression was considerably depleted in 90a and 91a cells compared

with K42 and K41 cells, with the TAP2 expression significantly suppressed in 91a cells. As shown in Figure 5c, d, the average ratio of TAP2/Calnexin (CNX) expression in 91a cells was 5% of that in K41, indicating that the TAP2 gene has been almost fully depleted. The silencing of tapasin in 91a cell was even more noticeable, with the ratio of tapasin against CNX roughly equivalent to zero. In 90a cells, the ratio of tapasin against CNX was approximately 12% of the ratio in K41, showing that the tapasin expression in 90a was also mostly depleted. In contrast, the levels of CNX in the four cell lines were unaffected (Figure 5c, d). The MHC- Ⅰ molecule surface expression on K42, 90a and 91a cells was as well significantly decreased compared with K41 cells, indicating that either the surface expression or the stabilization of MHC- Ⅰ surface molecules was impaired as a consequence of PLC component deficiency within the cells. However, the differences of MFI among K42, 90a or 91a are insignificant (Figure 5a, b).

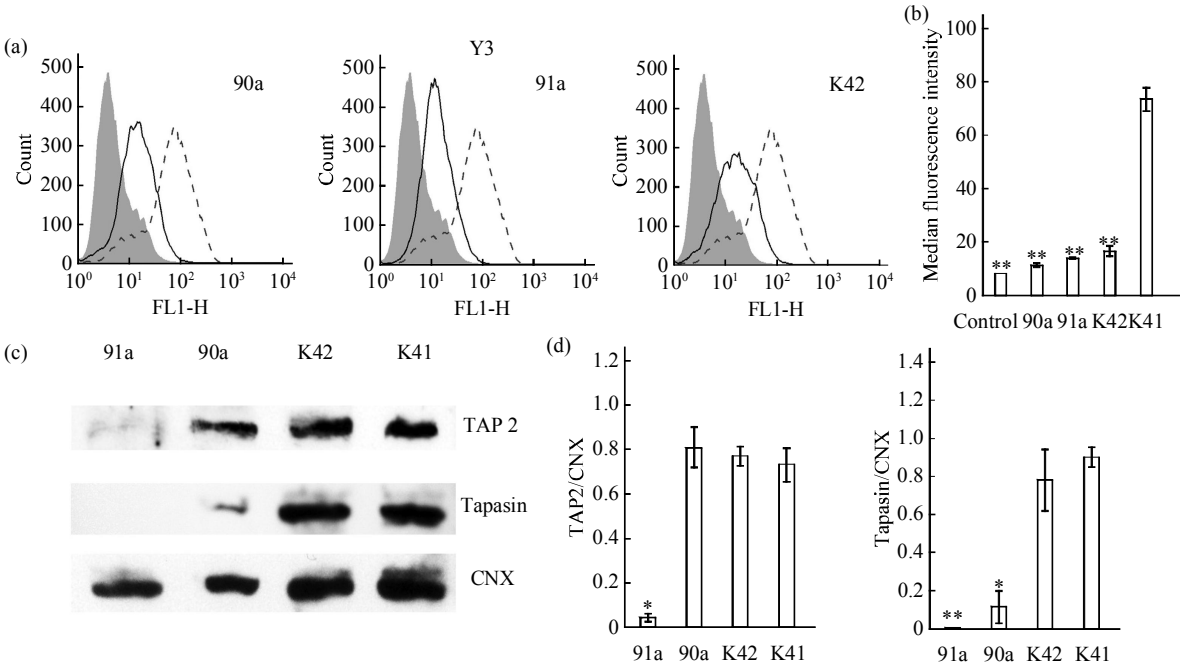

Fig. 5 Flow cytometry and Western blotting confirmation of PLC components deficiency

(a, b) Flow cytometry analysis using mAb Y3, a mAb specific to H-2 Kb. Cells were co-cultured with mAb Y3 for 30 min on ice and then with FITC-conjugated goat anti-mouse IgG Ab. The level of green fluorescence was analyzed by FACS (black line). K41 cells were used as positive control (grey dashed line). K41 cells not treated with Y3 and stained only by FITC-IgG Ab were used as negative control (filled area in each panel). The Median fluorescence intensity (MFI, arbitrary units) of the samples is shown in (b). $^{**}P < 0.01$ *vs* the K41 group. (c, d) Western blotting analysis of TAP2 and tapasin expression in K41, 90a, 91a and K42 cells. TAP2 and tapasin expression was probed by rabbit polyclonal antiserum against the components of tapasin and TAP2, respectively. Calnexin (CNX) expression was used as an internal control and probed by rabbit polyclonal antiserum against human CNX, which cross-reacts with mouse CNX. The results shown in (c) are representative of those obtained in three separate experiments. The blots were scanned and analyzed quantitatively with Software Quantity One 4.6.2 (Bio-RAD). The plots with normalization against CNX were shown in (d), $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* the K41 group.

3 Discussion

The classic MHC class Ⅰ antigen processing and presentation pathway is crucial for immune surveillance mediated by cytotoxic T lymphocytes[3, 23–24]. Through the PLC of the MHC class Ⅰ pathway, peptides generated in the cytosol are edited, loaded, transported to the ER, and bound to MHC class Ⅰ molecules to form stable complexes[23–25]. Our present study is the first time that the importance of these constituents to the MHC-Ⅰ pathway are compared and ranked. First, we established a novel system that could quantitatively deliver intact proteins into the cytosol, and analyzed MHC- Ⅰ antigen processing efficiency. Furthermore, we used this system to study MHC- Ⅰ antigen processing efficiency in K41 cells and several

related PLC constituent-deficient cell lines. It was found that the CRT deficient cell line K42 exhibited a significantly lower efficiency than the TAP2 and tapasin double silencing cell line 91a and the tapasin silencing cell line 90a, suggesting that CRT, compared with tapasin and TAP2, might play a more important role in determining the efficiency of the PLC in K41 fibroblasts. Our novel antigen-delivery system and findings will improve the understanding of the PLC of MHC-Ⅰ and expand the methodology for investigating the MHC class Ⅰ antigen processing and presentation pathway.

TAP2, tapasin and CRT are all crucial constituents of the PLC, although the functions and mechanisms of these proteins vary within the PLC, most likely as a function of the importance of each protein in MHC-Ⅰ processing [2, 4, 25–28]. However, methodological barriers have prevented investigators from ranking these proteins in order of importance to MHC-Ⅰ processing [29]. Cells deficient in TAP2, tapasin or CRT all exhibited reduced MHC class Ⅰ molecule expression on the cell surface and impaired MHC presentation efficacy[6, 8, 12–13, 24, 30]. Our results also showed that similar to 91a and K42 cells, the tapasin-deficient cell line 90a exhibited a lower level of MHC class Ⅰ molecule expression compared with the wild type K41 cells (Figure 5a), which is consistent with previous reports. Nevertheless, by using the H-2K^b specific mAb Y3, we did not find any significant differences among K42, 90a and 91a cells regarding the surface expression of MHC-Ⅰ and MHC-Ⅰ / SIINFEKL complexes (P > 0.05 for differences among K42, 90a and 91a cells regarding MFI). Similarly, by using the 25-D1.16 mAb, we observed no significant differences in processing efficiency among K42, 90a and 91a cell lines. Despite sufficient complex expression in K42, 90a and 91a cells, there was minimal cell surface staining by the 25-D1.16 mAb, indicating that the 25-D1.16 mAb was also not an ideal candidate for detecting differences in processing efficiency among these PLC component-deficient cells.

In this study, we applied the widely used B3Z endogenous ovalbumin presentation assay for the detection of processed and presented OVA SIINFEKL/H-2K^b, which reflects the efficiency of the MHC-Ⅰ pathway[16]. We also applied a novel method, using suilysin to deliver intact antigen into cells, to quantify the level of OVA in the cytosol while maintaining similar OVA concentrations in different cells. There were significant differences in MHC-Ⅰ antigen processing and presentation efficiencies among K42, 90a and 91a cells, showing the largest reduction in efficiency is in the CRT knockout K42 cells. The CRT deficiency cell line K42 showed significantly lower levels of B3Z activation efficiency under E∶T ratios from 1 to 32, even though in 91a cells both the tapasin and TAP2 had been almost completely depleted. As a leucine chaperone, CRT may be expected to be less important than TAP2 or tapasin in the MHC-Ⅰ pathway, resulting in a lesser reduction of antigen presentation efficiency. Previous reports also noted that in the absence of CRT, H2-D^b and H2-K^b could interact with the peptide-loading complex [8]. Quite opposite to our expectations, our results indicated that CRT had a significantly stronger influence on antigen presentation efficiency (Figure 4) than the other two constituents. Our results suggested that CRT, traditionally recognized only as a chaperone in the conventional MHC-Ⅰ pathway, might have undiscovered functions. Conversely the chaperone function of CRT, in conjunction with Calnexin and Erp57, may be more important for antigen processing than first suspected. Such a strong influence may partially result from the increasingly revealed "new" functions of CRT both in and out of the ER [31–32]. Further investigation into these mechanistic differences is needed in the future.

In addition, peptides and OVA should be applied to further investigate the constituents, and the importance of Erp57 within the ranking still requires investigation. Although the peptide loading efficiency within the PLC is optimized in the presence of tapasin, there are some class-Ⅰ allotypes that are reported to be independently presented without tapasin, and others rely on tapasin to varying degrees[6–7, 13, 24, 30]. The TAP heterodimer is integral for the conventional MHC class Ⅰ pathway, but a TAP-independent processing pathway can sufficiently compensate for the loss of the conventional pathway [33]. As such, studies based on peptides for class Ⅰ allotypes other than the admittedly high-affinity peptide OVA SIINFEKL can be of great value in future investigation.

The SLY delivery system adopted in our research has exhibited its suitability for investigating MHC class Ⅰ molecules and their hierarchical importance. The delivery of antigens of interest into the cytosol is widely considered to be crucial and necessary for the

investigation of the MHC class Ⅰ molecules. Microinjection and electroporation of whole proteins, recombinant vaccinia virus transduction, and transfection of cDNA constructs are traditional and common delivery systems, but they all have different flaws and limitations, such as the high cell death rate associated with electroporation [34], the individual cell-based operation, specialized equipment, and sophisticated procedures for microinjection [35–36], the unsuitability of vaccinia virus expression for the OVA-B3Z system [14], and the varied efficiency of transfection. In 2008, a novel method of delivering intact exogenous proteins into cells by SLO was described[14, 37–38]. This system was proved to be efficient for OVA peptides and intact proteins, and therefore can potentially be adopted for research into the MHC class Ⅰ pathway [14]. Compared with virus infection which is the most commonly used method in the investigation of the MHC- Ⅰ pathway [39], the advantages of using SLO to transport proteins into cells are the efficiency, specificity, time-saving and cost efficiency. However, with the relatively high price of commercialized SLO, the cost-efficiency of SLO treatment is still far from ideal. Also the SLO should be activated by DTT in Ca^{2+}/Mg^{2+} for 2 h at 37℃ before perforating the cells[15].

In our study, instead of SLO, we used SLY, a homologous protein to SLO and another member of CDC family[40–41]. SLY is a 54 ku protein secreted by a virulent pig-specific pathogen *Streptococcus suis* [40, 42], the crystal structure of which has recently been solved [43]. Like other family members, SLY has the typical functions of CDC family proteins: to bind to the membrane of target cells, oligomerize, and change conformation to form transmembrane pores [44-45]. Its function, like those of other CDC family members, depends on membrane receptors including cholesterol, thus making its perforating efficiency vary between different cells. In our study, SLY exhibited high efficiency for perforating K41 fibroblast cells(Figure 1) and other cells, including T2 cell and murine spleen cells (Figure S1 in **Supplementary marerial**). The death rate of cells treated by SLY was slightly lower than that of cells treated by SLO, and SLY treatment was exempt from DTT pre-treatment for activation. The stability of SLY was also impressive. During our research, SLY exhibited stable activity for up to 6 months, and a concentration of 1 mol/L K^+, Na^+, Mg^{2+}, Ca^{2+} or Cl^- solution did not affect the activity of SLY. These ion concentrations are higher than that of PBS or RPMI 1640, conceivably proving that ion concentrations in the culture medium have little impact on the efficiency of SLY. In addition, SLY is a soluble protein that can be expressed on a large scale by an *E. coli* expression system with a tremendously high yield. In terms of the economic aspects, such an advantage may be extremely important. Taken together, we conclude that SLY can replace the SLO reagent in studies of the MHC-Ⅰ pathway.

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Supplementary marerial Figure S1 is available at PIBB website(http://www. pibb.ac.cn)

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MHC-Ⅰ类肽组装复合体组分重要性的定量分析*

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摘要 TAP2、tapasin 和 calreticulin (CRT) 是 MHC-Ⅰ类内源性抗原加工和递呈途径肽组装复合体(PLC)中的 3 个重要蛋白质．虽然对于肽组装复合体及其单个蛋白组分在抗原加工递呈中的作用，国内外已有大量的研究，但是由于研究手段的缺乏，其在复合体中的相对重要性还未得到清楚和定量的分析．本文中我们建立了一种新型的抗原递送方法，利用猪链球菌溶血酶(SLY)对鼠纤维母细胞系 K41 及其 CRT 缺陷细胞系 K42、tapasin 缺陷细胞系 90a 及 tapasin、TAP2 双缺陷细胞系 91a 进行穿孔并定量递送入鸡卵清白蛋白(ovalbumin，OVA)，使其被内源性加工处理后的多肽 SIINFEKL 与小鼠 H2-K^b 装配成复合物并被递呈至细胞表面．通过利用小鼠 T 杂交瘤细胞 B3Z 和单克隆抗体 25D1.16 识别并检测不同细胞表面 SIINFEKL 与小鼠 H2-K^b 复合物，我们发现 CRT缺陷细胞系 K42 的抗原提呈能力相比 91a 及 90a，较 K41 有着更大幅度的下降，说明 CRT 在提呈过程中可能有着较预期更重要的作用．本文是首个对抗原递呈途径蛋白相对重要性的研究，其研究结果对于进一步研究 MHC-Ⅰ类抗原加工和递呈途径相关蛋白的作用提供了理论依据与研究手段．

关键词 MHC-Ⅰ，肽组装复合体，猪链球菌溶血酶，定量分析
学科分类号 Q2，Q7 **DOI**: 10.3724/SP.J.1206.2014.00060

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Supplementary material

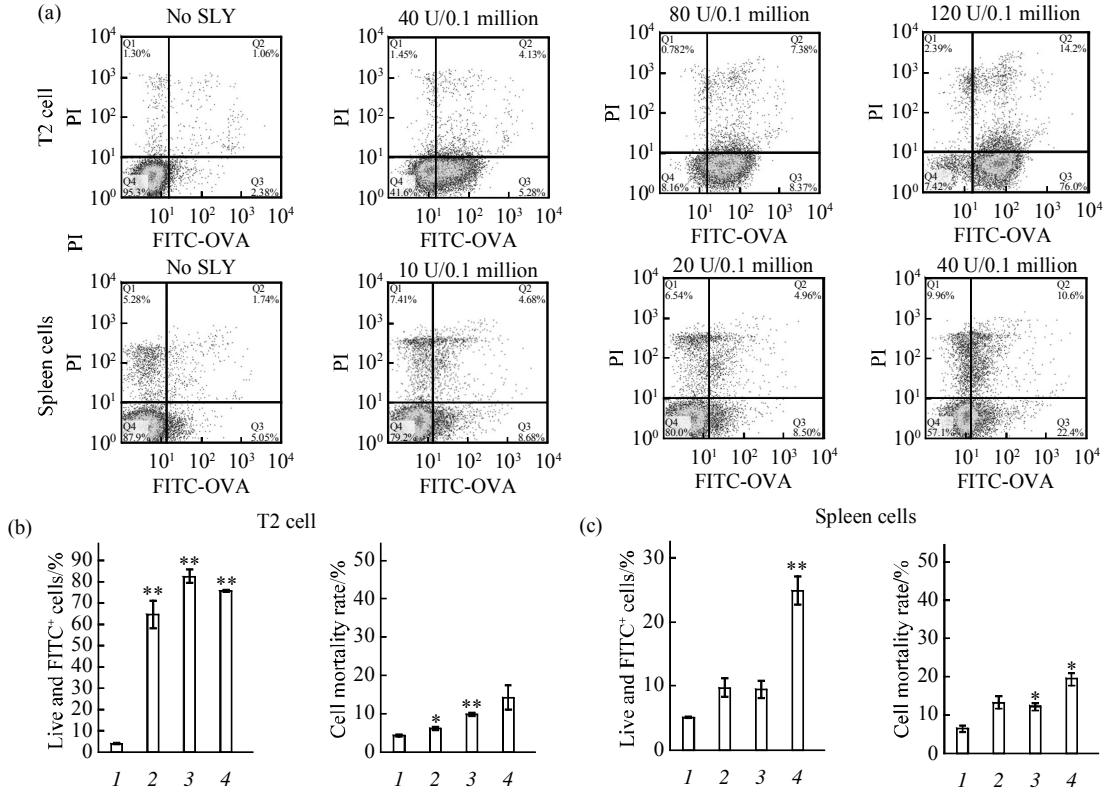

Fig. S1　Flow cytometry analysis of cell permeabilization in different cell lines

(a) Human T2 cells and murine spleen cells were treated with different concentrations of suilysin under the same other conditions (30 min, 37℃). Cells were co-incubated with 100 g/L fluorescein isothiocyanate (FITC)-tagged ovalbumin (OVA). Cells were stained with PI after extensive washing. We performed flow cytometry for the presence of FITC and/or PI-labeled cells. (b, c) percentage of live cells with FITC-tagged OVA ($FITC^+$ PI^-) and cell mortality (PI^+ cells) of flow cytometry results shown in Figure S1a, S1b is for T2 cell, S1c is for murine spleen cells. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* the control group(no sly). Data are representative of five independent experiments. (b)*1*: No SLY; *2*: 40U; *3*: 80U; *4*: 120U. (c)*1*: No SLY; *2*: 10U; *3*: 20U; *4*: 40U.