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In Vitro Studies of MHC Class I Peptide Loading and Exchange

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Summary

In the endoplasmic reticulum (ER), MHC class I molecules associate with several specialized proteins, forming a large macromolecular complex referred to as the “peptide-loading complex” (PLC). In the PLC, antigenic peptides undergo a stringent selection process that determines which antigen becomes part of the repertoire presented by MHC class I molecules. This ensures that the immune system elicits robust CD8+ T-cell responses to viruses and solid tumors. The ability to reconstitute in vitro MHC class I molecules in association with key proteins of the PLC provides a mean for studying at the molecular level how antigenic peptides are selected for presentation to CD8+ T-cells. Here, we describe practical procedures for generating a cell-free system made up of MHC class I molecules and tapasin that can be used for mechanistic studies of peptide loading and exchange.

Keywords

MHC class I molecule; peptide association; peptide dissociation; tapasin; peptide-loading complex; fluorescence anisotropy; peptide exchange

1. Introduction

Proteins expressed at high levels in *Escherichia coli* are often found in an insoluble form called inclusion body (IB) (1). IBs require high concentrations of denaturants to be solubilized. Proteins expressed as IBs also require precise conditions under which the solubilized IBs can be refolded to a native three-dimensional structure. The complexity of this process is even greater for proteins that are made up of more than one subunit such as

4. Freezing/thawing ensures that cells are completely lysed. Care should be taken not to let the IB mixture warm up in this process.

20. A fluorescein-derivatized lysine residue can be introduced into the peptide amino acid sequence at any positions where the side chain points up (into the solvent), as informed by x-ray crystallography. This strategy avoids steric interferences during reconstitution of peptide-filled MHC class I F0 molecules (see Subheading 3.2) (3).

21. For experiments carried out in the absence of tapasinJun, fit the observed averaged intrinsic anisotropy data to an exponential decay function of the form: $y(t)_{int} = y(t)_{pn} + \sum_i A_i \exp(-k_i t)$, where $y(t)_{pn}$ is the baseline offset, the summation is over the number of phases i , A_i and k_i ($k_i = 0.693/t_i$) are the amplitude and rate constant for phase i , respectively, and t is time (3).

22. For experiments carried out in the presence of tapasinJun, fit the averaged observed anisotropy data to an exponential decay function described by the sum of tapasinJun-dependent (tpn) and intrinsic dissociation data: $y(t)_{tpn} = y(t)_{pn} + f_{tpn} \cdot \sum_i A_i \exp(-k_i t) + ((1 - f_{tpn}) \cdot (y(t)_{int} - y(t)_{pn}))$, where f_{tpn} represents the fraction of dissociation reaction under the action of tapasinJun, $y(t)_{int}$ is the intrinsic decay function of the MHC class I molecule as computed in Note 21, and other parameters are as described in Note 21. The dissociation rate enhancement factor is defined as $t(\text{intrinsic; slow phase})/t(\text{tapasinJun})$ (3).

23. For experiments carried out in the absence of tapasinJun, the averaged observed intrinsic anisotropy data were fitted to an exponential function of the form: $y(t)_{int} = y(t)_{pn} + \sum_i A_i (1 - \exp(-k_i t))$, where parameters are as defined in Note 21 and Note 22 (3).

MHC class I molecules. Here we describe experimental procedures for reconstituting soluble MHC class I molecules from the refolding of IBs of heavy chain, β_2m and an antigenic peptide (2). Importantly, we discuss a strategy to stabilize interaction between the weakly associated MHC class I/tapasin complex (3) based on leucine zippers (3–7). The attachment of leucine zippers to the C-terminal ends of class I heavy chain and tapasin has greatly facilitated studies of peptide association and dissociation from MHC class I molecules in the context of tapasin (3). A similar strategy was used more recently to characterize the tapasin-related protein TAPBPR, a tapasin homolog (8). We also describe a fluorescence anisotropy assay to determine equilibrium and kinetics parameters between peptide ligands and MHC class IFos molecules in the presence and absence of tapasinJun. Overall, this chapter describes the main techniques and strategies for generating a soluble and functional MHC class IFos/tapasinJun complex that can be used as a cell-free system for biochemical and mechanistic studies of peptide loading and exchange.

2. Materials

2.1 Consumables and Lab Equipment

1. Glass homogenizer.
2. Stainless steel beaker.
3. Water bath.
4. Plastic pail.
5. 6,000–8,000 MWCO membrane dialysis bags.
6. Stirred cell.
7. Centriprep-10.
8. Gel filtration columns; 10 kDa spin columns; and Ni-NTA affinity columns.
9. Gel electrophoresis materials.
10. 1-cm fluorescence cuvettes and a stopped-flow spectrofluorometer.

2.2 Buffers

1. Resuspension buffer: 50 mM Tris, pH 8.0, 25% sucrose, 1 mM EDTA, 0.1% sodium azide, 10 mM DTT.
2. Lysis buffer: 50 mM Tris, pH 8.0, 1% triton-X100, 1% sodium deoxycholate, 100 mM NaCl, 0.1% sodium azide, 10 mM DTT.
3. Purification buffer 1: 50 mM Tris, pH 8.0, 0.5% triton-X100, 100 mM NaCl, 1 mM EDTA, 0.1% sodium azide, 1 mM DTT and
4. Purification buffer 2: 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% sodium azide, 1 mM DTT.
5. Solubilizing buffer: 25 mM MES, pH 6.0, 8 M urea, 10 mM EDTA, 0.1 mM DTT.

6. Oxidative refolding buffer: 50 mM Tris, pH 8.0, 400 mM L-arginine, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride.
7. FPLC running buffer: 20 mM Tris, pH 7.5, 150 mM NaCl.
8. Denaturation buffer: 20 mM Tris, pH 7.5, 6 M guanidinium hydrochloride, 150 mM NaCl.
9. Dialysis buffer 1: 20 mM Tris, pH 7.5, 8 M urea, 150 mM NaCl.
10. Dialysis buffer 2: 20 mM Tris, pH 7.5, 150 mM NaCl.
11. Dialysis buffer 3: 10 mM Tris, pH 8.0, 200 mM NaCl.
12. Dialysis buffer 4: 10 mM Tris, pH 8.0, 20 mM imidazole, 200 mM NaCl.
13. Equilibration buffer: 10 mM Tris, pH 8.0, 20 mM imidazole, 200 mM NaCl.
14. Washing buffer: 10 mM Tris, pH 8.0, 25 mM imidazole, 500 mM NaCl.
15. Elution buffer: 10 mM Tris, pH 8.0, 110 mM imidazole, 500 mM NaCl.
16. Incubation buffer: 20 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol.
17. Native PAGE gel: loading buffer, 50 mM Tris, pH 6.8, 0.1% bromophenol blue, 10% glycerol.
18. Running buffer: 25 mM Tris, pH 8.3, 200 mM glycine.
19. Dissociation and association buffer: 20 mM Hepes, pH 7.5, 150 mM NaCl.

2.3 Common Reagents

1. DNase I: stock solution at 2 mg/mL.
2. Liquid nitrogen.
3. Lysozyme: stock solution at 50 mg/mL.
4. $MgCl_2$: stock solution at 4 M.
5. EDTA: stock solution at 0.5 M.
6. Glycerol.
7. Synthetic peptide: stock solution at 10 mg/mL in water or DMSO.
8. DTT: stock solution at 1 M.
9. Coomassie Blue.

3. Methods

3.1 Preparation of purified inclusion bodies of class I heavy chainFos and β_2m

1. Add resuspension buffer to the bacterial cell pellets of class I heavy chainFos and β_2m (5–10 mL buffer/L cell culture) and transfer the mixture into a glass homogenizer. Resuspend the cell pellets to homogeneity (*see* Note 1).

2. Transfer the resuspended cell pellets into a stainless steel beaker (*see* Note 2) and add lysis buffer (2.5 mL/mL resuspension buffer) followed by lysozyme (to 1 mg/mL, *see* Note 3). Stir the mixture intermittently. The mixture will become gradually viscous as the DNA is released from the cells. After ~25 minutes, add 0.5 mL DNase I (stock solution, 2 mg/mL) followed by MgCl₂ (to 5 mM). The viscosity of the mixture will decrease gradually under the action of DNase I. After ~20 minutes, add EDTA (to 5 mM).
3. Freeze the lysed cells by placing the stainless steel beaker in liquid nitrogen. This is followed up by a thawing step in which the beaker is transferred into a warm water bath, swirl the mixture gently as it thaws (*see* Note 4). When the mixture is thoroughly thawed, add MgCl₂ (to 10 mM) to reactivate the DNase. Stir the mixture gently. After ~15 minutes, add EDTA again (to 10 mM).
4. Centrifuge the mixture at 8,000 rpm for 20 minutes at 4°C. Discard the supernatant and transfer the crude IBs into a glass homogenizer.
5. Resuspend the IBs in ~30 mL of purification buffer 1 and homogenize thoroughly (*see* Note 1). Centrifuge the mixture at 8,000 rpm (JA-14 rotor) for 20 minutes at 4°C. Discard the supernatant and repeat the washing step two more times. Resuspend the IBs in ~30 mL of purification buffer 2 and repeat the washing and centrifugation steps three times.
6. Dissolve the purified IBs in solubilization buffer (~2 mL buffer/L cell culture). Centrifuge in a tabletop centrifuge for ~10 minutes at 4°C (*see* Note 5). Aliquot the solubilized IBs (*see* Note 6) and store at –80°C.

3.2 Reconstitution of peptide-filled MHC class IFos molecules

1. Prepare 1 L of oxidative refolding buffer and cool it down thoroughly in an ice-water bath.
2. Under vigorous stirring, add synthetic peptide (to 10 μM) (*see* Note 7) followed by solubilized IBs of β₂m (to 2 μM) and class I heavy chainFos (to 1 μM) (*see* Note 8). Incubate the refolding buffer at 10°C for 48 hours.
3. Dialyze the refolding mixture in a plastic pail filled with FPLC running buffer using a 6000–8000 MWCO membrane at 4°C for 12 hours.
4. Concentrate the refolding mixture to ~1–2 mL using both a stirred cell and centrprep-10. Purify the crude MHC class IFos molecules on a gel filtration

¹The use of a motor-driven Teflon pestle allows to resuspend and wash thoroughly the cell pellets.

²A stainless steel beaker is used because of the subsequent freezing/thawing cycles (*see* Note 4).

³Lysozyme is added to the buffer as a clear solution, rather than in a powder form, to prevent the formation of lysozyme clumps.

⁴Freezing/thawing ensures that cells are completely lysed. Care should be taken not to let the IB mixture warm up in this process.

⁵Avoid freezing the urea solution by using short centrifugation times.

⁶The concentration of protein IBs can be determined by the Bradford protein assay.

⁷Synthetic peptides do not have to be purified for this experiment. Many peptides restricted to MHC class I molecules are hydrophobic and DMSO can be used for dissolving these peptides. The final concentration of DMSO in the refolding buffer should be less than ~5%. *See* Note 20 on fluorescein-labelled peptides.

⁸The solubilized IBs should be added under vigorous stirring to ensure that they are rapidly and thoroughly dispersed into the buffer; this is important to promote the correct refolding of class I heavy chainFos and β₂m subunits. Use a stock solution of class I heavy chainFos IBs at less than 0.3 mM for the addition step.

column in the FPLC running buffer. Collect the fractions corresponding to MHC class IFos molecules (*see* Note 9). Concentrate the combined fractions (*see* Note 10) and store the purified protein at -80°C .

3.3 Preparation of peptide-deficient MHC class IFos molecules

Peptide-deficient MHC class IFos molecules are prepared from the denaturation of peptide-filled MHC class IFos molecules after removal of the bound peptide (10).

1. Incubate peptide-filled MHC class IFos molecules in the denaturation buffer for 4 hours at room temperature (*see* Note 11).
2. Transfer the mixture to a 10 KDa spin column and wash extensively with the denaturation buffer to dilute away the peptide. Collect the retentate containing the denatured class I heavy chainFos and $\beta_2\text{m}$ subunits and dilute it with the denaturation buffer to 0.1 mg/mL protein (based on the starting concentration of peptide-filled MHC class IFos molecules).
3. Dialyze the mixture in 500 mL of dialysis buffer 1 using a 6000–8000 MWCO membrane at 15°C for 12 hours. Continue dialysis for an additional 12 hours with fresh 500 mL of dialysis buffer 1.
4. Add 30% molar excess of folded $\beta_2\text{m}$ (relative to the starting concentration of peptide-filled MHC class IFos molecules) (*see* Note 12) to the dialysis bag. Dialyze the solution in 1 L of dialysis buffer 2 at 4°C for 24 hours. Continue dialysis for an additional 18 hours with fresh 1 L of dialysis buffer 2.
5. Add glycerol (to 15%) to the dialyzed solution (*see* Note 13), mix well, and concentrate to $\sim 1\text{ mL}$.
6. Purify the crude peptide-deficient MHC class IFos molecules on a gel filtration column in the FPLC running buffer. Collect the fractions of peptide-deficient MHC class IFos molecules and add glycerol (to 15%) immediately to each collected fraction. Concentrate the combined fractions (*see* Note 14) and store the purified protein at -80°C . Determine the activity of peptide-deficient MHC class IFos molecules (*see* Note 15).

3.4 Expression and purification of soluble tapasinJun

TapasinJun is expressed in High Five cells using a recombinant baculovirus system (3,11).

1. Dialyze the concentrated supernatant of cell culture containing crude tapasinJun in Buffer 1 at 4°C , include two changes of dialysis buffer 3 over 48 hours.

⁹.MHC class IFos molecules elute from a calibrated gel filtration column at $\sim 50\text{ KDa}$.

¹⁰.Stock solutions of MHC class IFos molecules should be less than 10 mg/mL to minimize dimer formation through Fos/Fos association. The protein concentration can be determined by the Edelhoch's method (9).

¹¹.The concentration of MHC class IFos molecules in the denaturation buffer should be $\sim 10\text{--}20\text{ mg/mL}$.

¹².A 30% molar excess of folded $\beta_2\text{m}$ is added to promote the correct pairing of class I heavy chainFos with $\beta_2\text{m}$.

¹³.Glycerol helps to stabilize the intrinsically unstable groove of peptide-deficient MHC class IFos molecules.

¹⁴.Stock solutions of peptide-deficient MHC class IFos molecules should be less than 10 mg/mL. The protein concentration can be determined by the Edelhoch's method (9).

¹⁵.It is recommended that the activity of peptide-deficient MHC class IFos molecules be determined quantitatively as described in Ref. 3 to assess the number of peptide-deficient molecules in solution capable of effectively binding peptides.

Change the dialysis buffer to dialysis buffer 4 and continue dialysis for an additional 24 hours.

2. At 4°C, apply the dialyzed mixture onto a Ni-NTA affinity column that was previously equilibrated with the equilibration buffer. Wash the column with the washing buffer to remove non-specifically bound proteins. Then, elute tapasinJun in the elution buffer. Add DTT (to 1 mM) to the eluate and concentrate it using both a stirred cell and centrprep-10.
3. Purify the eluate containing crude tapasinJun on a gel filtration column in the FPLC running buffer (*see* Note 16). Collect the fractions corresponding to tapasinJun and add DTT (to 1 mM) and glycerol (to 10%) immediately to each collected fraction. Concentrate the combined fractions (*see* Note 17) and store the purified protein at –80°C.

3.5 Formation of MHC class IFos/tapasinJun complexes; a cell-free system

The Fos/Jun leucine zippers help tethering MHC class I molecules to tapasin generating a biologically active cell-free system. This cell-free system has been very helpful for mechanistic studies of how tapasin acts as a chaperone protein towards peptide-deficient MHC class I molecules and a catalyst of peptide dissociation (Subheading 3.6) and association (Subheading 3.7) (3) (*see* Note 18). The ability of tapasin to influence the peptide repertoire is rooted in these two critical functions.

1. Incubate peptide-filled or peptide-deficient MHC class IFos molecules (10 µg) and tapasinJun (10 µg) (1:1 molar ratio) on ice in the incubation buffer for 30 minutes.
2. Add native gel loading buffer (*see* Note 19) and run the samples on 8% native PAGE gel at 4°C (Fig. 1). Stain the gel with Coomassie Blue.

3.6 Studies of peptide dissociation from peptide-filled MHC class IFos molecules

Fluorescein-labelled peptides are used to monitor the dissociation of ligands from MHC class IFos molecules (3). The fluorescence anisotropy-based assay described here (and in Subheading 3.7) shows good reproducibility and requires small amounts of MHC class IFos molecules. Similar methodologies have been described for determining equilibrium and kinetics parameters for MHC class I/peptide interaction (13–15).

1. To a 1-cm fluorescence cuvette, add under stirring MHC class IFos molecules (40 nM), loaded with a tagged peptide (*see* Note 20), in the dissociation buffer containing nontagged peptide (40 µM) (to block the rebinding of dissociated

¹⁶. This additional purification step removes any remaining protein impurities and aggregates of tapasinJun.

¹⁷. Stock solutions of tapasinJun should be less than 10 mg/mL to minimize dimer formation through Jun/Jun association. The protein concentration can be determined by the Edelhoch's method (9).

¹⁸. A cell-free system in which peptide-deficient MHC class I molecules, tapasin, calreticulin, and ERp57 are incorporated together has been described in the literature for its effect on influencing peptide selection (12).

¹⁹. The total volume of each sample should not exceed 20 µL for loading into the native gel wells.

²⁰. A fluorescein-derivatized lysine residue can be introduced into the peptide amino acid sequence at any positions where the side chain points up (into the solvent), as informed by x-ray crystallography. This strategy avoids steric interferences during reconstitution of peptide-filled MHC class IFos molecules (*see* Subheading 3.2) (3).

tagged peptide) and β_2m (4 μM) (to avoid complications due to dissociation of β_2m from MHC class IFos molecules) in the absence or presence of tapasinJun (400 nM).

2. Monitor peptide dissociation kinetics, at 20°C, by fluorescence anisotropy using a spectrofluorometer. Set the polarization wavelength at 524 nm and excitation wavelength at 495 nm. Repeat the dissociation experiment 3 to 5 times using different batches of peptide-filled MHC class IFos molecules and tapasinJun.
3. See Note 21 and Note 22 for processing fluorescence anisotropy data.

3.7 Studies of peptide association onto peptide-deficient MHC class IFos molecules

1. Using a stopped-flow spectrofluorometer, mix automatically equal volumes (125 μL) from chamber A containing peptide-deficient MHC class IFos molecules (active concentration of 800 nM) and β_2m (80 μM) and chamber B containing a tagged peptide (160 nM) with or without tapasinJun (1,200 nM).
2. Monitor peptide association kinetics, at 20°C, by fluorescence anisotropy. Set the polarization wavelength at 510 nm and excitation wavelength at 495 nm. Average together the fluorescence polarization signals of ~3 independent experiments.
3. See Note 23 and Note 24 for processing fluorescence anisotropy data.

3.8 Studies of tapasin-mediated peptide exchange

The availability of peptide-filled and peptide-deficient MHC class IFos molecules and tapasinJun, and the use of fluorescence anisotropy-based assays for monitoring kinetics of peptide dissociation and association using a MHC IFos/tapasinJun cell-free system provides a valuable mean for studying tapasin-mediated peptide exchange. Tapasin-mediated effects on peptide exchange critically shape the repertoire of peptides presented by MHC class I molecules. An example of how to set-up a tapasin-mediated peptide exchange experiment is provided here (see Note 25). Briefly, in these experiments, a tapasin-sensitive peptide

²¹For experiments carried out in the absence of tapasinJun, fit the observed averaged intrinsic anisotropy data to an exponential decay function of the form: $y(t)_{int} = y_{int} + \sum_i A_i \exp(-k_i t)$, where y_{int} is the baseline offset, the summation is over the number of phases i , A_i and k_i ($k_i = 0.693/t_i$) are the amplitude and rate constant for phase i , respectively, and t is time (3).

²²For experiments carried out in the presence of tapasinJun, fit the averaged observed anisotropy data to an exponential decay function described by the sum of tapasinJun-dependent (tpn) and intrinsic dissociation data: $y(t)_{tpn} = y_{tpn} + f_{tpn} \sum_i A_i \exp(-k_i t) + (1 - f_{tpn}) \cdot (y(t)_{int} - y_{int})$, where f_{tpn} represents the fraction of dissociation reaction under the action of tapasinJun, $y(t)_{int}$ is the intrinsic decay function of the MHC class I molecule as computed in Note 21, and other parameters are as described in Note 21. The dissociation rate enhancement factor is defined as $t(\text{intrinsic; slow phase})/t(\text{tapasinJun})$ (3).

²³For experiments carried out in the absence of tapasinJun, the averaged observed intrinsic anisotropy data were fitted to an exponential function of the form: $y(t)_{int} = y_{int} + \sum_i A_i (1 - \exp(-k_i t))$, where parameters are as defined in Note 21 and Note 22 (3).

²⁴For experiments carried out in the presence of tapasinJun, the averaged observed anisotropy data were fitted to an exponential function described by the sum of tapasinJun-dependent (tpn) and intrinsic association data: $y(t)_{tpn} = y_{tpn} + f_{tpn} \sum_i A_i (1 - \exp(-k_i t)) + (1 - f_{tpn}) \cdot (y(t)_{int} - y_{int})$, where f_{tpn} represents the fraction of dissociation reaction under the action of tapasinJun, and other parameters are as described in Note 23 (3).

²⁵IC₅₀ values are determined as follows. Incubate peptide-deficient MHC class IFos molecules (100 nM in the absence of tapasinJun and 30 nM in the presence of tapasinJun due to the effect of tapasin on increasing the concentration of active peptide-deficient molecules in solution (3)) with a tapasin-insensitive control fluorescein-tagged peptide (100 nM) and different doses of a tapasin-sensitive competitor nontagged peptide (0–10 μM) together with an excess of β_2m (1 μM) in the absence or presence of tapasinJun (100 nM). After 20 h incubation, record the fluorescence anisotropy value of each sample. Calculate percentages of inhibition from the observed anisotropy signals in the absence, r_0 , and presence, r , of the competitor peptide: % Inhibition = $(r_0 - r)/(r_0 - r_f) \cdot 100$, where r_f , the minimum anisotropy signal corresponding to free control peptide, was measured in a separate experiment. The concentration of competitor peptide required for 50% inhibition of control peptide represents the IC₅₀ value. Changes in IC₅₀ values are used as a measure of tapasin function in “editing” peptides in favor of those that form kinetically stable peptide-filled MHC class I molecules.

competes for binding onto peptide-deficient MHC class I molecules with a tapasin-insensitive control peptide in the presence of tapasin (3). Changes in IC_{50} values, determined as described in Note 25, are used to assess the effects of tapasin on peptide loading.

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Figure 1.

Formation of MHC class IFos/tapasinJun complexes. HLA-B*0801Fos (10 μ g) and tapasinJun (10 μ g) (1:1 molar ratio) were incubated on ice in the incubation buffer for 30 minutes. Samples were analyzed on native gel (8%): lane 1, tapasinJun; lane 2, peptide-filled HLA-B*0801Fos; lane 3, peptide-deficient HLA-B*0801Fos; lane 4, mixture of tapasinJun and peptide-filled HLA-B*0801Fos; lane 5, mixture of tapasinJun and peptide-deficient HLA-B*0801Fos; and lane 6, tapasinJun/peptide-deficient HLA-B*0801Fos complex incubated with an excess of FLRGRAYGL peptide. The gel was run at 4°C in 25 mM Tris, pH 8.3, 200 mM glycine. The gel was stained with Coomassie Blue. The solid arrow indicates the position of MHC class IFos/tapasinJun complexes.