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Title: Stability and Structure of Bat Major Histocompatibility Complex Class I with Heterologous β_2-Microglobulin

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Author Questionnaire

1. Microscopy: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as possible.

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **65**

NOTE: 2 days of filming, Beijing and Shanghai, one on 07/21 and the other on 07/28

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **William J. Liu**: We use bat MHC I as a model to summarize the methodology and to evaluate the feasibility of a hybrid MHC I complexed with heterologous beta-2-microglobulin **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Jianxun Qi**: Previous studies have shown that mammalian beta-2-microglobulin substitution does not significantly affect peptide presentation and that hybrid MHC I can exhibit a similar structure and function to the original molecule **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Kefang Liu**: These techniques can be used to facilitate the functional and structural study of MHC I and for T cell response evaluation during infectious disease and tumor immunotherapy **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. *E. coli* Transformation

2.1. For *E. coli* culture transformation, add 10 nanograms of plasmid containing bat or human MHC (M-H-C) class 1 beta-2-microglobulin hydrogen chain to 100 microliters of *E. coli* suspension **[1-TXT]** and bathe the suspension in ice for 30 minutes **[2]**.

2.1.1. WIDE: Talent adding plasmid to suspension, with plasmid container visible in frame **TEXT: MHC: major histocompatibility complex**

2.1.2. Talent placing bacteria on ice

2.2. At the end of the incubation, heat shock the bacteria for 20 seconds at 42 degrees Celsius **[1]** and return the culture to the ice bath for 2 minutes **[2]**.

2.2.1. Talent placing bacteria at 42 °C

2.2.2. Talent placing bacteria on ice

2.3. Next, add 800 microliters of lysogeny broth to the culture **[1-TXT]** and shake the suspension on a rocking platform for 20 minutes at 37 degrees Celsius and 200 rotations per minute **[2]**.

2.3.1. Talent adding broth to culture **TEXT: See text for all broth/medium/buffer preparation details**

2.3.2. Suspension shaking on shaker

2.4. At the end of the incubation, spread 100 microliters of the bacteria onto an appropriate antibiotic-resistant culture plate **[1]**.

2.4.1. Bacteria being spread onto plate

3. Culture Inoculation

3.1. The next morning, pick a single recently transformed bacterial clone **[1]** and inoculate the clone in 3 milliliters of LB (L-B) with antibiotic medium **[2]**.

3.1.1. WIDE: Talent picking clone from plate

3.1.2. Talent adding clone to broth

3.2. Place the culture on the rocking platform at 37 degrees Celsius for 12-16 hours **[1]**.

3.2.1. Talent placing culture onto rocking platform

3.3. The next morning, transfer 500 microliters of the activated bacterial stock into 50 milliliters of LB with antibiotic medium **[1]** and return the bacteria to the rocking incubator overnight **[2]**.

3.3.1. Talent adding bacterial to broth

3.3.2. Culture shaking on shaker

3.4. Split the remaining activated bacterial stock into 500-microliter aliquots **[1]** and add each aliquot to 500-microliter volumes of 20% glycerol for minus 80-degree Celsius storage **[2]**.

3.4.1. Talent adding stock to container(s)

3.4.2. Talent adding glycerol to tube(s), with glycerol container visible in frame

3.5. To generate large amounts of recombinant protein, the next morning, transfer the bacterial preparation into 2 liters of LB with antibiotic medium at a 1:100 ratio **[1]** and return the culture to the rocking incubator until an absorbance of 0.6 at 600 nanometers is achieved **[2]**.

3.5.1. Talent adding bacteria to LB

3.5.2. Talent adding sample to spectrophotometer

3.6. Then add 1-millimolar IPTG to the culture to induce expression of the transgene **[1-TXT]**.

3.6.1. Talent adding IPTG, with IPTG container visible in frame **TEXT: isopropyl-1-thio-beta-D-galactopyranoside**

4. Bacteria Harvest

4.1. After 4-6 hours of induction, transfer the bacteria culture to bottles for centrifugation **[1-TXT]** and resuspend the bacterial cell pellets in 60 milliliters of PBS per bottle **[2]**.

4.1.1. WIDE: Talent adding bacteria to bottle(s) **TEXT: 20 min, 2000 x g, 4 °C**

4.1.2. Shot of pellet if visible, then PBS being added to bottle, with PBS container visible in frame

4.2. Then liberate the expressed recombinant protein by ultrasonic cell disruptor 99 times for 6 seconds and interval 12 seconds at 300 watts per sonication **[1]**.

4.2.1. Talent liberating protein

5. Inclusion Body Purification

5.1. For inclusion body purification, collect the sonicated bacteria by centrifugation **[1-TXT]** and resuspend the pellets in an appropriate volume of washing buffer for two additional centrifugations **[2-TXT]**.

5.1.1. WIDE: Talent adding tube(s) to centrifuge **TEXT: 30 min, 12,000 x g, RT**

5.1.2. Shot of pellet if visible, with washing buffer container visible in frame **TEXT: 10-20 min, 12,000 x g, 4 °C, x2**

5.2. After the second wash, resuspend the inclusion bodies in resuspension buffer **[1]** and set aside a 20-microliter aliquot of the sample for SDS-PAGE (S-D-S-page) to test the inclusion body purity **[2-TXT]**.

5.2.1. Talent adding resuspension buffer to tube, with resuspension buffer container visible in frame

5.2.2. Talent adding aliquot to tube **TEXT: SDS-PAGE: sodium dodecyl sulfide-polyacrylamide gel electrophoresis**

5.3. Centrifuge the remaining sample **[1]** and weigh the inclusion-body containing pellet **[2]**.

5.3.1. Talent adding tube(s) to centrifuge

5.3.2. Talent weighing pellet

5.4. Add dissolution buffer to the pellet to a final concentration of 30 milligrams of inclusion body/milliliter of buffer **[1]** and use a magnetic stirrer to slowly stir the solution at 4 degrees Celsius until the inclusion bodies are dissolved in the dissolution buffer **[2]**.

5.4.1. Talent adding buffer to tube, with buffer container visible in frame

5.4.2. Solution being stirred

5.5. After discarding the precipitates, store the inclusion bodies at minus 20 or minus 80 degrees Celsius **[1]**.

5.5.1. Talent placing tube(s) into freezer

6. MHC Complex Refolding

6.1. For MHC complex refolding, add 5-millimolar reduced glutathione and 0.5-millimolar oxidized glutathione to 250-300 milliliters of 4-degree Celsius refolding buffer **[1]** and slowly stir the solution on a magnetic stirrer at 4 degrees Celsius for 10-20 minutes **[2]**.

6.1.1. WIDE: Talent adding GSH and/or GSSG to tube, with GSH and GSSG containers visible in frame

6.1.2. Solution being stirred

~~6.2. **[1 TXT]**.~~

~~6.2.1. Talent adding inclusion bodies and/or peptides **TEXT: Prepare peptide folding complex preparation in same manner**~~

7. MHC H Chain and Beta$_2$m Injection and Dilution

7.1. For MHC hydrogen chain and beta-2-microglobulin dilution, load the inclusion body into a 1-milliliter syringe **[1]** and inject the entire 1-milliliter volume of inclusion body

solution into 1 liter of refolding buffer near the stir bar to obtain a fast and efficient dilution **[2-TXT]**.

7.1.1. WIDE: Talent loading solution into syringe

7.1.2. Talent injecting inclusion bodies into refolding buffer *Videographer: Difficult step* **TEXT: Beta2m refolds easily and remains stable in absence of H chain**

7.2. Next, dissolve 5 milligrams/milliliter of peptide in dimethyl sulfoxide **[1]** and quickly inject 200 microliters of peptide into the refolding solution as just demonstrated **[2]**.

7.2.1. Peptide being dissolved, with DMSO container visible in frame

7.2.2. Peptide being injected *Videographer: Difficult step*

7.3. After 10-20 minutes of slow stirring, inject 3 milliliters of H chain inclusion bodies into new 1-liter volume of refolding buffer **[1]** and allow the refolding to proceed at 4 degrees Celsius for 8-10 hours **[2]**.

7.3.1. Talent injecting H chain inclusion bodies, with H chain inclusion bodies container visible in frame *Videographer: Difficult step*

7.3.2. Solution being stirred

8. Refolded Protein Concentration Determination

8.1. To determine the refolded protein concentration, add exchange buffer to a pressurized chamber with a 10-kilodalton multicopper oxidase membrane **[1]** and concentrate the buffer to 30-50-milliliter volume **[2]**.

8.1.1. WIDE: Talent adding buffer to chamber

8.1.2. Talent concentrating buffer

8.2. Transfer the refolding solution to a centrifuge tube **[1]** and remove the precipitates by centrifugation **[2-TXT]**.

8.2.1. Talent adding solution to tube

8.2.2. Talent placing tube(s) into centrifuge **TEXT: 30 min, 2300 x g, 4 °C**

8.3. Carefully transfer the supernatant to the chamber **[1]** and further concentrate the buffer to a final volume of approximately 1 milliliter **[2]**.

8.3.1. Talent transferring supernatant to chamber

8.3.2. Buffer being concentrated

8.4. Remove any final contaminants by centrifugation **[1-TXT]** and transfer the supernatant to a sterile tube **[2]**.

8.4.1. Talent placing tube into centrifuge **TEXT: 10-20 min, 12,000 x g, 4°C**

8.4.2. Talent adding supernatant to tube

8.5. Use a 10/300 GL (G-L) size-exclusion column to purify the proteins **[1]**, collecting the samples at the peak **[2]** and analyzing them using SDS-PAGE **[3]**.

8.5.1. Talent adding supernatant to column

8.5.2. Sample being collected

8.5.3. Talent adding sample to gel

8.6. Collect the MHC complex peak **[1]** and concentrate the protein to a final concentration of 15 milligrams/milliliter **[2]**.

8.6.1. Talent collecting peak

8.6.2. Talent adding sample to chamber

8.7. Then dilute the complex to 7.5 milligram/milliliter concentration **[1-TXT]**.

8.7.1. Talent diluting complex **TEXT: Dilute to 7.5 mg/mL and 15 mg/mL for crystallization**

9. Crystallization, Data Collection, and Processing

9.1. To perform crystallization of the complexed MHC and peptide using the sitting drop vapor diffusion technique, add 0.8 microliters of the sample to a commercial crystal board **[1]** and seal the board **[2]**.

9.1.1. WIDE: Talent adding sample to crystal board

9.2. Equilibrate the sample solution against 100 microliters of reservoir solution at 4 or 18 degrees Celsius **[1]** and use a microscope to regularly observe the crystal growth over the next 6 months **[2-TXT]**.

9.2.1. Talent placing the crystal plate in the environment chamber

9.2.2. Talent at microscope, checking growth **TEXT: *i.e.*, at 3 d, 1 wk, 2 wks, 1 mo, 3 mos, 6 mos**

9.3. For cryogenic protection, at the end of the experiment, transfer the crystals to a storage solution containing 20% glycerol **[1]** before rapidly cooling the samples in a 100-degree-Kelvin gaseous nitrogen stream **[2]**.

9.3.1. Talent transferring the crystals to solution, with glycerol container visible in frame

9.3.2. Talent cooling crystals with nitrogen stream

9.4. Then collect X-ray diffraction data according to standard protocols.

9.4.1. Talent collecting X-ray diffraction data OR LAB MEDIA: To be provided by Authors: Representative x-ray diffraction data

10. Structure Determination and Analyses

10.1. To determine the structure of the crystal complexes, open the high-resolution structural X-ray diffraction data in the Denzo program within the HKL-2000 (H-K-L-two thousand) software package **[1]** and select an initial model in the Protein Data Bank **[2]**.

10.1.1. WIDE: Talent operating software, with monitor visible in frame

10.1.2. SCREEN: To be provided by Authors: Model being selected. NOTE: Didn't see the SC uploaded during postshoot processing, may need to remind the authors if they don't upload.

10.2. To determine the structure of the protein, open the data in the Phaser MR (M-R) program in CCP4 (C-C-P-4) **[1]** and use the refined X-ray model for the initial joint refinement **[2]**.

10.2.1. SCREEN: To be provided by Authors: Phase MR program being opened

10.2.2. SCREEN: To be provided by Authors: Refined X-ray model being used for initial refinement

10.3. Then use the Phenix refine alone and joint modes for the X-ray alone refinement **[1]** and the joint neutron for the X-ray refinement **[2]**.

10.3.1. SCREEN: To be provided by Authors: X-ray alone refinement being performed with Phenix refine lone and joint modes

10.3.2. SCREEN: To be provided by Authors: X-ray refinement being performed with joint neutron

10.4. After each round of refinement, manually check the model against the Fo-Fc (F-oh-F-C) and 2Fo- Fc positive nuclear density maps in Coot **[1]**.

10.4.1. SCREEN: To be provided by Authors: Model being checked against Fo-Fc and 2Fo-Fc maps

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

Authors: you can list 4-6 individual steps from the protocol, not entire sections

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?

7.1.-7.3. MHC can be renatured correctly. It is very important to obtain MHC complex with high refolding efficiency. Therefore, it is crucial to pay attention to select suitable peptides and increase the purity of inclusion bodies.

Results

11. Results: Representative Bat MHC I Beta2-Microglobulin Stability and Structure Analysis

11.1. In these representative experiments **[1]**, the binding capacities of the hendra virus-derived hendra virus-one peptide to bat MHC class one hydrogen chains with homologous bat beta-2-microglobulin **[2]** and heterologous human beta-2-microglobulin light chains were evaluated **[3]**.

11.1.1. LAB MEDIA: Figures 1C and 1D
11.1.2. LAB MEDIA: Figures 1C and 1D *Video Editor: please emphasize P3/beta-2-m peak in Figure 1C*
11.1.3. LAB MEDIA: Figures 1C and 1D *Video Editor: please emphasize P3/beta-2-m peak in Figure 1D*

11.2. In both analyses, crystals with a high resolution were formed through renaturation with the beta-2-microglobulin light chain **[1]**.

11.2.1. LAB MEDIA: Figures 1E and 1F *Video Editor: please add/emphasize black arrows in images*

11.3. In the absence of beta-2-microglobulin **[1]**, these complexes do not form **[2]**.

11.3.1. LAB MEDIA: Figure 1A *Video Editor: please emphasize P3 peak*

11.4. In the MHC class one hydrogen chain-hendra-virus-1-human beta-2-microglobulin structure **[1]**, conserved residues H31 (H-thirty-one), D53, W60, and Y63 of human beta-2-microglobulin, which correspond to bat beta-2-microglobulin **[2]**, make contact with the bottom of the peptide binding groove and conserved Q8, Y10, R12, N24 residues, which correspond to the beta-2-microglobulins bound to the alpha-3 domain **[3]**.

11.4.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize left structure*
11.4.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize H3, D53, W60, and Y63 residues in right squares*
11.4.3. LAB MEDIA: Figure 2A *Video Editor: please emphasize Q8, Y10, R12, N24 residues in right squares*

11.5. In the overall bat **[1]** and human structures **[2]**, the average root-mean-square deviation of residues 1-184 of the hydrogen chains is 0.248 under all of the carbon-

alpha atoms superpositions, indicating that there are no differences between these two complexes **[3]**.

11.5.1. LAB MEDIA: Figure 3A *Video Editor: please emphasize green sections of structure*
11.5.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize grey sections of structure*
11.5.3. LAB MEDIA: Figure 3A

11.6. The structure of the peptide alignment shows that the conformations of hendra virus-1 peptides in these two complexes are quite similar **[1]**.

11.6.1. LAB MEDIA: Figure 3B

11.7. Sequence alignment also shows that the amino acids of beta-2-microglobulin from different species are highly conserved **[1]**.

11.7.1.1. Figure 4 *Video Editor: please emphasize beta2 columns*

Conclusion

12. Conclusion Interview Statements

12.1. **Dan Lu**: It is important to obtain MHC complexes with a high refolding efficiency. Therefore, it is crucial to select suitable beta-2-microglobulins and to use highly purified inclusion bodies **[1]**.

12.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (7.1.-7.3.)

12.2. **Di Zhang**: This protocol can be used to obtain stable MHC I complexes through potential beta-2-microglobulin substitutions in other species and to assess their MHC I structure and function **[1]**.

12.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

12.3. **Penyan Wang**: The data obtained from these studies can be used to assess T cell responses during infectious disease and tumor immunotherapy **[1]**.

12.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*