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**Supplementary Data**

**Structural analysis of conformational changes in the mpox virus A7 protein**

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**Materials and Methods**

**Plasmid construction**

The constructs of MPXV *A7* (Genbank: NC\_063383.1), *A7N121* (residues 1–121), *A7N137* (residues 1–137) and *A7C* (residues 122–372) were amplified by PCR using the plasmid *pET28a-A7* (GENERAL BIOL. Anhui, China) as the template. These PCR products were then ligated into the modified *pET28a* plasmids, containing an N-terminal hexa-histidine tag and a subsequent Tobacco Etch Virus (TEV) protease cleavage site. The corresponding primers used for these constructs are listed in Supplementary Table S1. All recombinant plasmids were verified by sequencing (Tsingke Biotechnology Co., Ltd., Chengdu, China).

**Protein expression and purification**

All plasmids for *in vitro* assays were expressed in the same approach. The sequenced plasmid was transformed into *E. coli* BL21(DE3) (Novagen). Then the cells were incubated overnight at 37°C in 50 mL Luria-Broth (LB) medium supplemented with kanamycin at a final concentration of 50 μg/mL. The cell culture was inoculated into 2 × 1 L LB medium on the next day. When the OD600 value of the culture reached 0.6, overexpression of the target gene was induced for 16 h with 0.5 mmol/L isopropyl-D-thiogalactoside (IPTG) supplementation at 18 °C. The 2 L culture was subsequently harvested by centrifugation for 15 min, 1,914 ×*g* at 4°C. The harvested *E. coli* BL21(DE3) cells were resuspended in 50 mL buffer A (20 mmol/L Tris-HCl, pH 7.5, 10 mmol/L imidazole, 500 mmol/L NaCl) and lysed by ultrasonication on ice. After centrifugation for 30 min at 38,759 ×g at 4°C, the supernatant of the lysed mixture was applied to Ni Sepharose™ Fast Flow beads (GE Healthcare). Next, the His-tagged protein was eluted by buffer B (20 mmol/L Tris-HCl, pH 7.5, 500 mmol/L imidazole, 500 mmol/L NaCl) with a step-gradient method. The His-tag of the target protein was further cleaved by TEV protease (prepared by ourselves). The corresponding protein was subsequently dialyzed against buffer C (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L dithiothreitol (DTT) and 5% Glycerol) overnight at 4 °C. Finally, all target proteins were purified again by size-exclusion chromatography (Superdex 75 Increase 10/300 GL, GE Healthcare) using the buffer C, and the quality of these proteins was assessed by SDS-PAGE.

**Lipid-binding assay**

MPXV A7, A7N121, and A7C (20 μL, approximately 2 mg/mL) were prepared in buffer D (20 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl and 5 mmol/L DTT) and mixed with 1 μL NBD-PE (N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-snglycero-3-phosphoethanolamine, triethylammonium salt) (Macklin, Shanghai, China) at 2 mg/mL in methanol. All the reaction systems were incubated on ice for 2 h, and subsequently analyzed by native PAGE. A Tanon-5200 Imager (Tanon Co., Ltd., Shanghai, China) was used for the visualization of the fluorescence and coomassie-stained protein bands from the gel.

**Crystallization and data collection**

Both purified A7N121 and A7N137 were concentrated to approximately 20 mg/mL in buffer C. Crystallization trials for these two samples were performed at 291 K by employing the sitting-drop vapor-diffusion method with 1 µL of protein plus 1 µL of reservoir. Crystals of A7N121 were observed under the condition of No.24 of the kit PACT *premier*TM (Molecular Dimensions): 0.01 mol/L Zinc chloride, 0.1 mol/L MES pH 6.0, 20% w/v PEG 6000. While crystals of A7N137 were obtained under condition of No.95 of the kit IndexTM (Hampton Research): 0.1 mol/L Potassium thiocyanate, 30% w/v Polyethylene glycol monomethyl ether 2000. All above crystals were shock-cooled in liquid nitrogen. The diffraction dataset of A7N121 (~ 1.7 Å) was collected with X-ray wavelength of 0.97915 Å at Shanghai synchrotron radiation facility (SSRF) beamline BL18U1, Shanghai, China. The diffraction dataset of A7N137 (~ 2.5 Å) was collected at a wavelength of 0.97851 Å at SSRF beamline BL19U1, Shanghai, China. Two data sets were processed by *XDS* (Kabsch, 2010) and scaled with *Aimless* in *CCP4* (Evans & Murshudov, 2013). The space group of A7N121 crystal is *C2* with unit-cell parameters: a = 76.0 Å, b = 31.3 Å, c = 49.8 Å, α = 90°, β = 112.34°, γ = 90°. While the space group of A7N137 is *C2221* with unit-cell parameters: a = 37.9 Å, b = 140.3 Å, c = 153.5 Å, α = β = γ = 90°. The detailed crystallographic statistics are presented in Table 1.

**Phase determination and model refinement**

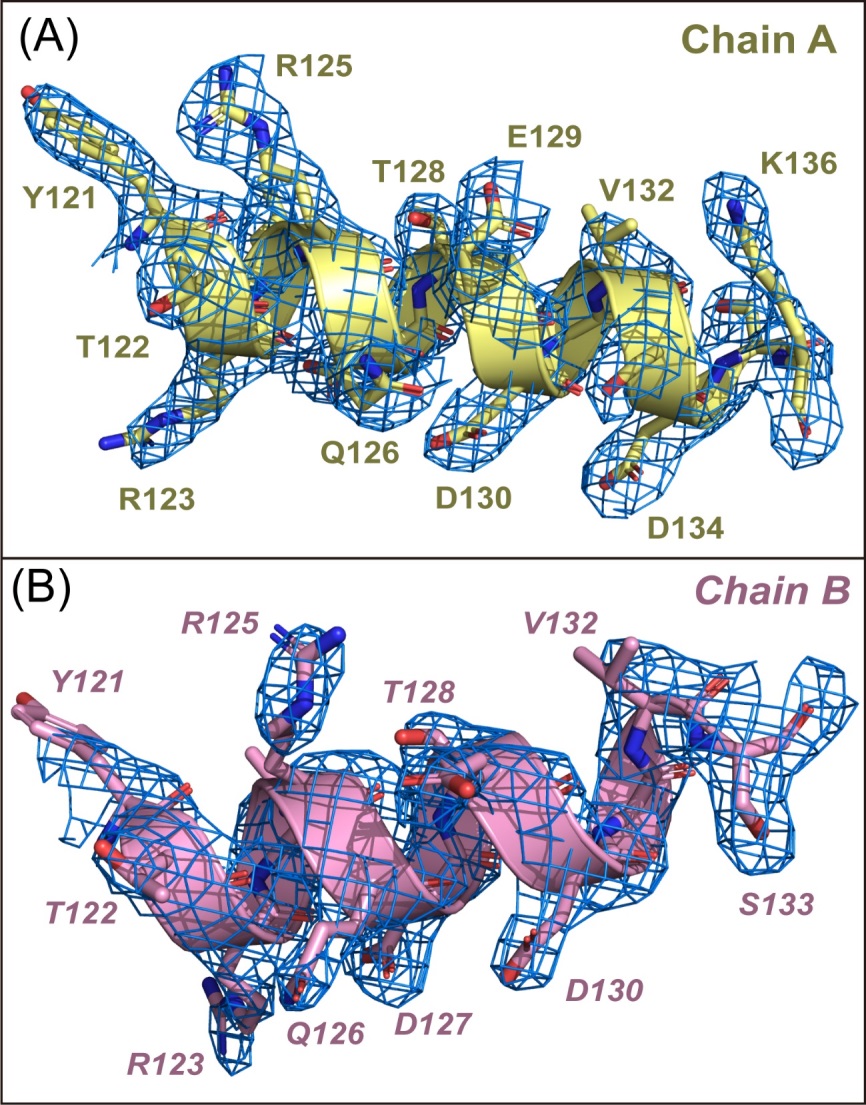
The structures of MPXV A7N121 and A7N137 were determined by molecular replacement (MR) method. The initial phase of MPXV A7N121 was determined by MR with program *MOLREP* (Vagin & Teplyakov, 2009), using VACV A6N (PDB code: 6CB6) as the search model. Next, the initial model of the MPXV A7N121 was rebuild and refined using programs *Coot* (Emsley et al., 2010) and *REFMAC5* (Kovalevskiy et al., 2018). The final *R*factor is 0.180, with *R*free = 0.205. Subsequently, the structure of A7N137 was determined with program *MOLREP* (Vagin & Teplyakov, 2009), using our A7N121 (PDB code: 8IZT) as the search model. The primary A7N137 model was continually rebuilt and refined using programs *Coot* (Emsley et al., 2010) and *PHENIX. refine* (Adams et al., 2010). The *R*factor and *R*free of A7N137 are 0.210 and 0.239, respectively. Please see Table 1 for the final refinement statistics.

**Differential scanning fluorimetry (DSF)**

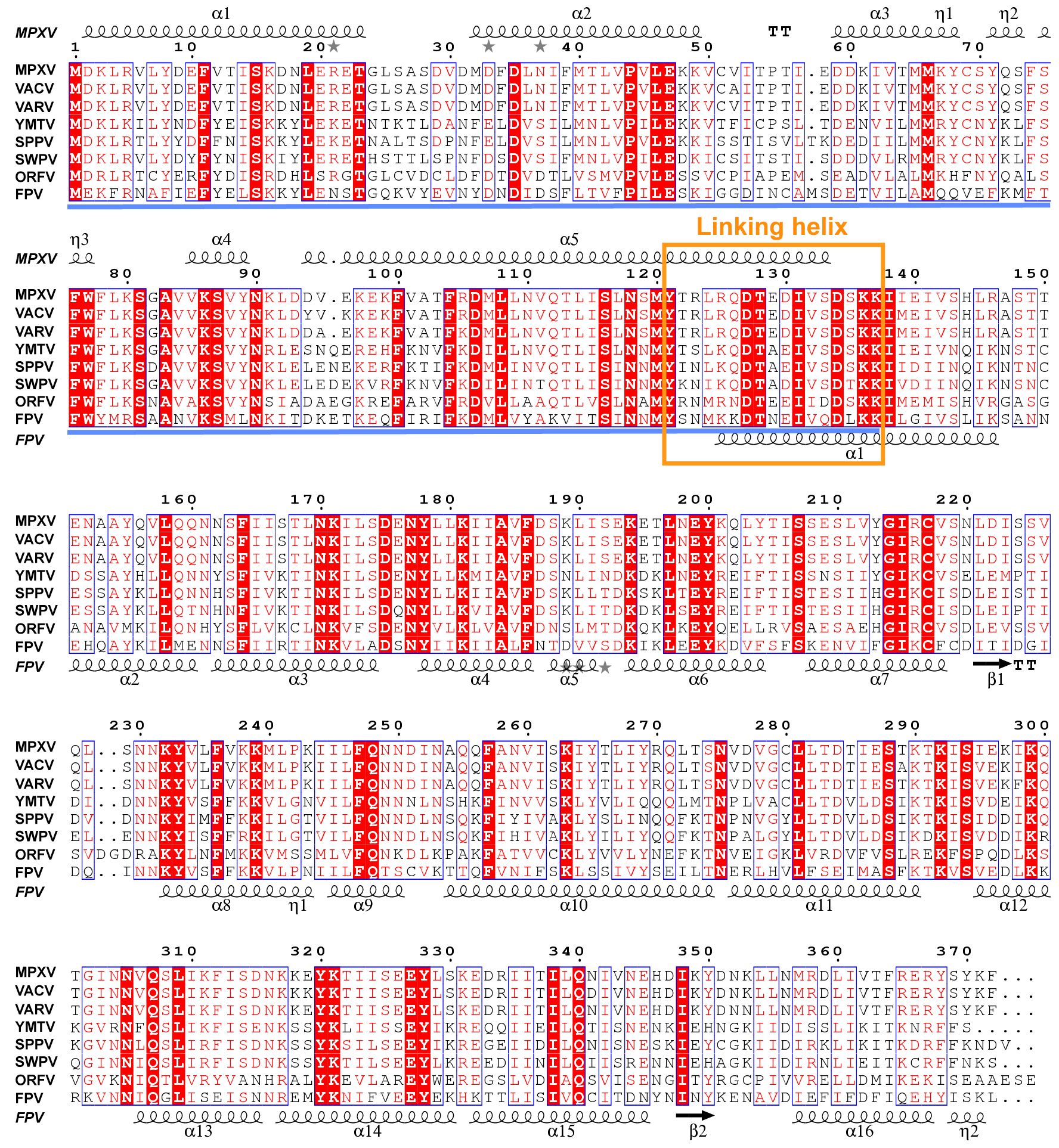
The structural integrity of the protein at increasing temperature was performed using SYPRO Orange (Sigma, USA) as an external fluorophore in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). Each reaction sample contained 1 μL SYPRO Orange fluorophore (diluted 1000-fold) and 9 μL target protein (final concentration at 10 μmol/L) in DSF buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl). Samples were then loaded in 96-well PCR plates and heated from 25 to 95 °C with a heating rate of 2 °C/min. Fluorescence intensity was analyzed using Bio-Rad CFX Maestro 1.1 software.

**Molecular dynamics (MD) simulation**

We performed 50 nanosecond (ns) MD simulation using the chain A of A7N137. The system was conveniently constructed by CHARMM-GUI (Jo et al., 2008), and the model of target protein was constructed and optimized by CHARMM force field (Best et al., 2012; Klauda et al., 2010). The system was solvated with the TIP3 water model and neutralized with potassium and chloride ions. Prior to the production simulation, a steepest descent algorithm was employed to minimize the system for 100 picosecond (ps) to eliminate any unnatural collisions. Subsequently, a 250 ps equilibration process was conducted, gradually releasing the scaling restraint until the force was fully removed. The MD simulation was performed under the isothermal-isobaric (NPT) conditions at a temperature of 303.15 K and a pressure of 1 atmosphere, using a time step of 2 femtosecond (fs) and constraining bonds involving hydrogen atoms with the linear constraint solver (LINCS) algorithm. The particle mesh Ewald (PME) method was used for calculating electrostatic interactions. Finally, the root mean square fluctuation (RMSF) was calculated for each residue of interest. All simulations were performed with the Gromacs software package, version 2018.8 (Abraham et al., 2015) and animated demonstration of molecular dynamics simulations were produced using VMD version 1.93 (Humphrey et al., 1996).



**Fig. S1** The *2Fo*−*Fc* electron density maps of the linking helices in two A7N137 chains. (**A**, **B**) The linking helices of the A7N137 chains A and B are shown in yellow and pink, respectively. Each residue of the linking helix is shown as sticks. The *2Fo*−*Fc* electron density map (σ = 1) of each helix is displayed in blue mesh. Figures were prepared using PyMOL (https://pymol.org).

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**Fig. S2** Structure-based multiple sequence alignment of MPXV A7 and its orthologs from different poxviruses. The corresponding sequence accession numbers are: MPXV, NC\_063383.1; VACV, NC\_006998.1; VARV, NC\_001611.1; YMTV, NC\_005179.1; SPPV, NC\_004002.1; SWPV, NC\_003389.1; ORFV, NC\_005336.1; FPV, NC\_002188.1. The truncation A7N137 is underlined in blue and the linking helix is marked by an orange box. The secondary structures of MPXV A7N137 (top, PDB code: 8IZU, chain A) and FPV170-C (bottom, PDB code: 6BR8, chain A) are indicated. This figure was prepared using the program ESPript (Gouet et al., 1999).

**Movie S1**. Molecular dynamics (MD) simulation to predict the movements of A7N137. A7N137 (chain A) was used as the initial conformation. The linking helix of A7N137 presents a significant up-and-down swing during the first 10 ns of MD simulation (Please see separate video file).

**Table S1.** Primers used for MPXV *A7* and *its truncations*.

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| --- | --- | --- |
| **Gene** | **Forward primer (5**′ **to 3**′**)** | **Reverse primer (5**′ **to 3**′**)** |
| *A7*  (1–372) | CTAGCTAGCATGGACAAACTTAGAGTTCTATACGATG | CCGCTCGAGTTAGAATTTATACGAATATCGTTCTCT |
| *A7N121*  (1–121) | CTAGCTAGCATGGACAAACTTAGAGTTCTATACGATG | CCGCTCGAGTTAATACATAGAGTTAAGAGAAATTAGAGT |
| *A7N137*  (1–137) | CTAGCTAGCATGGACAAACTTAGAGTTCTATACGATG | CCGCTCGAGTTATTTTTTGGAATCGGATACTATATCTTCGG |
| *A7C*  (122–372) | CTAGCTAGCACTAGACTGCGTCAAGATACCGAAG | CCGCTCGAGTTAGAATTTATACGAATATCGTTCTCT |

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