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Title	Structural comparison of the C-terminal domain of functionally divergent lyssavirus P proteins
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Supplementary methods

Construction of Ni-P CTD expression plasmid

The DNA fragment encoding Ni CTD (amino acids 186–297) was amplified from cDNA using 5′-AGGAATTCCATATGTGGTCTGCTACTAATGAGGAGGATG-3′ as a forward primer and 5′-CGGAATTCTTAGCAAGATGCATAGCGATTCAAATCATC-3′ as a reverse primer. The DNA fragment encoding DUVV-P CTD (amino acids 187–298) was amplified from cDNA using 5′-GGGAATTCCATATGTGGACTGCCAGCAATGAAGC -3′ as a forward primer and 5′-CCGGAATTCTTAATAGGTCAGGTATTTGTCAAGAT-3′ as a reverse primer. The resultant fragment was digested with *Nde*I and *Eco*RI, and ligated into the modified expression plasmid pET-28(a) (Merck) in fusion with N-terminal 10 × His-tag. The modified pET-28(a) contained the downstream box sequence (ATGAATCATA) before the start codon. To generate Ni-P CTD K214A, the whole expression vector was amplified with 5′-AGTAC*GC*GTTTCCTTCTCGATCTTCAG-3′ as a forward primer and 5′-AAGGAAACGCGTACTTCTTAGAAAAGCTTTC-3′ as a reverse primer. To generate Ni-P CTD W265G, the whole expression vector was amplified with 5′-ACTGGGAGGGGTCGCTCTGG-3′ as a forward primer and 5′-GCGACCCCTCCCAGTACACATC-3′ as a reverse primer.

Expression and purification

Expression and purification of Ni-P-CTD (wild, K214A and W265G mutants)

Escherichia coli strain C43 (DE3) (Thermo Fisher Scientific) was transformed using each expression vector. A single colony of the transformant was inoculated into 5 mL of LB medium (with 25 μg/ml of Kanamycin) and incubated at 37°C with shaking at 150 rpm. Then, all of the pre-cultures were inoculated into 1 L of LB medium in 2 L of baffled flasks and shaken at 150 rpm at 37°C. I PTG (1 mM final concentration) was added when the culture reached the OD600 = 0.9, followed by incubation at 25°C for 24h (wild-type and K214A), or 16°C for 24h (W265G). The cells were washed twice with buffer A (50 mM Na-phosphate, pH 8.0, 500 mM NaCl, 20 mM Imidazole, 10% glycerol). Finally, the cells were harvested by centrifugation. The cells were resuspended to 0.2 mg/mL (final

concentration) of cell lysis buffer (buffer A, DNase I (Merck), hen-egg lysozyme (Wako)) and disrupted using a sonicator. The cell lysate solution was centrifuged at 10°C and 40,000 × *g* for 30 minutes, and the supernatant was collected. After filtration, Ni-P CTD (or K214 or W265G mutant) was applied to the first Ni-affinity chromatography using His-Trap column 5mL (GE healthcare). The buffer A was used for the binding and the elution was conducted using a linear gradient against the buffer B (50 mM Na-Pi pH 8.0, 10% Glycerol, 1M NaCl, 500 mM Imidazole). After elution, the His-tag of the proteins was removed using His-tag-fused TEV protease during dialysis with dialysate buffer (20 mM Tris-HCl pH7.4, 200 mM NaCl, 10% Glycerol, 1 mM DTT). The proteins were further purified with Ni-Affinity chromatography with buffer B (50 mM Na-Pi pH 8.0, 10% Glycerol, 1M NaCl, 500 mM Imidazole); eluting at 5% B buffer, followed by size-exclusion column chromatography using a HiLoad 16/60 Superdex™ 75 prep grade column equilibrated with buffer C (10 mM HEPES-NaOH pH 7.4, 150 mM NaCl) was used. The solution including Ni-P CTD was concentrated with an Amicon 3000 (Millipore). The purity of the protein was assessed on a 15% SDS-PAGE, showing a single band near 13 kDa.

Expression and purification of DUVVP CTD

Escherichia coli strain BL21 star (DE3) (Thermo Fisher Scientific) was transformed using the expression vector. Bacteria cell culture, expression induction, and cell lysis were conducted as described for Ni-P CTD expression. After the disruption and filtration with buffer A, Ni-NTA agarose (GE Healthcare) was added into the soluble fraction and resuspended for 2h. After washing with buffer A, DUVV-P CTD was eluted from the resin using with 270 mM or 500 mM Imidazole in buffer A. After removal of the His-tag, following the same procedure as Ni-P CTD, the proteins were further purified via Ni-affinity chromatography with buffer D (50 mM Na-Pi pH 8.0, 10% Glycerol, 1M NaCl, 5 mM imidazole) and size-exclusion column chromatography using a HiLoad 16/60 Superdex™ 75 prep grade column equilibrated with buffer E (10 mM HEPES-NaOH pH 7.4, 150 mM NaCl). Then, the solution including DUVV-P CTD was concentrated with

an Amicon 3000 (Millipore). The purity of the protein was assessed on a 15% SDS-PAGE, showing a single band around 12 kDa.

The solubility of Ni-P CTD K214A, Ni-P CTD W265G, and DUVV-P CTD (WT) was observed to be low compared to Ni-P CTD wild type. In the purification procedure of Ni-P CTD K214A, a micro crystal (one length, ~ 5 μm) appeared in the buffer containing 10 mM HEPES-NaOH pH 7.4 and 150 mM NaCl. Precipitation of Ni-P CTD W265G and DUVV-P CTD was also observed after concentration (~ 50 μM) in the same buffer. The instability of these proteins precluded quantification of interaction with STAT1.

Crystallization

Microcrystals of Ni-P CTD K214A appeared during the purification procedure because of the low solubility in a buffer containing 10 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 3mM EDTA, and 0.05% Tween 20.

DUVV-P CTD was concentrated to a final concentration of 0.4 mg/ml in a buffer containing 10 mM HEPES-NaOH (pH 7.4), 150 mM NaCl before crystallization trials. Concentration was performed using a Millipore centrifugal filter device (Amicon Ultra-15, 3 kDa cutoff; Millipore). Screening for crystallization was performed using JCSG I, II, III, IV, JCSG + suite, MPD suite, classics suite, PACT suite, and PEGs and PEGs II suite (Qiagen) by the sitting-drop vapor diffusion method in 96-well plates (SWISSCI MRC 2 Well, Jena Bioscience). A drop of 0.2 μl of the sample was mixed with an equal volume of reservoir solution, and the mixture was equilibrated against 0.3 μl of reservoir solution at 293 K. Crystals were grown from JCSGI #14 (0.1 M HEPES pH7.5, 20% PEG 8000), JCSGII # 18 (0.1 M Tris pH8.0, 20% PEG 6000), JCSG III # 11 (0.1 M Tris pH8.5, 20% PEG 1000), # 33 (10% Glycerol, 0.1 M HEPES pH7.5, 5% PEG 3000, 30% PEG 400), classics suite # 61 (0.2 M Magnesium formate), # 44 (0.1 M HEPES Sodium salt pH7.5, 1.4 M Sodium citrate), # 62 (0.1 m MES pH6.5, 1.6M Magnesium sulfate), PEGs # 44 (0.1 M Tris pH8.5, 25% PEG 4000), # 46 (0.1 M Tris pH8.5, 25% PEG 8000), # 47 (0.1 M Tris pH8.5, 20% PEG 10000), # 48 (0.1 M Tris pH8.5, 15% PEG 20000), # 96 (0.2 M di-Ammonium citrate, 20% PEG 3350) and

PEGs II suite # 16 (0.1 M Tris pH8.5, 30% PEG 1000), # 80 (0.01 M tri-Sodium citrate, 16% PEG 6000).

Circular Dichroism spectrophotometry

J725 CD spectrometer (Jasco) was used for spectrophotometry. The protein samples were adjusted to 15µM in 10 mM HEPES-NaOH, 150 mM NaCl, pH 7.4 at 25°C using a quartz cuvette (0.1 cm path length). For each protein, eight scans were recorded, averaged, and subtracted from the eight averaged buffer scans. Mean residue ellipticity (MRE) (deg.cm^2.dmol^{-1}) was calculated using MRE = θ.MRW/10.l.c, where θ is the ellipticity (millidegrees), l is the pathlength (cm), c is the protein concentration (mg/mL), and MRW is the Mean Residue Weight calculated as MRW = Mr/(N-1), where Mr is the MW of the protein (Da) and N is the number of residues.