Supporting Information

Experimental Procedures

Circular Permutation of Chicken Interleukin (IL)-1β. The gene for wild-type (WT) chicken IL-1β was cloned as described[1] and used as the template to construct the circularly permutated IL-1β mutants.[2, 3] Initially, two copies of the IL-1β gene, tandemly connected by one of three peptide linkers, $(GAS)_5$, $(TAQT)_2$, or $GT(GGS)_8$, were PCR generated. These tandem-linked genes were then used to construct circularly permutated IL-1β mutants with the linker connecting the original N- and C- termini. The placement of circular permutation (CP) sites was predicted by CPred.[4] *B*-factor analysis was also performed to identify potential CP sites. Positions preferred by CP sites lie at the flexible regions with higher *B*-factors. Primers from Mission Biotechnology (Taiwan) for the PCR amplifications were designed with nucleoside sequences appropriate for the new N- and C- termini. The circularly permuted genes were individually cloned into vector pET-28a(+) (Promega, WI), with a His_6 and T7 tag at the N-terminus. The gene sequences were confirmed by DNA sequencing (Mission Biotechnology).

Protein Expression and Purification. Recombinant WT chicken IL-1β was expressed and purified as described.[1] Similar methods, with certain modifications, were used to obtain the circularly permutated IL-1β mutants. *Escherichia coli* BL21(DE3)-CodonPlus cells (Stratagene, Amsterdam, The Netherlands) bearing PET-28a(+) containing a gene for circularly permuted IL-1β mutant were cultured in 500 ml of LB broth, 50 μg/ml ampicillin at 37°C. Isopropyl β-D-1-thiogalactopyranoside (final concentration, 0.4 mM) was added to induce protein expression when the OD_{600} of each culture reached 0.6. After 20 h of incubation, the cells were harvested by centrifugation (8,000 × *g* for 20 min at 4°C). Each cell pellet was suspended in 25 mM Tris-HCl, 500 mM NaCl, pH 7.4 and lysed by sonication. Each lysate was centrifuged at 100,000 × *g* for 30 min at 4°C, and the recombinant His-tagged protein was purified using a 100-ml Co^{2+} affinity column (BD Biosciences, CA). The column was equilibrated with 25 mM Tris-HCl, 500 mM NaCl, pH 7.9. After loading a protein onto the column, it was eluted in the same buffer that also contained 75 mM imidazole. To remove the imidazole and concentrate the protein, a Centricon

Plus 20 centrifugal filter device (Millipore, Billerica, MA) was used. The final products were characterized by 12% (w/v) SDS-PAGE, and the protein concentrations were determined using Bio-Rad Bradford Protein Assay kit reagents (Bio-Rad, CA) with bovine serum albumin as the standard.

Circular Dichroism (CD) Spectroscopy. An Aviv 202 circular dichroism spectropolarimeter (Aviv Biomedical Inc., Lakewood, NJ) was used to obtain the CD spectra for CP36 and WT chicken IL-1β.[5] For secondary structure characterization, far-UV CD spectra of 10 μM samples in 10 mM potassium phosphate, pH 7.4, were recorded from 260 to 195 nm at 25°C using a 1-mm path length cuvette. Three CD scans for each sample were averaged and are reported as mdeg.

The thermostability of CP36 and WT chicken IL-1β was determined at temperatures between 4°C and 96°C by recording the changes in the ellipticity at 217 nm of their CD spectra.[1, 6] The temperature was raised in 2°C increments at a heating rate of 2°C/min. Prior to recording the ellipticity values, the temperature of the protein sample was equilibrated for 1.5 min.

For the guanidine-HCl unfolding experiment, protein samples (10 μM) in 10 mM potassium phosphate, pH 7.4, were mixed with solutions containing various concentrations of guanidine-HCl and their ellipticity values were measured at 25°C. Three independent unfolding curves were obtained.

Spectrofluorimetry. The intrinsic fluorescence of 1 mM solutions of CP36 and WT chicken IL-1β each in 25 mM Tris-HCl, 100 mM NaCl, pH 7.4, at 25°C was measured from 290 nm to 400 nm using an F-7000 fluorescence spectrofluorometer (Hitachi, Tokyo, Japan) and a 1-cm path length cuvette (excitation at 280 nm).

To characterize potential interactions between CP36 or WT IL-1β (10 μM each) and the fluorescent hydrophobic dye, 1,8-anilinonaphthalenesulfonate (20 μM), [1, 7, 8] in 25 mM Tris-HCl, 100 mM NaCl, pH 7.4, at 25°C, the fluorescence of the dye was measured from 385 to 600 nm (excitation at 365 nm). Each reported spectrum is the average of three scans and was plotted using KaleidaGraph software (Synergy Software, Reading, PA, USA).

Surface Plasmon Resonance. To measure the binding affinity of CP36, WT chicken IL-1β, or WT human IL-1β for the chicken IL-1 receptor at 25°C, surface plasmon resonance measurements were acquired using a BIAcore 3000 system (GE Healthcare).[9] The receptor (5 μg/ml) in 10 mM sodium acetate, pH 5.5, was immobilized (final condition, ~370 response units) on a research-grade CM5 sensor chip using the standard amine-coupling method[10]. Various concentrations of each protein in 10 mM HEPES, pH 7.2, 150 mM NaCl, 0.001% (v/v) Tween-20 were individually washed over the flow cell at a rate of 30 μl min^{-1}. The binary complexes were allowed to associate for 90 s and then to dissociate for 90 s. Kinetic data were fit with a 1:1 Langmuir binding model by the BIAcore 3000 evaluation software BIAevaluation 4.1.

Functional Assays. The bioactivity assay was performed as described.[1] Briefly, chicken DF-1 fibroblasts were cultured in Dulbecco minimum essential medium supplemented with 4% (v/v) fetal calf serum. CP36, WT chicken IL-1β, WT human IL-1β (100 ng/ml each) were then individually added into a culture and incubated for 2 h. The K60 mRNA levels were then detected as the measure of bioactivity using RT-PCR kit reagents (SuperScript III One-Step RT-PCR System, Invitrogen). β-Actin mRNA levels served as the internal control. The PCR primer sequences have been described.[1] PCR products were identified after electrophoresis through 1.5% (w/) agarose gels.

To measure the *in vivo* activity of CP36 and WT chicken IL-1β, they were individually injected at a concentration of 10 μg/kg body mass into adult specific-pathogen-free white leghorn chickens (Animal Health Research Institute, Taiwan).[1] After 1 h, the plasma cortisol level was measured using a Roche E170 Modular immunoassay analyzer (Roche Diagnostics, Mannheim, Germany). In addition, protein samples were heated at different temperatures for 10 min and cooled to 25°C before injection into chickens. The animal-use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), National Tsing Hua University, Taiwan. Three independent experiments for each protein that used three chickens were performed.

Structural Modeling of CP36. The CP36 structural model was built at the Robetta server (http://robetta.bakerlab.org/).[11] The model was optimized using the CHARMM[12] force-field and a steepest descendent minimization protocol in the Discovery Studio 2.0 (Accelrys Inc., San

Diego, CA). The quality of the structure was assessed using PROCHECK v.3.5.4.[13] VOIDOO was used to calculate the internal pocket volume.[14] The comparison of the CP36 model and the X-ray structure of IL-1β[1] were performed by Discovery Studio 2.0. Figures were drawn using PyMOL (DeLano Scientific; http://www.pymol.org).

Limited Proteolysis assay. Trypsin treatment of purified WT chicken IL-1β and CP36 were performed at room temperature in 20 mM Tris, 125 mM NaCl buffer. Trypsin (10 ng) was added to the 100 μL WT chicken IL-1β or CP36 (0.5 mg/mL) and incubated. Aliquots of 8 μL were removed from the reaction mix at time points 0, 3, 10, 20, 30, 60, 180, 360, and 1200 min and stopped the reaction by boiling with equal volume of SDS sample buffer for 10 min. The protein samples were placed on ice and then separated in 15% SDS-polyacrylamide gels and stained with Coomassie Blue.

Analytical Ultracentrifugation. Analytical sedimentation velocity ultracentrifugation was performed with 16.5 μM samples of WT chicken IL-1β and CP36 using a Beckman XL-A optima analytical ultracentrifuge equipped with an absorbance optics unit (280 nm) and a Ti-60a titanium rotor (Beckman Coulter, Inc., Brea, CA, USA). The buffer was 25 mM Tris–HCl, pH 7.4, with 100 mM NaCl, and the temperature was 20 °C. The sedimentation coefficient distributions were calculated using SEDFIT85.[15]

References

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Table S1. Protein structure analysis. VOIDOO and NOC (http://noch.sourceforge.net/) were used to analyze the non-polar pocket volume and solvent-accessible surface area, respectively.

	Pocket size (Å^3)	Solvent-accessible surface area (Å^2)	
		All protein	Aromatic residues
WT IL-1β	50.5	8470.7	444.6
CP36	454.0	10674.1	820.1

Fig. S1

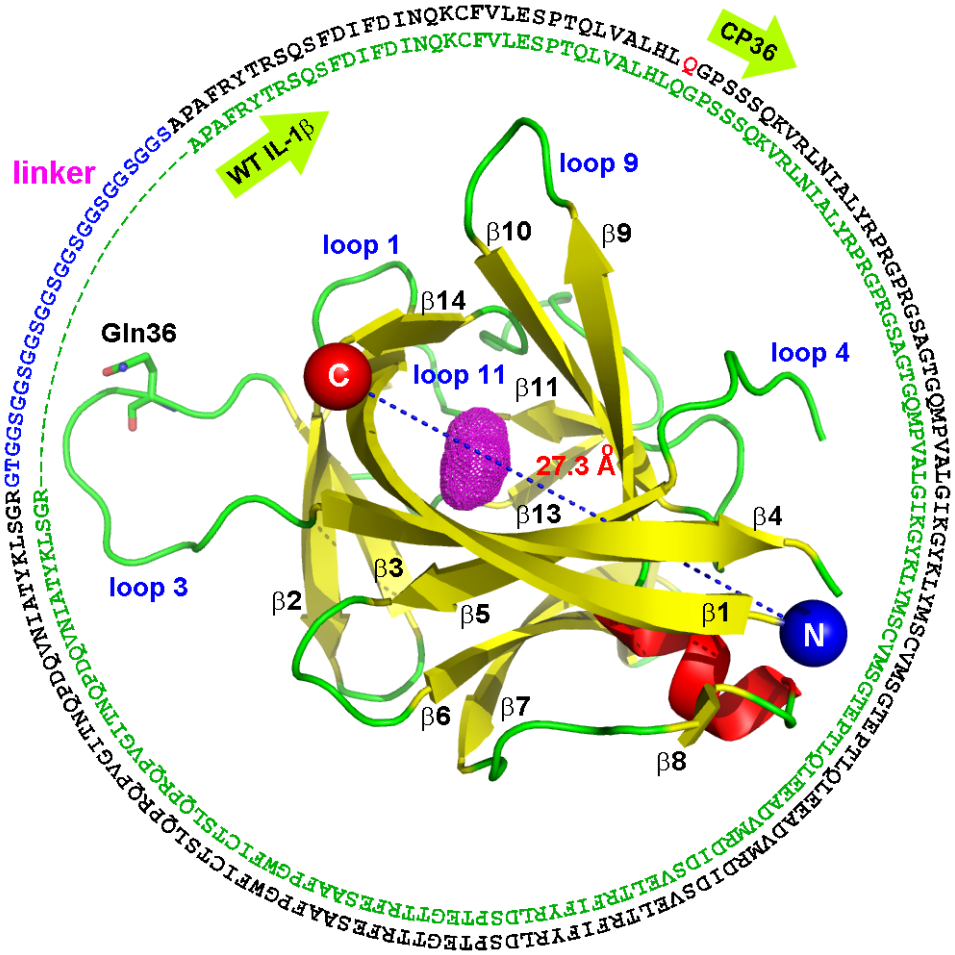


Fig. S1 Circular permutation of WT chicken IL-1β. The aligned sequences of WT chicken IL-1β (inner circle) and CP36 (outer circle). The positions of the N-terminal residue and the linker in CP36 are indicated. A ribbon diagram of the WT chicken IL-1β structure and its non-polar pocket demarked by purple mesh are shown. The dashed line represents the distance between the N- and C-termini (27.3 Å). The functional loops 1, 3, 4, 9, and 11 involved in receptor activation are labeled.

Fig. S2

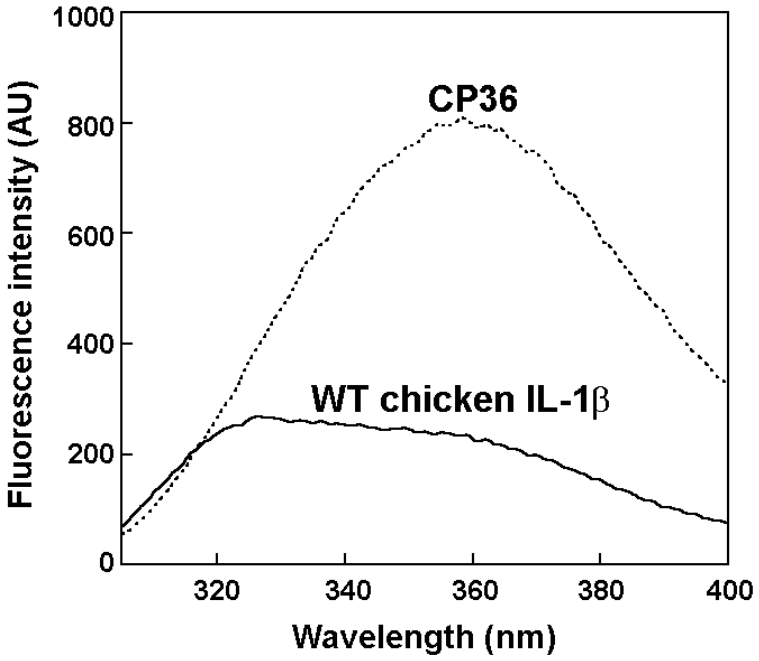


Fig. S2 Intrinsic fluorescence of WT chicken IL-1β and CP36. The intrinsic fluorescence of each protein was recorded between 305 nm and 400 nm at 25°C (excitation wavelength, 280 nm). Solid line, WT chicken IL-1β; dotted line, CP36. AU, arbitrary unit.

Fig. S3

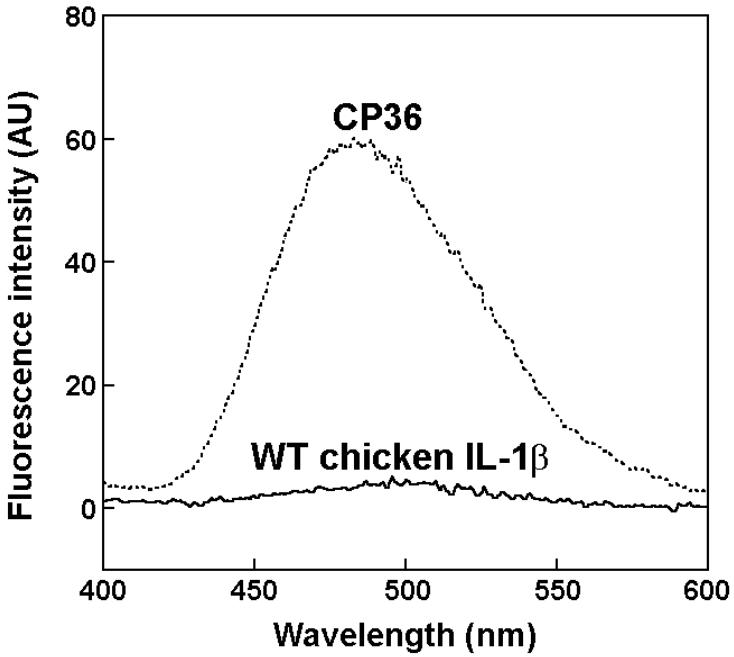


Fig. S3 ANS-binding assay. The extrinsic fluorescence of 1,8-anilinonaphthalenesulfonate (ANS) was used to assess the packing of hydrophobic residues in WT chicken IL-1β and CP36. Fluorescence intensity was recorded between 385 nm and 600 nm (excitation wavelength, 365 nm). The ANS fluorescence was greater in the presence of CP36 than in the presence of WT IL-1β, implying that CP36 has a loosely packed non-polar core or a larger exposed non-polar surface area. AU, arbitrary unit.

Fig. S4

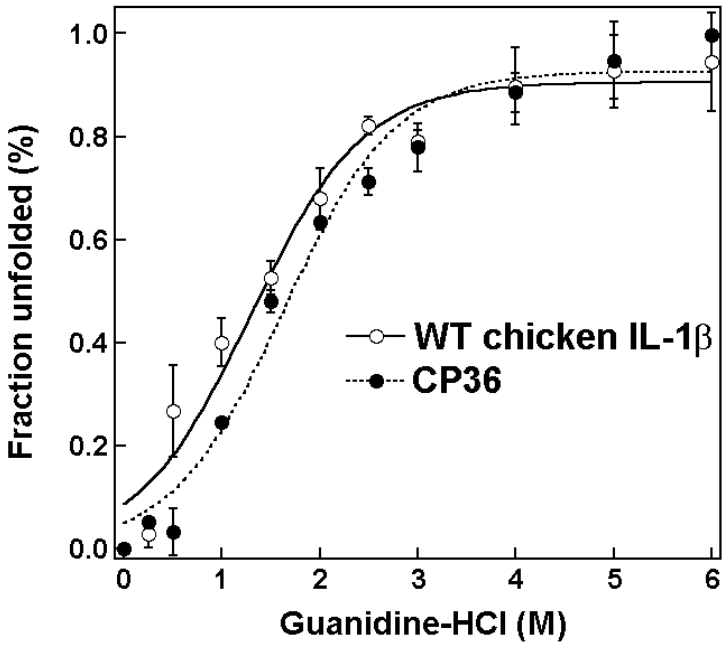


Fig. S4 Guanidine-HCl-induced unfolding curves. Unfolding was monitored at different guanidine-HCl concentrations by following the change in ellipticity at 217 nm. The fraction unfolded is calculated as: $f = (\theta - \theta_F) / (\theta_U - \theta_F)$, where θ is the observed ellipticity at 217 nm, at a given guanidine-HCl concentration, and θ_U and θ_F are the ellipticities of the denatured and native states, respectively. Open circles, WT IL-1β; solid circles, CP36.

Fig. S5

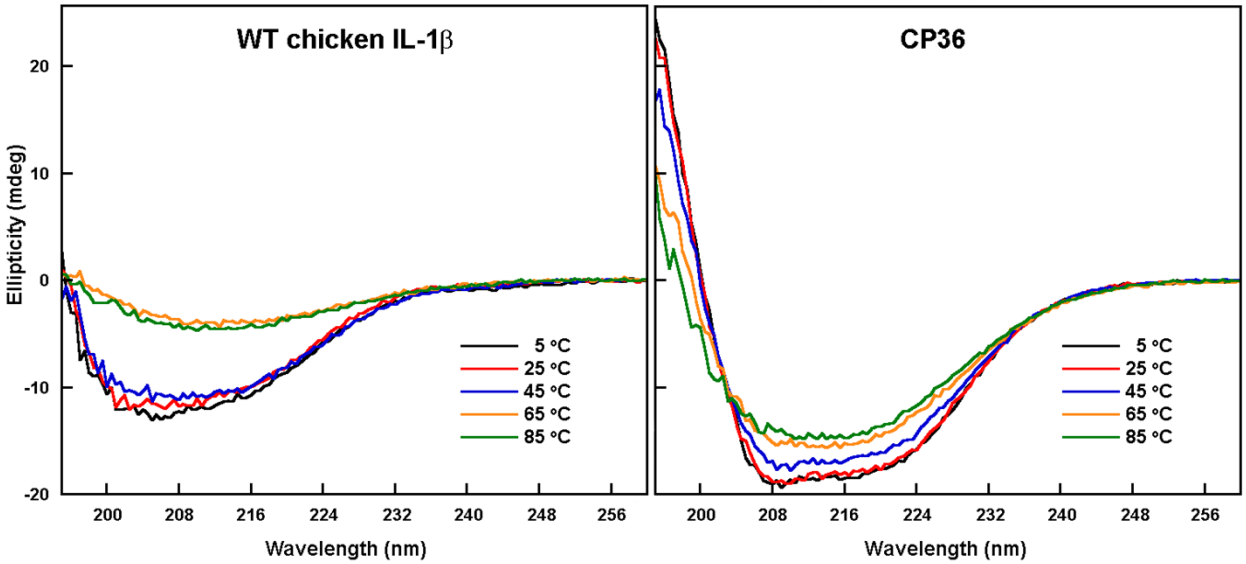


Fig. S5 Thermal stabilities of WT chicken IL-1β and CP36. Far-UV CD spectra of WT chicken IL-1β and CP36 recorded between 260 and 195 nm at different temperatures are presented. Three CD scans for each sample were averaged and are reported as mdeg.

Fig. S6

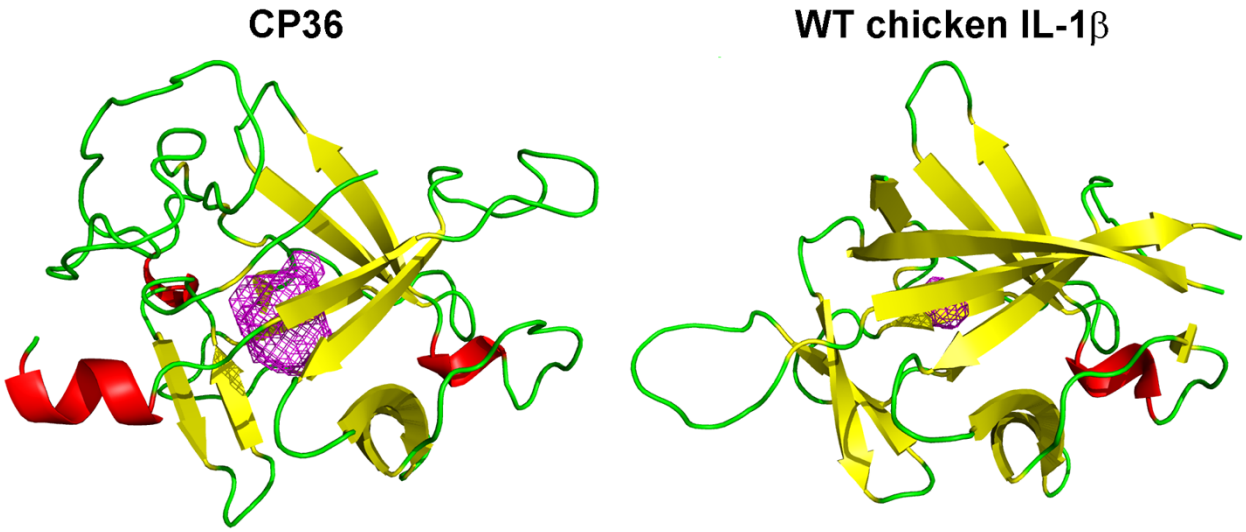
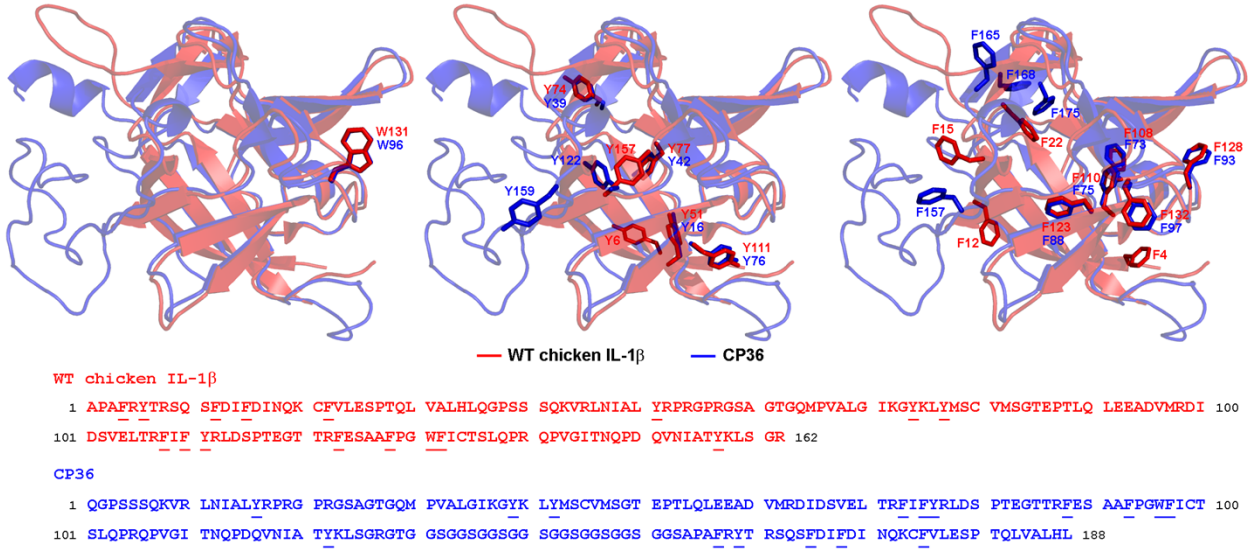


Fig. S6 The non-polar pockets of WT chicken IL-1β and CP36. The structure of WT chicken IL-1β (right panel) was aligned with that of CP36 (left panel). The structures are shown as ribbon diagrams. The α-helices and β-strands are shown as helices and arrows, respectively. The boundaries of their internal non-polar pockets are shown as a purple mesh.

Fig. S7



WT chicken IL-1β

1 APAFRYTRSQ SFDIFDINQK CFVLESPTQL VALHLQGPSS SQKVRLNIAL YRPRGPRGSA GTGQMPVALG IKGYKLYMSC VMSGTEPTLQ LEEADVMRDI 100

101 DSVELTRFIF YRLDSPTEGT TRFESAAFPG WFICTSLQPR QPVGITNQPD QVNIATYKLS GR 162

CP36

1 QGPSSSQKVR LNIALYRPRG PRGSAGTGQM PVALGIKGYK LYMSCVMSGT EPTLQLEEAD VMRDIDSVEL TRFIFYRLDS PTEGTTRFES AAFPGWFICT 100

101 SLQPRQPVGI TNQPDQVNIA TYKLSGRGTG GSGGSGGSGG SGGSGGSGGS GGSAPAFRYT RSQSFDIFDI NQKCFVLESP TQLVALHL 188

Fig. S7 Comparison of the positions of the aromatic residues in CP36 with those in WT chicken IL-1β. To compare the locations and orientations of the aromatic residues, WT chicken IL-1β (red, PDB entry, 2WRY) was superimposed onto CP36 (blue). The CP36 structure was modeled at the Robetta server (http://robetta.bakerlab.org/). The positions of the tryptophan, tyrosines, and phenylalanines are shown in the left, middle, and right panels, respectively. The primary sequences of the two proteins are presented beneath the structures, and the aromatic residues are underlined. The structures were drawn by PyMOL.

Fig. S8

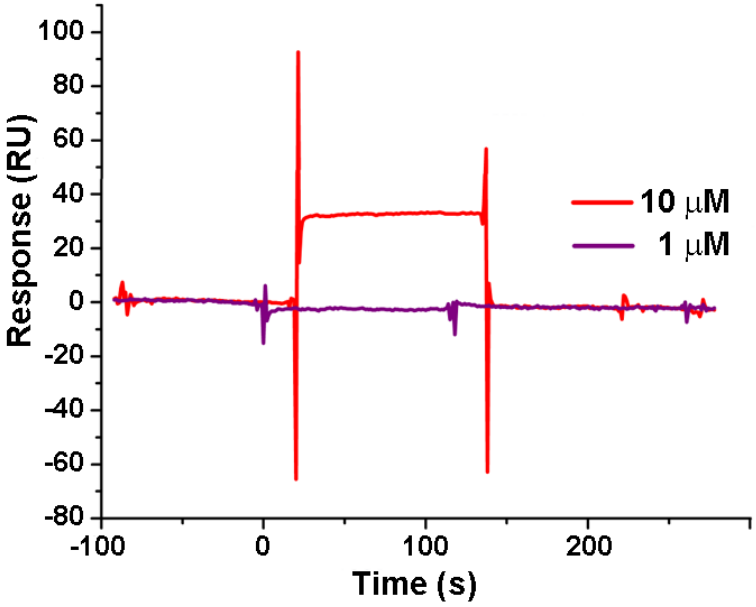


Fig. S8 Surface plasmon resonance of the binding of human IL-1β to the chicken IL-1 receptor. Sensorgrams were obtained while injecting 1 or 10 μM WT human IL-1β over a receptor-immobilized CM5 chip. Even at 10 μM, binding of WT human IL-1β to the receptor was not detected. RU, response unit.

Fig. S9

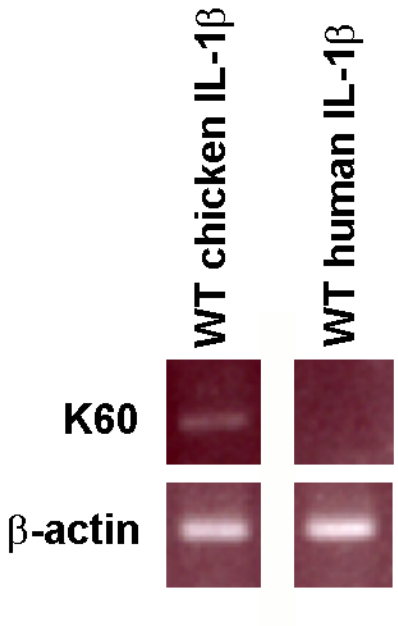


Fig. S9 Functional assay for WT human and chicken IL-1βs. To determine if WT human and chicken IL-1β function in chicken fibroblasts, they were incubated with chicken fibroblasts, and the expression of fibroblast K60 mRNA induced by each IL-1β was assessed. The expression level of β-actin mRNA served as the internal control.

Fig. S10

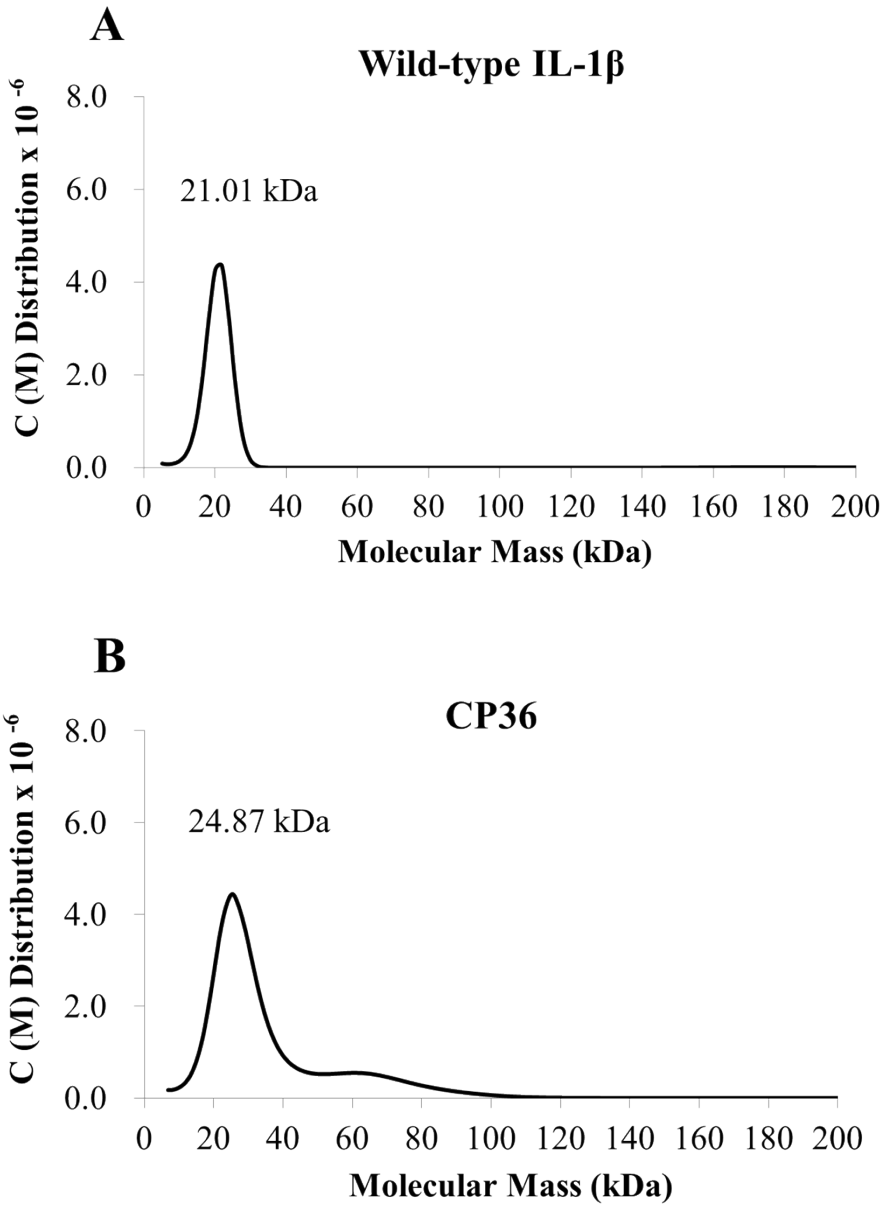


Fig. S10. Oligomeric states of WT chicken IL-1β and CP36. Analytical ultracentrifugation results of (A) WT chicken IL-1β and (B) CP36. The continuous molar mass distribution C (M) for each protein is plotted as a function of molecular mass and was calculated using SEDFIT85.[15]

Fig. S11

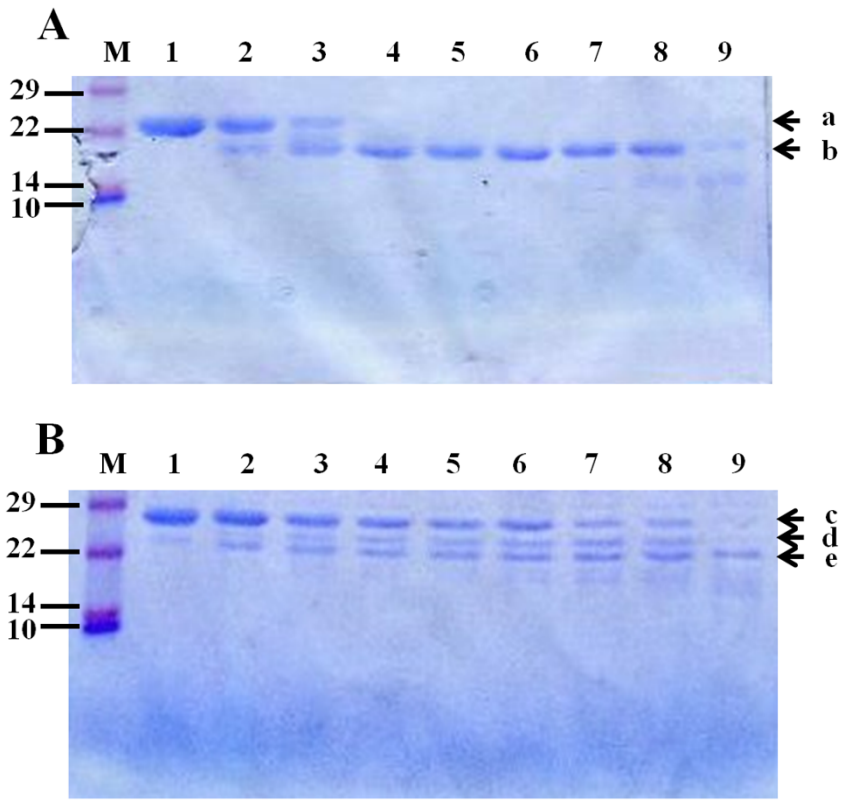


Fig. S11. Characterization of the stability of WT chicken IL-1β and CP36 by limited proteolysis assays. WT chicken IL-1β (A) or CP36 (B) were incubated with trypsin at room temperature. Aliquots of the reaction were removed after 0 (lanes 1), 3 (lanes 2), 10 (lanes 3), 20 (lanes 4), 30 (lanes 5), 60 (lanes 6), 180 (lanes 7), 360 (lanes 8) and 1200 min (lanes 9), respectively. 8μL of each reaction products were separated in 15% SDS-PAGE and visualized proteins using Coomassie Blue staining. Marker proteins (lane M) are indicated in kDa. Bands a and c illustrate the parent (black arrow) proteins of WT chicken IL-1β and CP36. Bands b, d and e illustrate the digested segments (black arrow) found in the WT chicken IL-1β and CP36 proteolysis reactions.