Electronic Supplementary Material (ESI) for ChemComm.

Supplementary information

Zinc clasp-based reversible toolset for selective metal-mediated protein heterodimerization

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Materials

N,N-Diisopropylethylamine (DIEA), Fmoc-protected amino acids (Fmoc-Ala-OH·H2O, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH, piperidine, *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), D-biotin, [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid (Fmoc-O2Oc-OH), and DL-dithiothreitol (DTT) were purchased from Iris Biotech GmbH. Trifluoroacetic acid (TFA), 1,2-ethanedithiol (EDT), thioanisole, anisole, triisopropylsilane (TIPS), COMU, Fmoc-Asp(OtBu)-(Dmb)-Gly-OH, Fmoc-Lys(Mtt)-OH, Dawson Dbz AM resin, 5(6)-carboxytetramethylrhodamine (TAMRA), guanidine hydrochloride (GdnHCl), 4-mercaptophenylacetic acid, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), reduced glutathione (GSH), ethylenediaminetetraacetic acid (EDTA), HCl (trace metal grade), 4-(2-pyridylazo)resorcinol (PAR), polyethylene glycol (PEG) 3,300, Triton X-100, bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), *N*-carboxymethyl-*N*′-(2-hydroxyethyl)-*N*,*N*′-ethylenediglycine trisodium salt (Na_3-HEDTA), boric acid, $[Cu(MeCN)_4]BF_4$, $NaClO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, biocytin, and bovine serum albumin (BSA) were from Merck KGaA. Diethyl ether, acetic anhydride, dichloromethane (DCM), NaCl, and KNO_3 were from Avantor Performance Materials Poland S.A. Tryptone, yeast extract, LB Broth, agar, agarose, isopropyl-β-D-1-thiogalactopyranoside (IPTG), sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were purchased from Lab Empire. Ampicillin, chloramphenicol and Tris base were from Roth, pTYB21 vector and chitin resin from New England BioLabs, Chelex 100 resin from Bio-Rad, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES) from Bioshop, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) from TCI Europe N.V., PEG 3,350 from Hampton Research, TentaGel R RAM and TentaGel S-NH_2 resins from Rapp Polymere GmbH, dimethylformamide (DMF) and acetonitrile (MeCN) from VWR. All of the experiments were performed in chelexed buffers and solutions. All buffers were prepared with Milli-Q water obtained with a deionizing water system (Merck KGaA).

Peptides synthesis and purification

Peptides were synthesized on a solid-phase support using the Fmoc strategy on TentaGel R RAM Amide Rink resin (0.18 mmol/g substitution) and a Liberty 1 Microwave Assisted Peptide Synthesizer (CEM) according to the previous published procedures.[1,2] Fmoc-Asp(OtBu)-(Dmb)-Gly-OH dipeptide was used for the synthesis of Lck peptide in order to prevent aspartimide formation. Peptides were acetylated at the N-terminus with acetate anhydride (8 eq.) and DIEA (10 eq.). On-resin coupling of TAMRA (4 eq.) was performed for Lck peptide on the N terminus using COMU (4 eq.) and DIEA in DMF (6 eq.). Resin cleavage was performed with a mixture of TFA/EDT/thioanisole/anisole/TIPS/H_2O (86:3:5:2:2:2, v/v/v/v/v/v) over a period of 4 h. After evaporation of the solution to the minimum volume under nitrogen atmosphere, peptides were precipitated with cold diethyl ether (-20°C). Crude peptide pellets were centrifuged (4°C) and washed with cold diethyl ether (5-7 times) after 2-3 minutes of sonication to get rid of the excess of scavengers.

Resin-bound and biotinylated Lck peptide (Lck(biot)) was synthesized on the TFA-resistant TentaGel S NH_2 resin (0.26 mmol/g substitution) and TentaGel R RAM resin, respectively, according to the above procedure. For molecular baits, DCM was gradually substituted by the buffer (see below) or resin was stored at 4°C after drying under a vacuum. Biotinylation was performed using the orthogonally blocked lysine residue with Mtt protection and its conjugation as the second-coupled amino acid in the synthesis route (Scheme S1). To monitor the conjugation of D-biotin (2.5 eq.) after deprotection of the targeted lysine (1% TFA/DCM, 3×3 min) using the ESI-MS instrument the additional amino acid (lysine) was coupled as the first in the synthesis route. After biotinylation a PEG-based O2Oc linker (2.5 eq.) was incorporated with a standard coupling procedure.

Peptide purification was performed on a C18 column (Phenomenex) with a gradient of acetonitrile and 0.1% TFA using a Dionex Ultimate 3000 HPLC system. Elution gradients with retention times of purified peptides are presented in Table S1 and the selected chromatograms are presented in Figure S10. The identity of peptides was confirmed with an API 2000 Applied Biosystems ESI-MS instrument and the identified mass values are listed in Table S1. Concentration of peptide stocks in 10 mM HCl was determined using a sulfhydryl group reactant, DTNB (ε = 14,150 M^{-1} cm^{-1} at 412 nm), prior to each experiment.[3]

Protein expression and purification

Fluorescently labeled Clover-CD4RARH and mRuby2-Lck proteins were obtained using molecular cloning procedures, *E. coli* expression system and intein-mediated purification (IMPACT Kit, New England BioLabs). Clover, mRuby2 plasmids and CD4RARH and Lck genes were ordered from Addgene (plasmids #40259 and #40260) and GenScript, respectively.[4] Amplification of DNA sequences was performed by PCR reaction with designed primers (Table S6) and Phusion High-Fidelity DNA Polymerase (Thermo Scientific) to get SapI-Clover-BamHI, SapI-mRuby2-BamHI, BamHI-CD4RARH-PstI and BamHI-Lck-PstI. Identity was confirmed by agarose gel electrophoresis and purification was performed with PCR clean-up kit (Thermo Scientific). Samples were digested with BamHI enzyme (Thermo Scientific), purified from agarose gel with GeneJET Gel Extraction Kit (Thermo Scientific) and ligated by T4 DNA ligase (Thermo Scientific) for further PCR amplification. pTyb21 vector and amplified PCR products were double-digested with SapI and PstI restriction enzymes (Thermo Scientific), ligated, and pTyb21-Clover-CD4RARH and pTyb21-mRuby2-Lck constructs were obtained. Concentration and purity were determined spectrophotometrically with NanoDrop 2000 (Thermo Scientific). Vectors were used to transform chemically competent *E. coli* DH5α strain, isolated from transformants with ampicillin resistance and verified with DNA sequencing. Plasmids encoding Clover-CD4RARH and mRuby2-Lck sequences were transformed into the host *E. coli* BL21(DE3)rhIL strain. A single colony from the freshly streaked plate was used to inoculate 100 ml of TB media supplemented with 150 μg/ml of ampicillin and 34 μg/ml of chloramphenicol and incubated at 37°C. After 5 hours 10 ml of starter culture was centrifuged (4,000×*g*, 2 min), resuspended in fresh medium and inoculated the main 1 l culture. Cells were grown at 37°C until the OD_{600} reached approx. 0.7, then the protein expression was induced by IPTG addition to a final concentration of 0.1 mM. After overnight incubation at 15°C the cultures were centrifuged (6,000×*g*, 20 min) and the cell pellets were resuspended in lysis buffer (20 mM Na_3-HEPES, pH 8.5, 0.5 M NaCl, 0.1% Triton X-100, 20 uM PMSF, 1 mM TCEP). Cell lysates were prepared by sonication on ice (50% amplitude, 25 min total time, 8 s pulses, 20 s rest) and centrifuged (12,000×*g*, 30 min) at 4°C.

Purification of proteins were performed according to the manufacturer procedure from IMPACT expression system. Proteins were finally purified using FPLC system (NGC Biorad) with ENRich SEC 10×300 Column (70 kDA) previously washed with 50 mM HEPES, 100 mM KNO_3, 400 μM TCEP and 10 mM EDTA. Protein samples with 10 mM EDTA were

purified in 50 mM HEPES, 100 mM KNO_3, 400 μM TCEP buffer and concentrated using Amicon Ultra-4 10 kDa centrifugal filters (Merck KGaA). The identity of purified proteins was confirmed using ABI 4800 MALDI TOF/TOF measurements (Applied Biosystems).

Native Chemical Ligation

Native chemical ligation was employed to obtain CD4RARH-tagged protein.[5] N-terminal Nbz derivative of CD4RARH and BBL protein domain with C-terminal cysteine were synthesized on Dawson Dbz AM and TentaGel R RAM resins, respectively, according to the standard Fmoc SPPS procedures [1,2]. Addition of Ser-Gly linker was conducted to ensure the flexibility and to minimize steric hindrance during the final ligation step (Table S1). To avoid acetylation of the N-acyl-benzimidazolinone moiety coupling of the Ac-Gly residue was performed. Conversion of Dbz to the Nbz group with *p*-nitrochloroformate was conducted according to the published procedure.[5] To purify CD4RARH(Nbz) 0.1% TFA isocratic elution with FPLC was applied (Superdex Peptide 10/300 GL column with 7 kDa resolution). The BBL domain was purified by HPLC with an MeCN gradient in 0.1% TFA (33 to 45% in 9 min, retention time 11 min) on an Aeris PEPTIDE 3.6 μm XB-C18 column. In the ligation experiment Nbz peptide was dissolved in 0.2 M phosphate buffer, 6 M guanidine hydrochloride, 0.2 M 4-mercaptophenylacetic acid, 50 mM DTT, pH 7.0 in 200 μl in the concentration range 1.5-5 mM. Buffer was degassed with nitrogen prior to use. The dissolved peptide sample was brought to pH 7 and the (Cys)BBL peptide was added in the ratio from 0.6 to 1 over Nbz peptide. The ligation mixture was incubated for 60 min and purified with a Superdex Peptide 10/300 GL column with isocratic 0.1 % TFA. The ligation yielded 30% (Figure S7).

Electronic absorption spectroscopy

Properties of protein fragments towards Zn(II) were examined by Zn(II) titration and subsequent absorption spectra collection. Formation of ligand-to-metal charge transfer bands was observed at the wavelength range of 200-260 nm (50 mM borate buffer, pH 7.4, 100 mM $NaClO_4$, 25°C).[6] All of the spectroscopic measurements were performed on a Jasco V-650 instrument in a 1 cm quartz cuvette. Each sample contained the TCEP reductant (3 eq.) over the total amount of cysteine residues.[7] Concentration of peptides in each sample was 30 μM and Zn(II) titration was performed to 2 molar equivalents of Zn(II) over peptide with 25-30 titration steps including 3 minutes incubation time, which was established experimentally. For

the data analysis the absorption at 220 nm wavelength was extracted with the reference value of 260 nm. Co(II) titrations were conducted for 50 μM $Co(Lck)_2$ complexes by stepwise addition of CD4 peptides and the collection of UV-Vis spectra in the 300-900 nm spectral range. Experiments were performed in 50 mM HEPES, 0.1 M KNO_3, 1 mM TCEP and pH 7.4 at 25°C.

Competition with PAR probe

The competition of peptides with chromophoric metal chelator PAR for Zn(II) (Equation 1) was performed by measuring the absorbance values of $Zn(H_xPAR)_2$ complexes at 492 nm (ε = 71,500 $M^{-1} \cdot cm^{-1}$) in each titration step after 3 minutes of equilibration.[8] Application of the PAR chelator probe allows one to detect complexes' stoichiometry and to compare their affinities. All samples contained 100 μM PAR solution partially saturated with $ZnSO_4$ (10 μM) in 50 mM HEPES, 100 mM KNO_3, 400 μM TCEP, pH 7.4. Titration was performed in 20-30 steps up to 2.7 peptide equivalents over Zn(II). Stock solution of 20 mM PAR was prepared in DMSO solution. Heterodimer conditional binding constant K_{12} has been calculated according to the published protocol.[8]

$$Zn(H_xPAR)_2 + CD4RARH + Lck \rightleftharpoons Zn(CD4)(Lck) + 2H_xPAR \quad \text{(Eq. 1)}$$

Zn(II)-buffered system

In order to obtain a more comprehensive view of Zn(CD4)(Lck) heterodimer stability, it was decided to acquire apparent dissociation constants with CD titration experiments in Zn(II)-buffered media. The series of 25 μM metal chelators (EDTA, HEDTA and EGTA) with different amounts of $ZnSO_4$ maintain the free zinc concentration range of 10-15 pZn ($-\log[Zn(II)_{free}]$). Zn(II) to peptide transfer during equilibration was included in the calculations since the peptide concentration in each sample was 5 μM. Table S2 presents pZn calculated in different ratios of chelator to Zn(II) with HySS2009 software using apparent dissociation constants of chelators and their stability constants with Zn(II).[9] To obtain apparent dissociation constants for protein derivatives - Zn(CD4RARH(Clover))(Lck(mRuby2)) and ZnCD4(RARH(Clover))(Lck(TAMRA)) - fluorimetric measurements were applied (Figure 2e). Chelator concentration was 40 μM and Zn(II) to peptides transfer was neglected due to the insignificant pZn changes of metal buffers in 0.5 μM peptide concentration (Table S3).

Concentration of free Zn(II) in buffered media was calculated using protonation and stability constants of chelators [10] and HySS2009 software.[9]

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded in the range of 195 to 260 nm in 20 mM Tris, 10 mM $NaClO_4$, pH 7.4 and TCEP (3 eq. over cysteine residues) with a Jasco J1500 spectropolarimeter at 25°C. Peptide concentration was set experimentally in such a way as not to exceed 600 V photomultiplier electric potential at 195 nm. Zn(II) titration experiments were performed from 0 to 2 equivalents of Zn(II) over peptides. For selectivity studies with Mg(II) and Ca(II) experiments were conducted in the same manner. In the Cu(I) titrations 10% MeCN was applied to diminish metal ion oxidation rate. The apparent dissociation constant of Zn(CD4)(Lck) heterodimer was determined in the competition experiments of peptides with chelators for Zn(II).[2,11] Samples consisted of 5 μM peptides, 25 μM metal chelator (EGTA, HEDTA or EDTA), TCEP (5 eq. over number of sulfhydryl moieties [7]) and a set of $ZnSO_4$ concentrations (Table S2). Each titration step involved 45 minutes of incubation before triple point measurements of ellipticity at 202 nm and 220 nm. Data were normalized to the 0-1 range of molar fraction of the complex (y) and fitted to the logarithmic version of Hill's equation (Equation 2) according to the previously published procedure.[12] $pZn_{0.5}$ stands for half of the complex formed (y = 0.5), y_{min} and y_{max} refer to minimal and maximal intensities and are 0 and 1, respectively, and n is the cooperativity factor.

$$y = y_{min}\left(\frac{[Zn(II)_{free}]^n}{[Zn(II)_{free}]^n + Zn_{0.5}}\right) + y_{max}\left(\frac{Zn_{0.5}}{[Zn(II)_{free}]^n + Zn_{0.5}}\right) \quad \text{(Eq. 2)}$$

Due to the complex stoichiometry of heterodimer the value of $Zn_{0.5}$ is not equal to the apparent binding constant, as in the case of the monomeric complex. Regarding equal concentration of complex and peptides at $Zn_{0.5}$, K_{12} values were calculated according to Equations 3 and 4:

$$Zn(II) + CD4RARH + Lck \rightleftharpoons Zn(CD4RARH)(Lck) \quad \text{(Eq. 3)}$$

$$K_{12}(Zn(CD4)(Lck)) = \frac{[Zn(CD4RARH)(Lck)]}{[Zn(II)_{free}] \cdot [CD4RARH] \cdot [Lck]} \quad \text{(Eq. 4)}$$

Fluorimetry

To monitor complex formation and determine apparent dissociation constants of Zn(CD4RARH)(Lck) heterodimer, fluorescently labeled CD4RARH(Clover), Lck(mRuby2) and Lck(TAMRA) served as two FRET pairs (Clover to mRuby2 and Clover to TAMRA). Measurements were performed at the constant temperature of 25°C with 514 nm excitation wavelength (1.0 nm slit) and 508-650 nm range in emission spectra (3.0 nm slit) in a quartz cuvette using a Jobin Yvon Fluoromax-3 spectrofluorimeter (Horiba). Protein or peptide samples were prepared in 0.5 μM concentration (50 mM HEPES, 100 mM KNO_3, 400 μM TCEP, pH 7.4) with 40 μM metal chelator and a set of Zn(II) concentrations up to 37 μM in order to maintain Zn(II) buffering in the pZn range of 9-15 (Table S3). Samples were incubated for 3 hours and measurements were performed as three averaged scans. Cu(I) ions were added directly to the equimolar mixture of CD4RARH(Clover) and Lck(mRuby2) with addition of 10% MeCN. Fluorescence intensities were fitted to the enhanced formula for ratiometric probes as described previously (Equation 5)[13] with the acceptor wavelengths of 600 and 583 nm for Clover and TAMRA, respectively. I_{1b}, I_{1u}, I_{2b} and I_{2u} are borderline intensity values for donor and acceptor, respectively.[13] $R_{1/2}$ stands for the ratio of donor to acceptor intensities at each measured point, K_d is the dissociation constant and n is Hill's coefficient. Calculated K_{12} values are presented in Table S4.

$$R_{1/2} = \frac{I_{1b}x^n + I_{1u}K_d^n}{I_{2b}x^n + I_{2u}K_d^n} \qquad \text{(Eq. 5)}$$

Size exclusion chromatography for determination of complexes formation

Heterodimerization studies were performed on the Superdex Peptide 10/300 GL and ENrich SEC 70 column for peptide and protein analysis, respectively, using the Bio-Rad NGC chromatography system. Experiments were performed in 100 mM Tris, 300 mM NaCl, pH 7.4, which were chelexed and degassed before use. Addition of 3 eq. of TCEP over sulfhydryl groups was used in each sample. Columns were preconditioned with 2 column volumes of buffer supplemented with 10 mM EDTA. Peptide samples were prepared in 30 μM concentration and incubated with an equimolar amount of Zn(II) for 45 min before injection

of a 100 µl sample. Analysis was run at 0.5 ml/min and the system was purged with 10 mM EDTA buffer after each run. CD4RARH(Clover) and Lck(mRuby2) complex formation was observed for 10 µM samples prepared in Zn(II)-buffered media (1 mM HEDTA supplemented with $ZnSO_4$ in the range of 0.1-0.9 mM, see above). Samples were incubated for 60 minutes before injection (100 µl) and run at 1.0 ml/min.

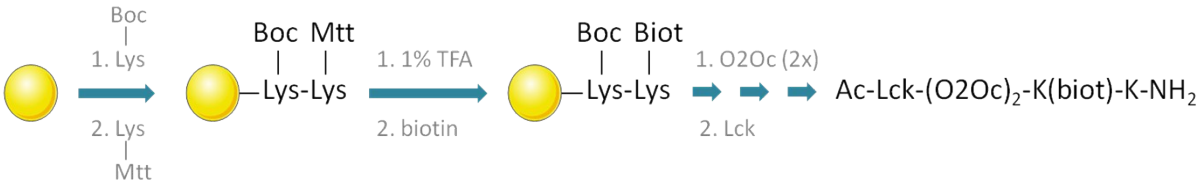
Molecular baits based on Lck-immobilized resin

Organic solvent in synthesized molecular baits (10 ± 1 mg) was replaced with 50 mM HEPES, 100 mM KNO_3, and 400 µM TCEP, pH 7.4 (binding buffer) with three washing steps of 75, 50 and 25% DCM in the buffer. Baits were then equilibrated for 12 hours to condition the resin and ensure the reduction of cysteine moieties with TCEP.[7] Quantitative determination of Zn(II)-binding efficiency was performed as a PAR assay and involved incubation with Zn(II), washing and elution steps (Scheme S2). Four independent experiments on 3 to 4 resin samples with immobilized Lck were performed, where unmodified resin was treated as a control. $ZnSO_4$ (1.2 and 2 molar equivalents over theoretical on-resin peptide capacity) in binding buffer (2 ml) was added to each resin sample and mixed for 2 to 4 hours. After the washing step with binding buffer (3 to 4 times), 10 mM HCl was added to elute bound Zn(II) (3 fractions). For all collected samples Zn(II) content was determined by absorption measurement of the $Zn(H_xPAR)_2$ complex with an absorption coefficient of 71,500 $M^{-1}\cdot cm^{-1}$ at 492 nm at pH 7.4.[8] To 100 µM PAR in binding buffer 4-5 aliquots of sample were added in a stepwise manner. Three results with the lowest standard deviation were averaged and combined for all washing and elution steps. A standard sample with the same amount of $ZnSO_4$ was prepared, measured by PAR assay and applied in the analysis with the recovery of 86.5 ± 1.7%. Average Zn(II)-binding capacity for 1 g of Lck-bound resin was calculated (0.23 ± 0.04 mmol/g). PAR assay performed after 6-month storage at 4°C showed no change in Zn(II)-binding capacity of baits. Binding of the CD4RARH and its conjugate with the BBL domain was accomplished for 7 resin samples with unmodified resin as a control in two independent experiments. Samples contained 50 µM and 20 µM (2 and 1 ml of binding buffer) CD4RARH and CD4RARH-BBL, respectively. Two resin samples were treated with 1.2 eq. of $ZnSO_4$ over CD4RARH, two with 1.2 eq. over Lck capacity and for two samples Zn(II)-controlled medium was applied (1 mM HEDTA, 0.8 mM $ZnSO_4$ in binding buffer). The control sample was represented by unmodified resin with 1.2 eq. of $ZnSO_4$ over resin capacity. Samples were mixed for 2 hours and further washing

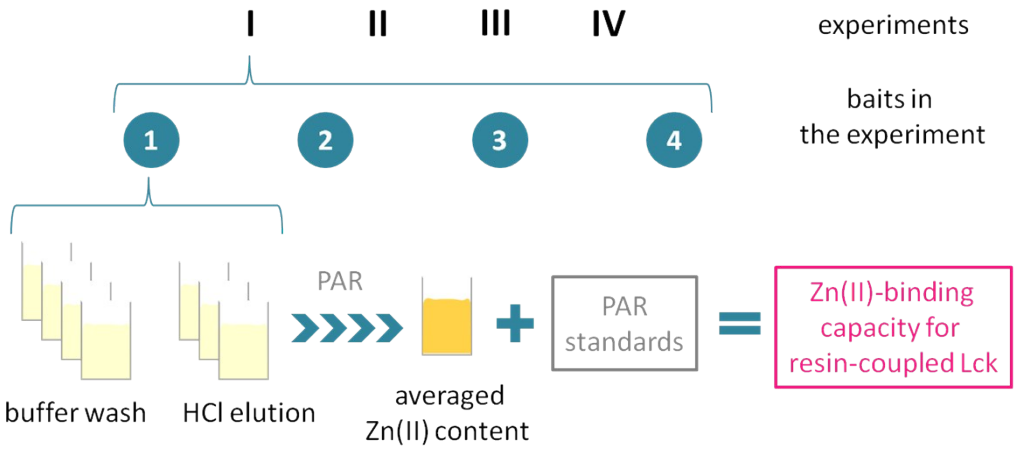
(binding buffer) and elution (10 mM HCl) steps were performed (4 and 3 fractions, respectively). Buffer samples were acidified with 10% TFA up to pH 2 and the HPLC analysis was performed as described above. Chromatogram peaks were integrated with Chromeleon 6.80 software and plotted against peptide concentration (Figure S10).

Biolayer interferometry (BLI)

To detect interprotein zinc heterodimer formation biolayer interferometry was applied (Octet K2, Pall ForteBio). With the optical analytical technique analyses of the interference pattern of white light reflected from two surfaces - a layer of immobilized Lck (biotin tagged, see above) on a streptavidin biosensor tip (ForteBio) and an internal reference layer – were performed. In black 96-well plates 0.1 μM biotinylated Lck peptide was loaded (300 s) with a total working volume of 200 μl per well so as to reach the signal intensity of 0.5 nm and ensure that sensors were not saturated. Within the study two buffers were used: buffer 1 (50 mM HEPES, 0.1 M KNO_3, 0.2% BSA (w/v), 0.1% PEG 3,300 (w/v), 0.05% Triton (v/v), 1 mM TCEP) and buffer 2 (buffer 1 supplemented with 1 mM HEDTA and 0.8 mM $ZnSO_4$, see above for details). Biosensor tips were pre-wetted in a buffer 1 (10 min) followed by equilibration with buffer 2 (400 s). The next steps involved: equilibration in buffer 2 (300 s), quenching with 0.04 mg/ml biocytin (60 s), equilibration (120 s), association (400 s) and dissociation (400 s). Afterwards, acidification was performed (10 mM HCl, 60 s) followed by buffer 1 wash (60 s) and buffer 2 equilibration (120 s) to perform the second association/dissociation stage. Investigated ligands (CD4RARH, CD4RARH(BBL), CD4RARH(Clover) and CD4RARH(Clover) lysate) were used in a 1 μM concentration, which was set experimentally (data not shown). As a control sample the reference sensor without loaded ligand was treated in the same manner. The mixing speed for each step was 1000 rpm and the test temperature was 25°C. All measurements were done in triplicate. Sensograms were fitted (local full) to a 1:2 heterogeneous model (Figure S9) from which the association (k_{on}) and dissociation (k_{off}) rate constants were calculated (Octet Data Analysis software, Table S5). Normalization (from 0 to 1) of binding response to maximum values for sensograms presentation was applied.



Scheme S1. Synthesis of biotinylated Lck fragment. Resin beads are presented as yellow dots. Orthogonal protection of lysine residues with Boc and Mtt groups allowed incorporation of the biotin moiety on the second Lys side chain. A double PEG-based linker (O2Oc) was coupled to separate the Lck sequence from the biotinylation site. Further synthesis steps were performed according to the standard Fmoc SPPS procedure.[1]



Scheme S2. Experimental workflow of PAR assay. Number of experiments and baits in each experiment are indicated in Roman and Arabic numerals, respectively. For each bait four buffer washing and three HCl elution steps were conducted. Determination of Zn(II) content was performed spectrophotometrically with PAR chelator.[8] PAR standards with the average recovery of 86.5 ± 1.7% were applied. Zn(II)-binding capacity from experiments I-IV was calculated for 1 g of Lck-bound resin (0.23 ± 0.04 mmol/g) with synthesis efficiency of 90 ± 17%.

Table S1. Experimental and theoretical mass values of synthesized peptides and their sequences. Purification gradients with peptide retention times are indicated; n/a stands for 'not applicable'.

Name	Peptide sequence	Purification gradient	Retention time	Mass experimental	Mass theoretical
CD4short	Ac-EKKTCQCPHRFQKT-NH_2	5.5-20 min 15-40%	14.4 min	1708.86	1709.03
CD4helix	Ac-RMSQIKRLLSEKKTCQCPHRFQKT-NH_2	5.5-20 min 16-39%	14.1 min	2989.01	2988.61
CD4wt	Ac-RCRHRRRQAERMSQIKRLLSEKKTCQCPHRFQKT-NH_2	5-13 min 15-40%	11.1 min	4338.42	4338.16
CD4RCRH	Ac-RCRHRRRQAERMSQIKRLLSEKKTCQCPARFQKT-NH_2	5-13 min 15-40%	11.2 min	4272.15	4272.09
CD4RCRA	Ac-RCRARRRQAERMSQIKRLLSEKKTCQCPARFQKT-NH_2	5-13 min 15-40%	11.4 min	4206.58	4206.03
CDRARH	Ac-RARHRRRQAERMSQIKRLLSEKKTCQCPARFQKT-NH_2	12-14 min 36,5-38%	12.7 min	4240.32	4240.03
Lck	Ac-SHPEDDWMENIDVCENCHYPIVPLDGKGT-NH_2	13-20 min 40-60%	15.2 min	3355.82	3355.69
Lck(biot)	Ac-SHPEDDWMENIDVCENCHYPIVPLDGKGT-$(O2Oc)_2$-K(biot)K-NH_2	9-13 min 48%	11.1 min	4128.30	4128.66
CD4RARH(BBL)	Ac-GRARHRRRQAERMSQIKRLLSEKKTCQCPARFQKTSGSGCGSQNNDALSPAIRRLLAEHNLDASAIKGTGVGGRLTREDVEKHLAKA-NH_2	n/a	n/a	9622.24	9621.89

Table S2. The chemical components of Zn(II)-buffered media used for the Zn(II) competition with chelators with CD4RARH and Lck and related pZn values. Chelator and peptide concentrations in each sample were 25 and 5 μM, respectively. Experiments were performed in 50 mM HEPES with I = 0.1 M (from KNO_3) at pH 7.4 and measurements were taken at 25°C.

Total Zn(II) [μM]	pZn[a] in EDTA[b]	pZn[a] in HEDTA[b]	pZn[a] in EGTA[b]
2.7	-	13.16	10.20
5.3	14.24	12.78	9.81
8.0	14.00	12.54	9.56
10.6	-	12.38	9.36
13.2	13.62	12.22	9.19
15.9	-	12.05	9.01
18.5	-	11.89	8.84
21.1	-	11.71	8.61
23.7	-	11.45	8.38
26.3	-	10.72	7.75

[a]Correction was made to take into account transfer of metal from Zn(II)-buffered media to peptides.
[b] Protonation and stability constants of EDTA: β_{HL} = 10.17, β_{H2L} = 16.28, β_{H3L} = 18.96, β_{H4L} = 20.96, β_{H5L} = 22.47, β_{ZnHL} = 19.44, β_{ZnL} = 16.44; HEDTA: β_{HL} = 9.81, β_{H2L} = 15.18, β_{H3L} = 17.78, β_{ZnL} = 14.60; EGTA: β_{HL} = 9.40, β_{H2L} = 18.18, β_{ZnL} = 12.60.[10]

Table S3. Chemical components of Zn(II)-buffered media used for the Zn(II) competition with chelators with equimolar mixture of CD4RARH(Clover) and Lck(mRuby2) as well as CD4RARH(Clover) and Lck(TAMRA) with related pZn values. Samples were prepared in 50 mM HEPES buffer with I = 0.1 (from KNO_3) at pH 7.4. Measurements were taken at 25°C. Chelator and protein concentrations in each sample were 40 and 0.5 μM, respectively, with supplementation of 400 μM TCEP. Zn(II) transfer was neglected due to the high molar excess of chelator over peptides.

Total Zn(II) (μM)	pZn in EDTA[a]	pZn in HEDTA[b]	pZn in EGTA[c]
1	15.24	13.78	10.79
2	14.93	13.46	10.48
3	14.74	13.28	-
4	-	13.14	10.16
5	14.49	13.03	-
6	-	12.93	-
8	14.25	12.79	9.80
10	-	12.67	-
12	14.02	12.56	-
14		12.46	-
16	13.84	12.37	-
18	-	12.29	-
20	-	12.20	-
22	13.58	12.12	-
24	-	12.03	9.03
25	13.41	11.94	-
27	13.33	-	-
28	13.28	-	-
29	13.23	11.75	8.78
30	13.17	-	-
33	12.97	11.50	-
37	12.56	-	8.11

[a]Protonation and stability constants of EDTA: βHL = 10.17, βH2L = 16.28, βH3L = 18.96, βH4L = 20.96, βH5L = 22.47, βZnHL = 19.44, βZnL = 16.44; HEDTA: βHL = 9.81, βH2L = 15.18, βH3L = 17.78, βZnL = 14.60; EGTA: βHL = 9.40, βH2L = 18.18, βZnL = 12.60.[10]

Table S4. Conditional binding constants (K_{12}) of heterodimerization for zinc clasp-based systems from experiments in Zn(II)-buffered media are gathered. Corresponding $pZn_{0.5}$ (half of the complex formed) and Hill's coefficient values (n) of binding isotherms are indicated.

System	$pZn_{0.5}$	K_{12}	n	Methodology
Zn(CD4RARH)(Lck)	-	$5.10 \cdot 10^{18}$	-	PAR competition[a]
	12.07 ± 0.06	$9.33 \cdot 10^{17}$	1.37	circular dichroism[b]
$Zn(CD4RARH)_2$	8.79 ± 0.04	$1.00 \cdot 10^{14}$	1.27	circular dichroism[b]
ZnCD4RARH(Clover) Lck(mRuby2)	12.06 ± 0.04	$4.57 \cdot 10^{18}$	0.97	fluorimetry[b]
ZnCD4RARH(Clover) Lck(TAMRA)	11.99 ± 0.02	$3.89 \cdot 10^{18}$	1.10	fluorimetry[b]

[a] Binding constant calculated according to published protocol, ref 8.

[b] Binding constants calculated acoording to Eqs 2-5 (see Fluorimetry and Circular dichroism above).

Table S5. Kinetic parameters obtained from biolayer interferometry experiments. Kinetic constants (k_{on} and k_{off}) were calculated from the local full fitting to the 1:2 heterogeneous binding model (Figure S9). R_{max} is the maximum binding levels expressed in response units.

Analyte	Analyte molecular weight [kDa]	k_{on} [$M^{-1}s^{-1}$]	k_{on} error	k_{off} [s^{-1}]	k_{off} error	R_{max}
CD4RARH	4.3	$4.80 \cdot 10^{3}$ $3.43 \cdot 10^{3}$	$5.20 \cdot 10^{5}$ $8.67 \cdot 10^{3}$	$6.76 \cdot 10^{-4}$ $<1.0 \cdot 10^{-7}$	$1.69 \cdot 10^{-2}$ $2.20 \cdot 10^{-3}$	1.59 0.81
CD4RARH(BBL)	9.6	$5.34 \cdot 10^{3}$ $5.45 \cdot 10^{3}$	25.6 29.2	$1.21 \cdot 10^{-3}$ $1.31 \cdot 10^{-3}$	$7.54 \cdot 10^{-6}$ $8.31 \cdot 10^{-6}$	0.97 0.89
CD4RARH(Clover)	30.8	$3.34 \cdot 10^{3}$ $3.14 \cdot 10^{3}$	14.2 14.8	$2.29 \cdot 10^{-3}$ $2.32 \cdot 10^{-3}$	$3.30 \cdot 10^{-6}$ $3.25 \cdot 10^{-6}$	5.03 4.92
CD4RARH(Clover) lysate	30.8	$4.54 \cdot 10^{2}$ $7.52 \cdot 10^{2}$	77.7 98.9	$5.32 \cdot 10^{-3}$ $5.64 \cdot 10^{-3}$	$1.69 \cdot 10^{-4}$ $1.56 \cdot 10^{-4}$	17.51 11.78

Table S6. Sequences of primers (green) and cDNA encoding sequences (purple) used in this study (Genomed S.A. Poland and GenScript USA). FRW and REV refer to forward and reverse primers, respectively.

SapI-Clover-BamHI	
FRW	CCTAATTGCTCTTCCAACATGGTGAGCAAGGGCG
REV	CGATGGATCCCTTGTACAGCTCGTCCATGC
BamHI-CD4RARH-PstI	
FRW	GCTAGGATCCCGTGCCCGTCATCGTC
REV	GCGACTGCAGTTACGTTTTTTGAAAGCGCGC
SapI-mRuby2-BamHI	
FRW	CTATTGCTCTTCCAACATGGTGTCTAAGGGCGAGGAGCTG
REV	CGATGGATCCCTTGTACAGCTCGTCCATCCCACC
BamHI-Lck-PstI	
FRW	GCTAGGATCCAGCCATCCGGAAGATGACTG
REV	GCGACTGCAGTTAGGTACCTTTGCCGTCCAG
CD4RARH (pUC57)	
CGTGCCCGTCATCGTCGCCGTCAGGCGGAACGCATGAGCCAAATTAAACGTCTGC TGTCTGAAAAGAAAACCTGCCAGTGTCCGGCGCGCTTTCAAAAAACGTAA	
Lck (pUC57)	
AGCCATCCGGAAGATGACTGGATGGAAAACATTGATGTGTGCGAAAATTGTCACT ATCCGATCGTTCCGCTGGACGGCAAAGGTACCTAA	

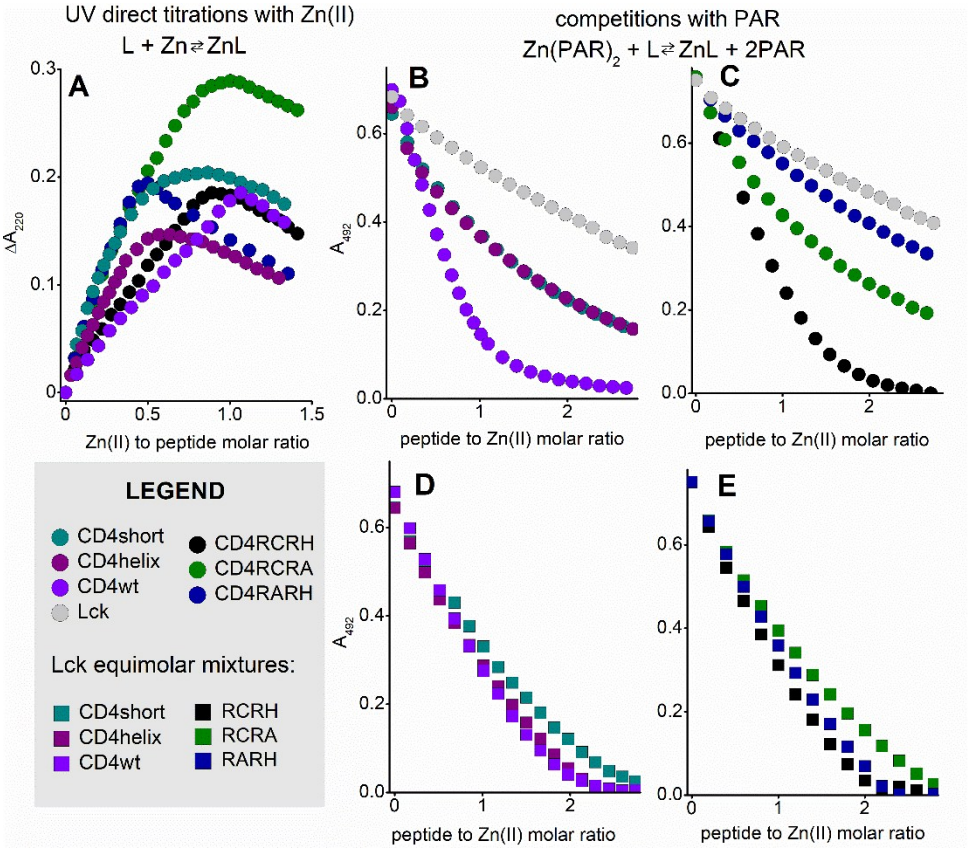


Figure S1. Spectroscopic experiments performed to determine stoichiometry and relative affinity of Zn(II) complexes. a) Stepwise titration of $ZnSO_4$ to the indicated peptide samples was conducted in the UV range (50 mM borate buffer, pH 7.4, 100 mM $NaClO_4$). b-e) Competition experiments with 100 µM chromophoric chelator PAR with 10 µM $ZnSO_4$ were performed in 50 mM HEPES, 100 mM KNO_3, and 400 µM TCEP, pH 7.4. Titration with equimolar mixture of CD4 and Lck resulted in a decrease of ZnH_xPAR_2 complex absorbance at 492 nm.[8]

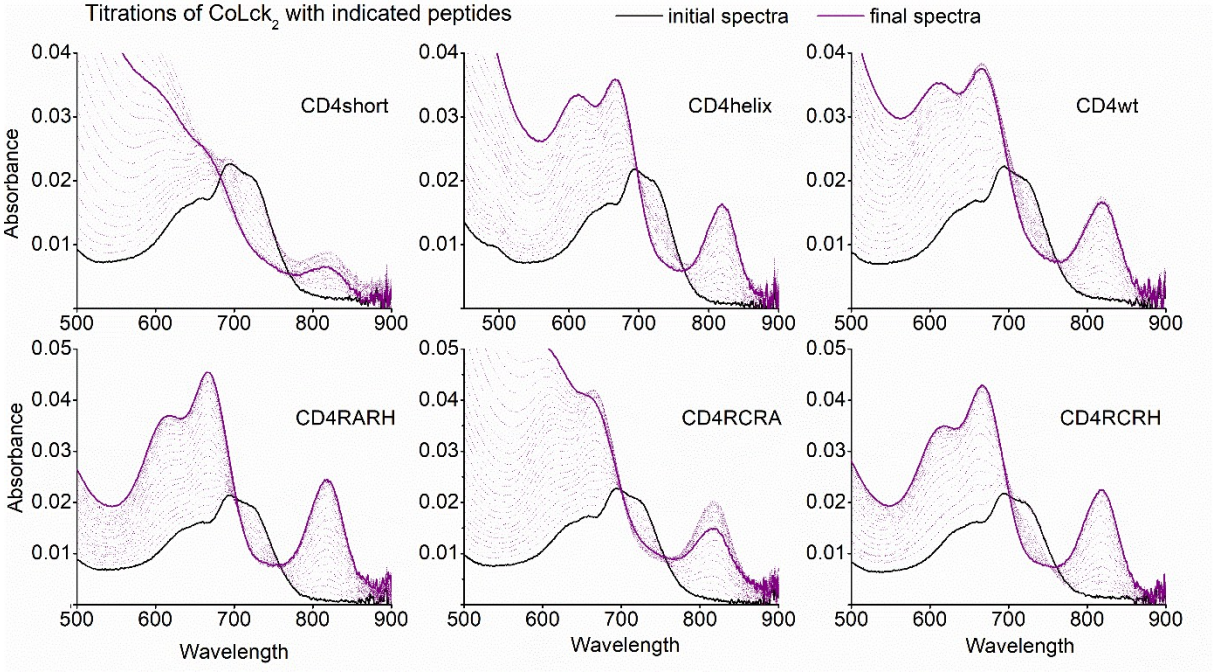


Figure S2. Spectra of 50 μM $Co(Lck)_2$ homodimer titrations with the indicated peptides in the spectral range of 500-900 nm (50 mM HEPES, 0.1 M KNO_3, 1 mM TCEP and pH 7.4 at 25°C). Initial and final spectra are presented in black and violet, respectively.

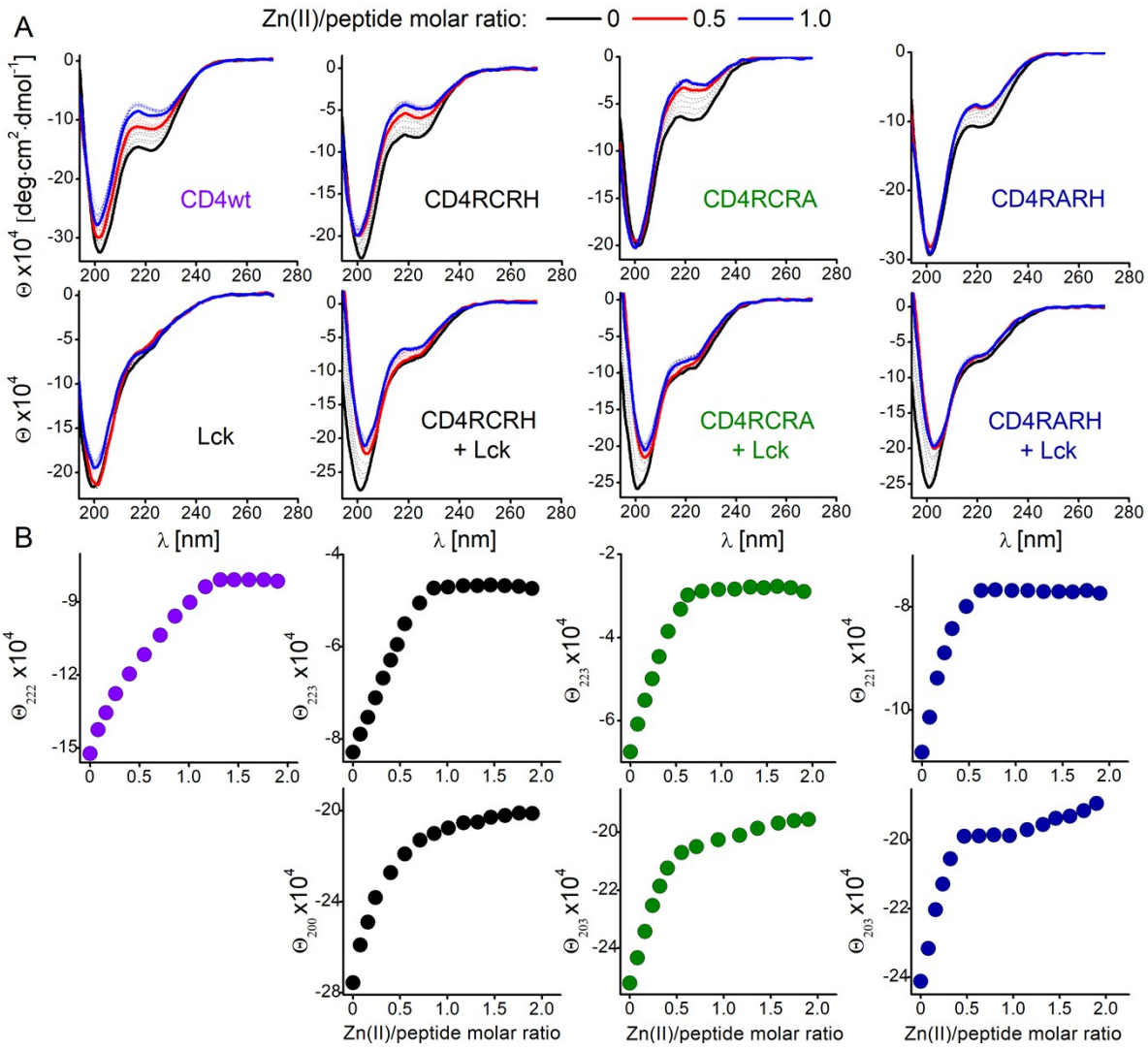


Figure S3. CD titrations of zinc clasp peptides with Zn(II) in 20 mM Tris·H_2SO_4 buffer, 0.1 M $NaClO_4$, TCEP (3 eq. over cysteine residues) and pH 7.4. A) Titrations of peptides with Zn(II) ions. Black color indicates initial spectra and red and blue color present the spectra at 0.5 and 1 Zn(II)-to-peptide molar ratio, respectively. B) The dependence of ellipticity at the selected wavelength according to the Zn(II) to peptide molar ratio. Order of graphs matches the subsection. B) Violet, black, olive and blue colors indicate the CD4wt, CD4RCRH, CD4RCRA and CD4RARH (or their Lck mixture), respectively.

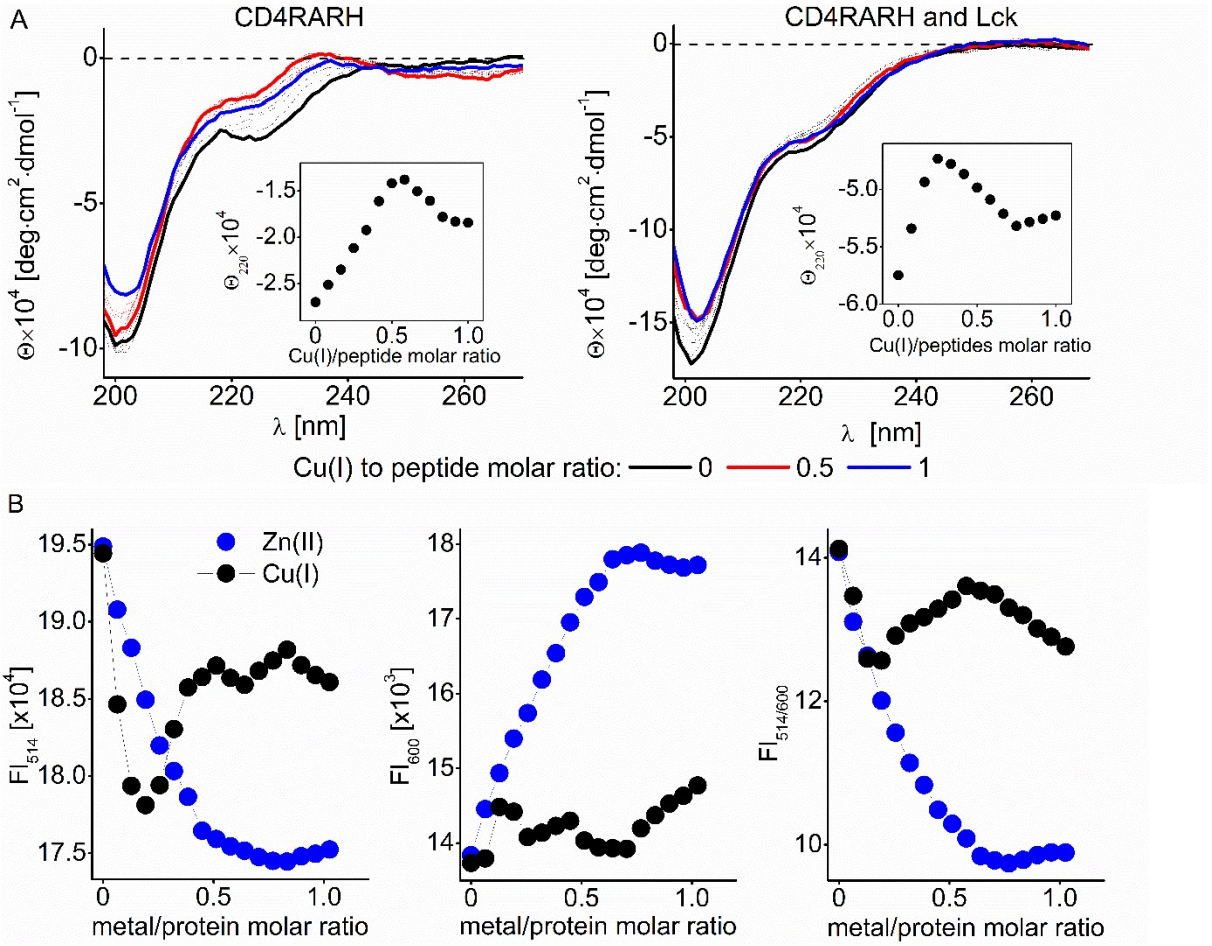


Figure S4. CD and fluorimetric titrations of CD4RARH and Lck peptides with Cu(I) in 20 mM Tris·H_2SO_4 buffer, 0.1 M NaClO4, TCEP (3 eq. over Cys residues), 10% MeCN and pH 7.4. A) CD-monitored titrations of CD4RARH and equimolar mixture of CD4RARH and Lck with Cu(I). Black color indicates initial spectra and red and blue color present the spectra at 0.5 and 1 Cu(I)-to-peptide molar ratio, respectively. Insets present changes of ellipticity at 220 nm as a function of Cu(I) to peptide molar ratio. B) Changes of fluorescence intensity presented at wavelengths of donor, acceptor and their ratio for titration of CD4RARH(Clover) and Lck(mRuby2) equimolar mixture with Zn(II) and Cu(I) ions (black and blue, respectively).

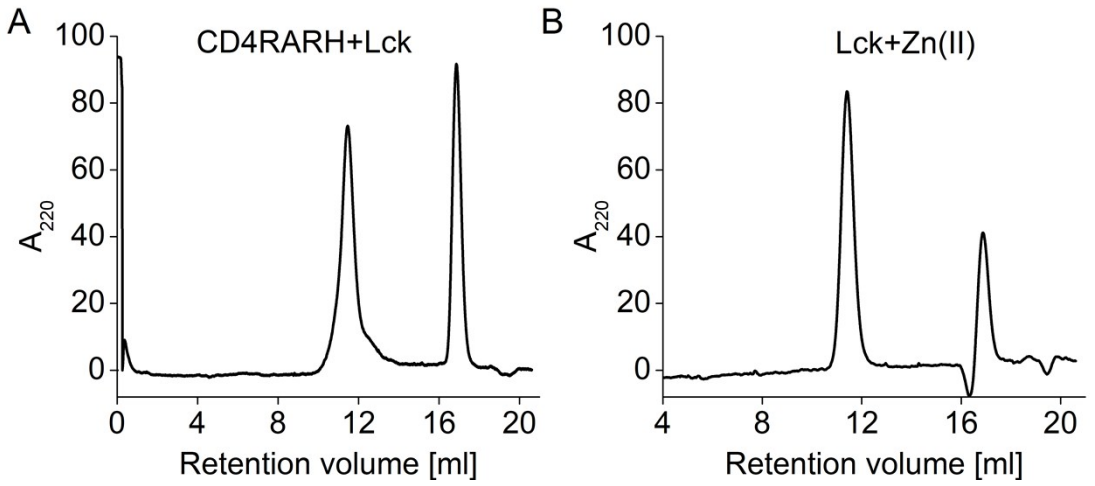


Figure S5. Negative controls for size exclusion chromatography of zinc clasp heterodimer formation. Addition of 3 eq. of TCEP over sulfhydryl groups was used in each sample. A) Chromatogram of CD4RARH and Lck equimolar mixture. B) Chromatogram of Lck sample with equimolar content of Zn(II). Samples were incubated for 45 min before injection (100 µl). Analysis was run at 0.5 ml/min in 100 mM Tris, 300 mM NaCl, pH 7.4.

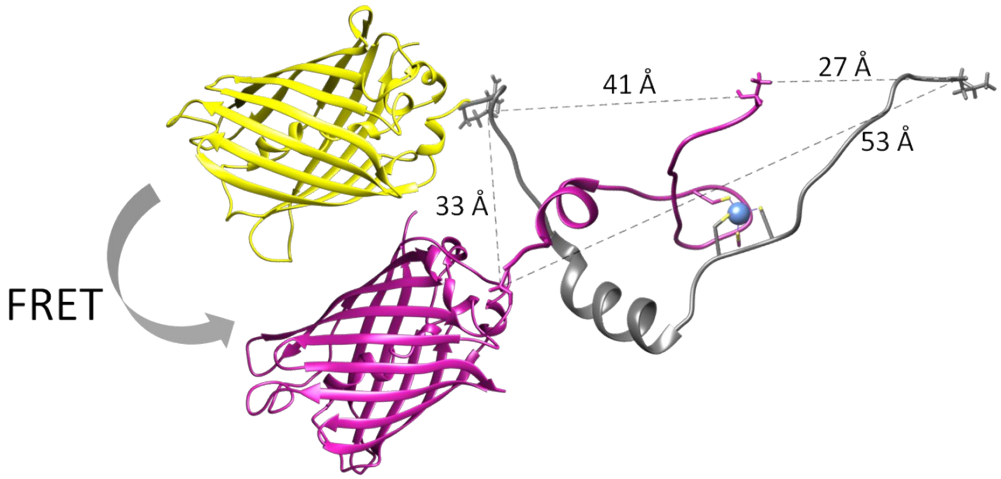


Figure S6. Schematic representation of fluorescently labeled CD4RARH(Clover) and Lck(mRuby2) in the complex with Zn(II). CD4RARH, Lck(mRuby2) and Clover are marked in grey, purple, and yellow, respectively. Distances between peptide ends are shown by dashed lines. Zn(II) is shown in blue. For the presentation the 1Q68 and 1GFL PDB file and UCSF Chimera 1.12 software were applied.

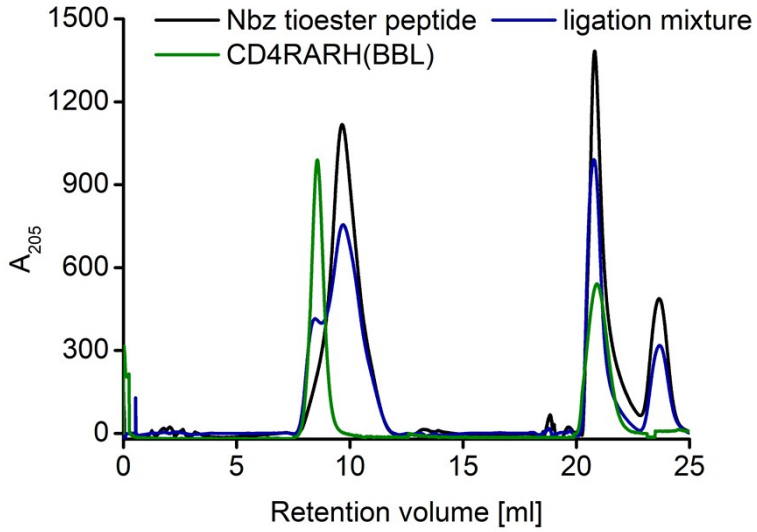


Figure S7. Size exclusion chromatograms of CD4RARH(Nbz) peptide in the ligation buffer (0.2 M phosphate buffer, 6 M guanidine hydrochloride, 0.2 M 4-mercaptophenylacetic acid, 50 mM DTT and pH 7.0)- black line. Blue and green line show the ligation mixture with (Cys)BBL addition in the 0.6-1 molar ratio and CD4RARH(BBL), respectively.[5] The isocratic gradient of 0.1% TFA with 0.5 ml/min was applied.

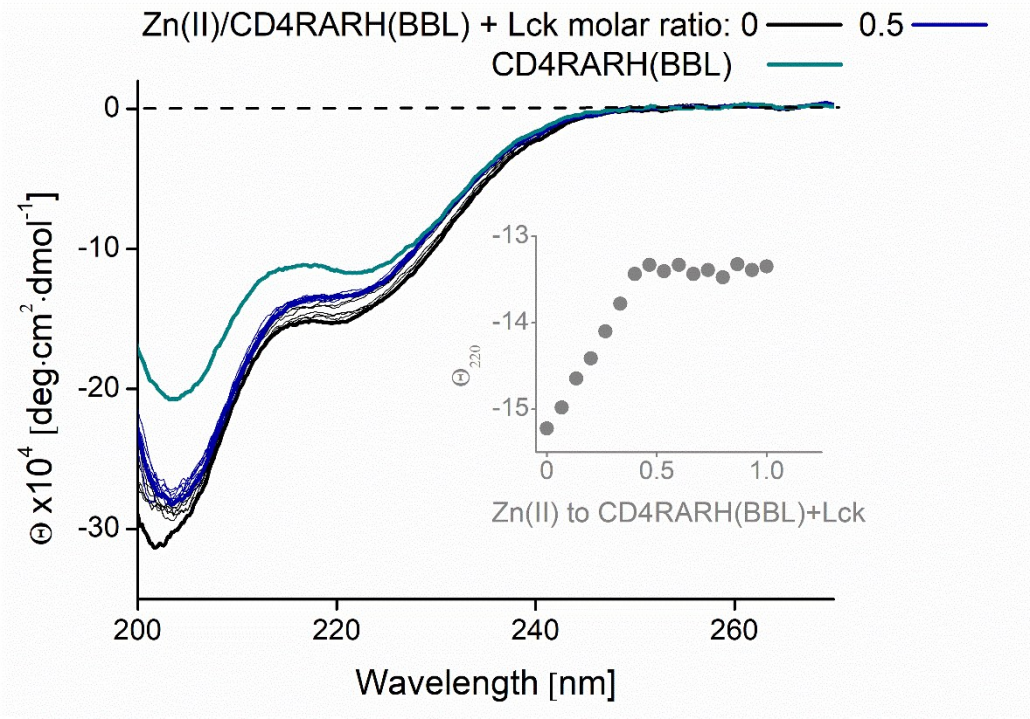


Figure S8. CD spectra of CD4RARH(BBL) and Lck equimolar mixture in a stepwise titration with Zn(II). Initial and final spectra are presented in black and blue, respectively. CD4RARH(BBL) spectrum is shown in cyan. The inset presents ellipticity changes at 220 nm with different ratios of Zn(II)-to-peptide mixture. Titration was conducted in 20 mM Tris, 10 mM $NaClO_4$, pH 7.4 at 25°C. Addition of 3 eq. of TCEP over sulfhydryl groups was used in each sample

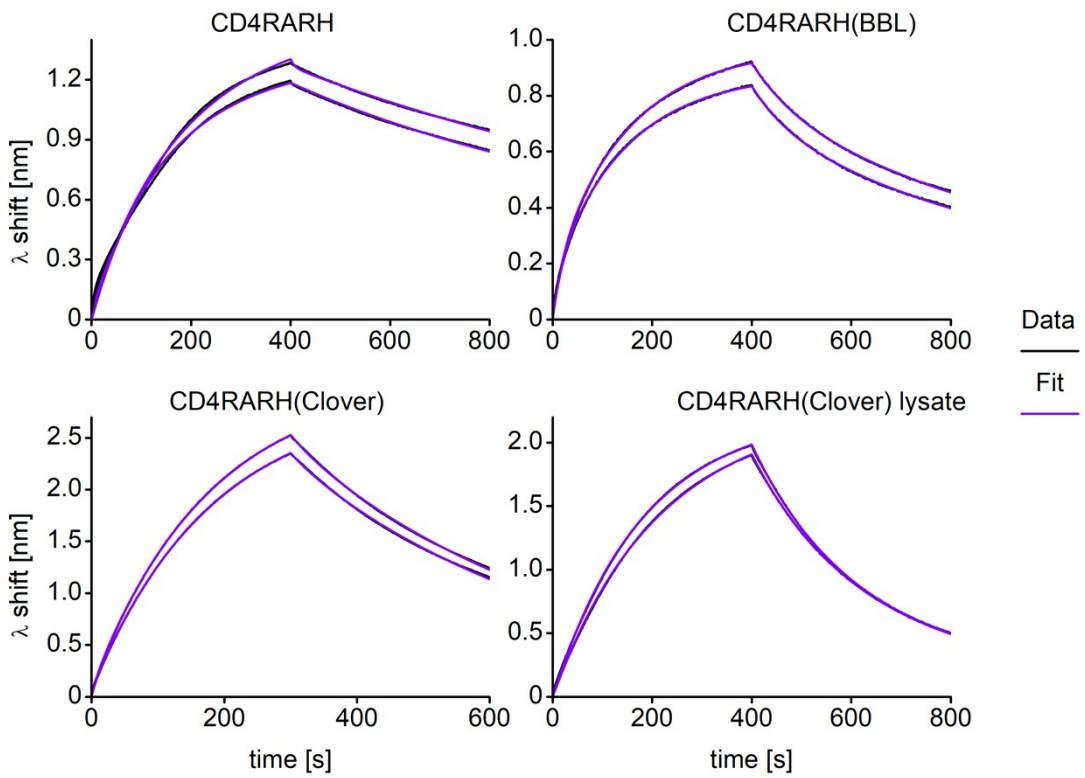


Figure S9. Raw data sensograms from biolayer interferometry measurements with real-time data acquisition of two-stage association and dissociation steps for CD4RARH, CD4RARH(BBL), CD4RARH(Clover) and the bacterial lysate with overexpressed CD4RARH(Clover) are shown in black. 1 μM ligands in 50 mM HEPES, 0.1 M KNO_3, 0.2% BSA (w/v), 0.1% PEG 3,300 (w/v), 0.05% Triton (v/v), 1 mM TCEP, 1 mM HEDTA, 0.8 mM $ZnSO_4$ with 1,000 rpm stirring at 25°C were used. Violet curves represent results of local full fitting to 1:2 heterogeneous model with Octet Data Analysis software.

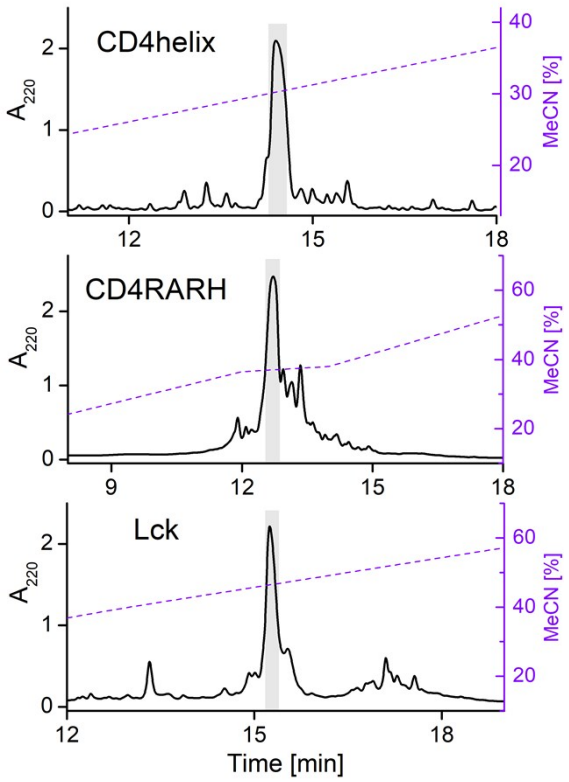


Figure S10. Example of chromatograms from CD4helix, CD4RARH and Lck peptides purification on a HPLC system in MeCN/0.1% TFA gradient (Table S1). Gray boxes indicate collection windows. Right Y-axis presents MeCN elution gradient (dashed line) and is colored in violet.

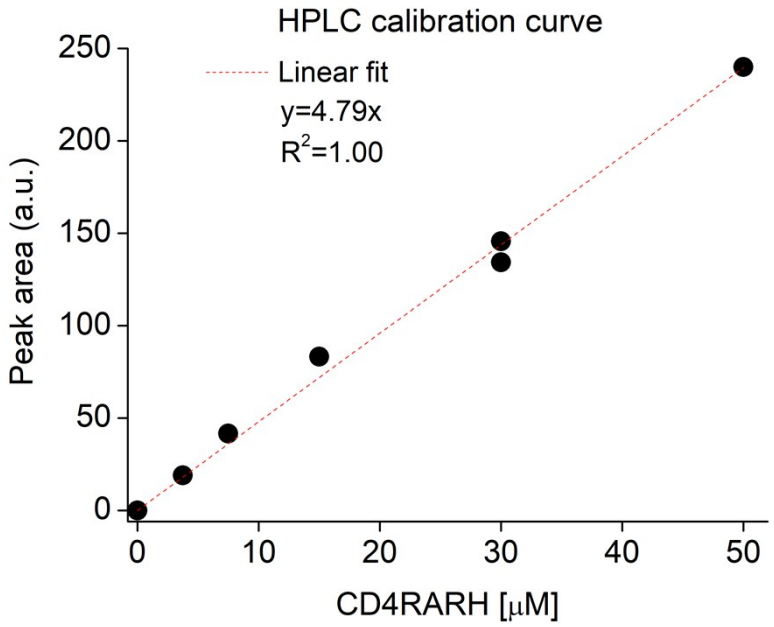


Figure S11. Calibration curve plotted with CD4RARH concentration against peak area. Samples were prepared in the range of 0-50 μM (50 mM HEPES, 100 mM KNO_3, 400 μM TCEP, pH 7.4) and acidified with 10% TFA to pH 2 before injection of 200 μl sample on C18 Column (Phenomenex, Dionex Ultimate 3000 HPLC system). Integration of peaks was performed with Chromeleon 6.80 software.

References:

[1] A. Kocyła, J. Adamczyk, A. Krężel, Interdependence of free zinc changes and protein complex assembly - insights into zinc signal regulation, *Metallomics*, 2018, **10**, 120-131.

[2] K. Kluska, J. Adamczyk, A. Krężel, Metal binding properties, stability and reactivity of zinc fingers, *Coord. Chem. Rev.*, 2018, **367**, 18-64.

[3] P. Eyer, F. Worek, D. Kiderlen, G. Sinko, A. Stuglin, V. Simeon-Rudolf and E. Reiner, Molar absorption coefficients for the reduced Ellman reagent: Reassessment, *Anal. Biochem.*, 2003, **312**, 224–227.

[4] A. Lam, F. St-Pierre, Y. Gong et al., Improving FRET dynamic range with bright green and red fluorescent proteins, *Nat. Methods*, 2012, **9**, 1005-1012.

[5] J. B. Blanco-Canosa, P. Dawson, An Efficient Fmoc-SPPS Approach for the Generation of Thioester Peptide Precursors for Use in Native Chemical Ligation, *Angew. Chem. Int. Ed.*, 2008, **120**, 6957-6961.

[6] K. Kluska, J. Adamczyk, A. Krężel, Metal binding properties of zinc fingers with a naturally altered metal binding site, *Metallomics*, 2018, **10**, 248-263.

[7] A. Krężel, R. Latajka, G. Bujacz, W. Bal, Coordination properties of Tris(2-carboxyethyl)phosphine, a newly introduced thiol reductant, and its oxide, *Inorg. Chem*., 2003, **42**, 1994-2003.

[8] A. Kocyła, A. Pomorski, A. Krężel, Molar absorption coefficients and stability constants of metal complexes of 4-(2-pyridylazo)resorcinol (PAR): Revisiting common chelating probe for the study of metalloproteins, *J. Inorg. Biochem.*, 2015, **152**, 82-92.

[9] L. Alderighi, P. Gans, A. Ienco, D. Peters, A. Sabatini, A. Vacca, Hyperquad simulation and speciation (HySS): a utility program for the investigation of equilibria involving soluble and partially soluble species, *Coord. Chem. Rev.*, 1999, **184**, 311-318.

[10] A. E. Martell and R. M. Smith, Critical Stability Constants, Plenum Press, New York, 1974.

[11] A. Krężel, W. Maret, The biological inorganic chemistry of zinc ions. *Arch Biochem Biophys*., 2016, **611**, 3-19.

[12] T. Kochańczyk, P. Jakimowicz, A. Krężel, Femtomolar Zn(II) affinity of minimal zinc hook peptides - a promising small tag for protein engineering, *Chem. Commun*. *(Camb.)*, 2013, **49**, 1312-1314.

[13] A. Pomorski, T. Kochańczyk, A. Miłoch and A. Krężel, Method for accurate determination of dissociation constants of optical ratiometric systems: Chemical probes, genetically encoded sensors, and interacting molecules, *Anal. Chem.*, 2013, **85**, 11479–11486.