Supplemental Information file

Post-translational acylation controls the folding and functions of the CyaA RTX toxin

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Supplemental Figure S1A: Annotated MS/MS spectrum showing the three distinct PTMs detected at residue K983 in the CyaA toxin: (A) unmodified residue, (B) myristoylated K983, (C) palmitoylated K983, and (D) palmitoleylated K983.



Supplemental Figure S1B: Annotated MS/MS spectrum showing the two PTMs detected at residue K960 in the CyaA toxin: (A) unmodified residue, (B) palmitoylated K860, and (C) palmitoleylated K860.



unmodified K860 (A) and K983 (B) residues in the pro-CyaA toxin.



Supplemental Figure S1D: Semi-quantification of K860 and K983 acylation of both CyaA and pro-CyaA monomers from high quality MS/MS spectra. The two lysine residues are always found unmodified in pro-CyaA and acylated in CyaA. We cannot rule-out the possibility that a small fraction of CyaA remains unmodified, but the abundance of such peptides appears too low to be detected by the latest generation of LC-MS Orbitrap instrument used in this study. Absence of PTM on the lysine residue is in blue, palmitoylation in red, palmitoyleylation in green and myristoylation in yellow. Maybe you can briefly explain how was done the quantification with the CID data set.



Supplemental Figure S2: Refolding of CyaA and pro-CyaA into a monomeric toxin. The protein is refolded by Size Exclusion Chromatography, using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare), as described in Karst *et al.*, 2014. Briefly, the Superdex 200 pg was equilibrated with buffer A (20 mM Hepes, 150 mM NaCl, pH 7.4), complemented with 2 mM CaCl₂. The sample consists of 5 mL of CyaA at 5 μ M or 5 mL of proCyaA at 2 μ M stored in 8 mM urea, 20 mM Hepes, pH 7.4. After elution, monomers and multimers were pooled separately. Proteins were then concentrated and their hydrodynamic radius measured. Proteins were aliquoted and stored at -20°C. Note the difference of elution volume between the two monomeric species, CyaA and pro-CyaA, in agreement with the AUC data (CyaA monomers are more compact than pro-CyaA monomers).

MQQSHQAGYANAADRESGIPAAVLDGIKAVAKEKNATLMFRLVNPHSTSLIAEGVATKGLGVHAKSSDWGLQAGYIPVNPNL	SKLFGRAPEVIARADNDVNSSLAHGHTAVDLTLSKERL
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80	85 90 95 100 105 110 115 120
DYLRQAGLVTGMADGVVASNHAGYEQFEFRVKETSDGRYAYQYRRKGGDDFEAVKVIGNAAGIPLTADIDMFAIMPHLSNFR	D S A R S S V T S G D S V T D Y L A R T R R A A S E A T G G L D R E R I D L
125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 200	205 210 215 220 225 230 235 240
L WK I A R A G A R S A V G T E A R R Q F R Y D G D M N I G V I T D F E L E V R N A L N R R A H A V G A Q D V V H G T E Q N N P F P E A D E K I F V V S A T G E S	QMLTRGQLKEYIGQQRGEGYVFYENRAYGVAGKSLFDD
245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320	325 330 335 340 345 350 355 360
G L G A A P G V P S G R S K F S P D V L E T V P A S P G L R P S L G A V E R Q D S G Y D S L D G V G S R S F S L G E V S D MA A V E A A E L E M T R Q V L H A G A	R Q D D A E P G V S G A S A HWG Q R A L Q G A Q A V A A A Q R L V H A I A
365 370 375 380 385 390 395 400 405 410 415 420 425 430 435 440	445 450 455 460 465 470 475 480
L M T Q F G R A G S T N T P Q E A A S L S A A V F G L G E A S S A V A E T V S G F F R G S S R W A G G F G V A G G A M A L G G G I A A A V G A G M S L T D D A P A G	Q K A A A G A E I A L Q L T G G T V E L A S S I A L A L A A A R G V T S G L
485 490 495 500 505 510 515 520 525 530 535 540 545 550 555 560	565 570 575 580 585 590 600
Q V A G A S A G A A A G A L A A L S P ME I Y G L V Q S H Y A D Q L D K L A Q E S S A Y G Y E G D A L L A Q L Y R D K T A A E G A V A G V S A V L S T V G A A V	SIAAAASVVGAPVAVVTSLLTGALNGILRGVQQPIIEK
605 610 615 620 625 630 635 640 645 650 655 660 665 670 675 680	685 690 695 700 705 710 715 720
LANDYARKIDELGGPQAYFEKNLQARHEQLANSDGLRKMLADLQAGWNASSVIGVQTTEISKSALELAAITGNADNLKSVDV	F V D R F V Q G E R V A G Q P V V L D V A A G G I D I A S R K G E R P A L T
725 730 735 740 745 750 755 760 765 770 775 780 785 790 795 800	805 810 815 820 825 840
FITPLAAPGEEQRRRTKTGKSEFTTFVEIVGKQDRWRIRDGAADTTIDLAKVVSQLVDANGVLKHSIKLDVIGGDGDDVVLA	NASRIHYDGGAGTNTVSYAALGRQDSITVSADGERFNV
845 850 855 860 85 870 875 880 885 900 905 910 915 920	925 930 935 940 945 950 955 960
R K Q L NN A N V Y R E G V A T Q T T A Y G K R T E N Y Q Y R H Y E L A R Y G Q Y E Y D T L E H Y Q H I I G G A G N D S I T G N A H D N F L A G G S G D D R L D G	G A G N D T L V G G E G Q N T V I G G A G D D V F L Q D L G V W S N Q L D G
965 970 975 980 985 990 995 1000 1005 1010 1015 1020 1025 1030 1035 1040	1045 1050 1055 1060 1065 1070 1075 1080
G A G V D T V K Y N V H Q P S E E R L E R MG D T G I H A D L Q K G T V E K W P A L N L F S V D H V K N I E N L H G S R L N D R I A G D Q D N E L WG H D G N D T	IRGRGGDDILRGGLGLDTLYGEDGNDIFLQDDETVSDD
1085 1090 1095 1100 1105 1110 1115 1120 1125 1130 1135 1140 1145 1150 1155 1160	1165 1170 1175 1180 1185 1190 1195 1200
I D G G A G L D T V D Y S A M I H P G R I V A P H E Y G F G I E A D L S R E WY R K A S A L G Y D Y Y D N Y R N Y E N Y I G T S M K D Y L I G D A Q A N T L MG Q G	G D D T V R G G D G D D L L F G G D G N D M L Y G D A G N D T L Y G G L G D
1205 1210 1215 1220 1225 1230 1235 1240 1245 1250 1255 1260 1265 1270 1275 1280	1285 1290 1295 1300 1305 1310 1315 1320
D T L E G G A G ND WF G Q T Q A R E HD V L R G G D G V D T V D Y S Q T G A H A G I A A G R I G L G I L A D L G A G R V D K L G E A G S S A Y D T V S G I E N V V	G T E L A D R I T G D A Q A N V L R G A G G A D V L A G G E G D D V L L G G
1325 1330 1335 1340 1345 1350 1355 1360 1365 1370 1375 1380 1385 1390 1395 1400	1405 1410 1415 1425 1430 1435 1440
DGDDQLSGDAGRDRLYGEAGDDWFFQDAANAGNLLDGGDGRDTVDFSGPGRGLDAGAKGVFLSLGKGFASLMDEPETSNVLR	N I E NA V G S A R DD V L I G D A G A N V L NG L A G N D V L S G G A G D
1445 1450 1455 1460 1465 1470 1475 1480 1485 1490 1495 1500 1505 1510 1515 1520	1525 1530 1535 1540 1545 1550 1555 1560
D V L L G D E G S D L L S G D A G N D D L F G Q G D D T V L F G V G Y G H D T I Y E S G G G H D T I R I N A G A D Q L W F A R Q G N D L E I R I L G T D A L T V	HDWYRDADHRVEIIHAANQAVDQAGIEKLVEAMAQYPD
1565 1570 1575 1580 1585 1590 1595 1600 1605 1610 1615 1620 1625 1630 1635 1640	1645 1650 1655 1660 1665 1670 1675 1680
P G A A A A P P A A R V P D T L M Q S L A V NW R 1685 1690 1695 1700 1705	
Total: 172 Peptides, 85.7% Coverage, 1.55 Redundancy	

Supplemental Figure S3: Peptide map of full-length CyaA in the presence of urea. A sequence coverage map was determined after 2 min digestion with pepsin. Each blue bar represents a single CyaA peptide. A linear sequence coverage of 85.7% was achieved. The position of the post-translational acylation sites at K860 and K983 are highlighted by gold stars.

MQQSHQAGYANAADRESGIP	A A V L D G I K A V A	A K E K N A T L M F	RLVNPHSTSL	I A E G V A T I	GLGVHAKS	SDWGLQA	G Y I P V N P N	LSKLFGF	8 A P E V I J	8 R A D N D V	/ N S S L A H G	H T A V D L	T L S K E R L
5 10 15 20	25 30	35 40	4550	55	60 65	70	75 80	85	90	95 10	0 105	110	115 120
D Y L R Q A G L V T G M A D G V V A S N	HAGYEQFEFR	/ KETSDGRYA	V Q Y R R K G G D D	FEAVKVI	5 N A A G I P L T	ADIDMFA	IMPHLSNF	R D S A R S S	V T S G D S	215 2	NRTRRAAS	E A T G G L	DRERIDL
125 130 135 140	0 145 150	155 160	165 17	0 175	180 185	190	195 200	205	210		10225	230	235 240
LWKIARAGARSAVGTEARRQ	FRYDGDMNIG	VITDFELEVR	NALNRRAHAV	G A Q D V V Q I	HGTEQNNPF	PEADEKI	F V V S A T G E	SQMLTRO	5 Q L K E Y 1	IGQQRGE	GYVFYEN	R A Y G V A	G K S L F D D
245 250 255 260	265 270	275 280	285 29	0 295	300 305	310	315 320	325	330	335 34	0 345	350	355 360
G L G A A P G V P S G R S K F S P D V L	ETVPASPGLRF	R P S L G A V E R Q	D S G Y D S L D G V	GSRSFSL	5 E V S D M A A V	EAAELEM	TRQVLHAG	A R Q D D A E	PGVSG	455 46	0 R A L Q G A Q	A V A A A Q	RLVHAIA
365 370 375 380	385 390	395 400	405 41	0 415	420 425	430	435 440	445	450		0 465	470	475 480
LMTQFGRAGSTNTPQEAASL	SAAVFGLGEAS	S A V A E T V S G	FFRGSSRWAG	G F G V A G G J	AMALGGGIA	A A V G A G M	SLTDDAPA	G Q K A A A G	570	QLTGGT\	ELASSIA	LALAAA	RGVTSGL
485 490 495 50	0 505 510	515 520	525 53	0 535	540 545	550	555 560	565	570	575 58	0 585	590	595 600
Q V A G A S A G A A A A G A L A A A L S P	MEIYGLVQQSH	HYADQLDKLA	Q E S S A Y G Y E G	DALLAQL	(RDKTAAEG	A V A G V S A	VLSTVGAA	V S I A A A A	690 S V V G A I	9 V A V V T S	ULTGALN	G I L R G V	Q Q P I I E K
605 610 615 620	0 625 630	635 640	645 65	0 655	660 665	670	675 680	685		695 70	0705	710	715 720
LANDYARKIDELGGPQAYFE	KNLQARHEQLA	ANSDGLRKML	ADLQAGWNAS	SVIGVQT	TEISKSALE	L A A I T G N .	A D N L K S V D	V F V D R F \	QGERV	GQPVVI	. D V A A G G I	DIASRK	GERPALT
725 730 735 740	745 750	755 760	76577	0 775	780 785	790	795 800	805	810	815 82	0 825	830	835 840
FITPLAAPGEEQRRRTKTGK	SEFTTFVEIVO	GKQDRWRIRD	GAADTTIDLA	KVVSQLVI	0 A N G V L K H S	I K L D V I G	G D G D D V V L .	A N A S R I H	H Y D G G A (G T N T V S Y	AALGRQD	SITVSA	DGERFNV
845 850 855 860	865 870	875 880	885 89	0 895	900 905	910	915 920	925	930	935 94	0 945	950	955 960
R K Q L N N A N V Y R E G V A T Q T T A	Y G K R T E N V Q Y F	R H V E L A R V G Q	V V E V D T L E H V	QHIIGGA	5 N D S I T G N A	H D N F L A G	G S G D D R L D	G G A G N D 1	L V G G E (G Q N T V I C	GAGDDVF	L Q D L G V	W S N Q L D G
965 970 975 980	985 990	995 1000	1005 101	10 1015	1020 1025	1030	1035 1040	1045	1050	1055 10	50 1065	1070	1075 1080
G A G V D T V K Y N V H Q P S E E R L E	RMGDTGIHADI	Q K G T V E K W P	ALNLFSVDHV	KNIENLH	5 S R L N D R I A	G D D Q D N E	L W G H D G N D	T I R G R G G	5 D D I L R (G L G L D 1	TLYGEDGN	D I F L Q D	DETVSDD
1085 1090 1095 110	0 1105 1110	1115 1120	1125 113	30 1135	1140 1145	1150	1155 1160	1165	1170	1175 11	80 1185	1190	1195 1200
I D G G A G L D T V D Y S A M I H P G R	IVAPHEYGFG1	IEADLSREWV	R K A S A L G V D Y	Y D N V R N V I	NVIGTSMK	D V L I G D A	Q A N T L M G Q	G G D D T V F	R G G D G D I) L L F G G E	GNDMLYG	D A G N D T	LYGGLGD
1205 1210 1215 122	0 1225 1230	1235 1240	1245 125	50 1255	1260 1265	1270	1275 1280	1285	1290	1295 13	00 1305	1310	1315 1320
D T L E G G A G N D W F G Q T Q A R E H	D V L R G G D G V D 1	TVDYSQTGAH.	AGIAAGRIGL	GILADLG	A G R V D K L G E	A G S S A Y D	T V S G I E N V	V G T E L A E	0 R I T G D /	QANVLF	8 G A G G A D V	L A G G E G	D D V L L G G
1325 1330 1335 134	0 1345 1350	1355 1360	1365 137	70 1375	1380 1385	1390	1395 1400	1405	1410	1415 14	20 1425	1430	1435 1440
D G D D Q L S G D A G R D R L Y G E A G	D D W F F Q D A A N A	AGNLLDGGDG	R D T V D F S G P G	RGLDAGA	KGVFLSLGK	G F A S L M D	E P E T S N V L	R N I E N A \	/ G S A R D I	0 V L I G D A	G A N V L N G	L A G N D V	LSGGAGD
1445 1450 1455 146	0 1465 1470	1475 1480	1485 149	90 1495	1500 1505	1510	1515 1520	1525	1530	1535 15	40 1545	1550	1555 1560
DVLLGDEGSDLLSGDAGNDD	L F G G Q G D D T Y I	FGVGYGHDT	I Y E S G G G H D T	IRINAGAI	Q L W F A R Q G	NDLEIRI	LGTDDALT	V H D W Y R C	0 A D H R V I	IIHAAN	IQAVDQAG	I E K L V E	АМАQҮРD
1565 1570 1575 158	0 1585 1590	1595 1600	1605 161	LO 1615	1620 1625	1630	1635 1640	1645	1650	1655 16	50 1665	1670	1675 1680
P G A A A A A P P A A R V P D T L M Q S 1685 1690 1695 170	LAVNWR 0 1705												

Total: 141 Peptides, 91.0% Coverage, 1.26 Redundancy

Supplemental Figure S4: Peptide map of native CyaA monomers. A sequence coverage map was determined after 2 min digestion with pepsin. Each blue bar represents a single CyaA peptide. A linear sequence coverage of 91.0% was achieved. The position of the post-translational acylation sites at K860 and K983 are highlighted by gold stars.



Supplemental Figure S5: Differences in amide hydrogen exchange of CyaA at varying urea concentrations. The structural organization of each domain is displayed in Panel A. Selected peptides displaying unique HDX-MS behaviors in each of the AC, HR and RD domains are given in Panel B. Buffer: buffer A, complemented with 2 mM CaCl2.



Supplemental Figure S6: Differences in amide hydrogen exchange of CyaA at varying urea concentrations in the absence of calcium. The structural organisation of each domain is displayed in Panel A. The HDX-MS behaviour of CyaA in various concentrations of urea is displayed in Panel B. It is evident that in the absence of calcium, no dynamic HDX-MS behaviour is observed in any region of the protein, regardless of the urea concentration. Buffer: buffer A (20 mM Hepes, 150 mM NaCl, pH 7.4), complemented with 2 mM EDTA.



Supplemental Figure S7: Effect of Calcium on the deuterium uptake behavior of CyaA in 6M urea. (A,B) Deuterium uptake profiles of CyaA monitored for each peptide in the presence of 2 mM EDTA (A) or 2 mM Calcium (B). (C) Fractional uptake differential plot generated by subtracting the uptake measured for each peptide and at each time point in the absence (EDTA) and presence of Calcium. A positive difference value indicates a calcium-induced reduction of solvent accessibility.



Supplemental Figure S8: Effect of Calcium on the deuterium uptake behavior of CyaA in 3M urea. (A,B) Deuterium uptake profiles of CyaA monitored for each peptide in the presence of 2 mM EDTA (A) or 2 mM Calcium (B). (C) Fractional uptake differential plot generated by subtracting the uptake measured for each peptide and at each time point in the absence (EDTA) and presence of Calcium. A positive difference value indicates a calcium-induced reduction of solvent accessibility.



Supplemental Figure S9: Effect of Calcium on the deuterium uptake behavior of CyaA in 1.6M urea. (A,B) Deuterium uptake profiles of CyaA monitored for each peptide in the presence of 2 mM EDTA (A) or 2 mM Calcium (B). (C) Fractional uptake differential plot generated by subtracting the uptake measured for each peptide and at each time point in the absence (EDTA) and presence of Calcium. A positive difference value indicates a calcium-induced reduction of solvent accessibility.



Supplemental Figure S10: Differences in amide hydrogen exchange of pro-CyaA at varying urea concentrations. The structural organisation of each domain is displayed in Panel A. The HDX-MS behaviour of pro-CyaA in various concentrations of urea is displayed in Panel B. At 6 M urea, no dynamic HDX-MS behaviour is observed throughout the protein. At 3 M urea, only the RD region displays dynamic activity. At 1.6 M urea, the AC, HR and RD domains have dynamic HDX-MS events. Buffer: buffer A, complemented with 2 mM CaCl2.



Supplemental Figure S11: Differences in amide hydrogen exchange of pro-CyaA at varying urea concentrations. The structural organization of each domain is displayed in Panel A. Selected peptides displaying unique HDX-MS behaviors in each of the AC, HR and RD domains are given in Panel B. Note that the uptake difference 6 M - 3 M in pro-CyaA shows no fractional uptake difference in HR (see the red box) while in CyaA it does (Supplemental Figure S5A). Buffer: buffer A, complemented with 2 mM CaCl2.



Supplemental Figure S12: Differences in HDX-MS behaviour between monomeric pro-CyaA and CyaA. Panel A displays peptides located in both the HR and AR domains and which give the greatest differences in HDX-MS activity between the two proteins. Panel B displays a Logit representation of the data. Peptides within the AR and HR domains cluster together and independently of each other, demonstrating significant and unique HDX-MS behaviours between the two.



Supplemental Figure S13: Hydrophobicity of the primary structure of the CyaA toxin computed using TMHMM (red trace) and PHD transmembrane helix (blue) prediction servers (1, 2). Major changes observed by HDX-MS between the hydrophobic regions of CyaA and pro-CyaA monomers (peptides 539-546, 571-582, 615-622, 682-696, 686-696 and 702-710 see Figures 5 and 6 for details) are located within the predicted hydrophobic regions. TMHMM is available here:

http://www.cbs.dtu.dk/services/TMHMM/

PHD transmembrane helix prediction is available here:

https://npsa-prabi.ibcp.fr/cgi-bin/npsa automat.pl?page=/NPSA/npsa htm.html

	Proteins	Assay conditions	Hemolysis (%)	Bound Activity (% total)	Internalized Activity (% total)
1	CyaA in urea	CaCl ₂	87 ± 8	2.14	1.55
		EDTA	< 2.0	0.02	< 0.01
2	Monomeric CyaA	CaCl ₂	100 ± 6	2.70	0.85
		EDTA	< 2.0	0.34	< 0.01
3	Multimeric CyaA	CaCl ₂	14 ± 9	2.93	< 0.01
		EDTA	< 2.0	0.39	< 0.01
4	Pro-CyaA in urea	CaCl ₂	< 2.0	0.03	< 0.01
		EDTA	< 2.0	< 0.01	< 0.01

Supplemental Table S1: Hemolytic and cytotoxic activities of the different CyaA species. Toxin binding and translocation into sheep erythrocytes were assayed essentially as described in Karimova et al., 1998 (3). Adenylate cyclase activity was measured as described in Ladant et al., 1988 (4): 1 unit of adenylate cyclase activity corresponds to 1 µmol of cAMP formed in min at 30°C and pH 8.0. The different CyaA preparations were diluted directly to final concentrations of 5.6-11 nM (1-2 µg/mL) into suspensions of sheep erythrocytes (5×10⁸/mL) in buffer A (20 mM Hepes-Na, pH 7.5, 150 mM NaCl) supplemented with either 2 mM CaCl₂ or 1 mM EDTA and incubated at 30°C for 20 min. An aliquot was removed to determine the total adenylate cyclase activity added to each sample. The cell suspensions were chilled on ice and centrifuged at 4°C, and the pelleted cells were resuspended in buffer A and separated into two batches. One batch was centrifuged again and the pelleted cells were lysed with 0.1% Tween 20. The enzymatic activity measured in this extract corresponds to that of the toxin bound to the cells and was expressed as a percentage of total activity added to the cells. To the second batch, 20 µg of TPCK (L-1-(tosylamino)-2phenylethyl chloromethyl ketone)-treated trypsin (Sigma) were added and the mixture was incubated for 10 min at room temperature to digest the adenylate cyclase that remained at the external surface of the erythrocytes. After addition of soybean trypsin inhibitor (5- fold excess), the erythrocytes were washed again with buffer A and then lysed with 0.1% Tween 20. The adenylate cyclase activity protected from trypsin digestion, corresponding to the internalized AC activity, was then measured and expressed as a percentage of total activity added. Activities are expressed as percentages of total CyaA activity added to the erythrocytes suspension (taken as 100%) and represent the average values (with standard deviations below 20 %) from at least two independent measurements. Results are in good agreement with prior studies that showed that about 2-3 % of total CyaA can bind to erythrocytes while 0.5-1.5 % can be internalized in the presence of calcium (3, 5). The hemolytic activity was measured after overnight incubation at 37°C with 60 nM of indicated proteins by quantifying the amount of hemoglobin released at 540 nm (and of intracellular content released at 405 nm). Complete lysis (corresponding to 100% lysis) was obtained by the addition of 0.1% Triton.

Protein species	CyaA monomers	Pro-CyaA monomers
Sedimentation coefficient, S	7.4 ± 0.1	7.1 ± 0.1
R _H , nm	5.5 ± 0.1	5.8 ± 0.1
Solvent density, g.mL ⁻¹	1.006	1.006
Solvent viscosity, g.cm ⁻¹ .s ⁻¹	0.013	0.013
Molecular mass, kDa	177	177
Partial specific volume, mL.g ⁻¹	0.725	0.725

Supplemental Table S2: Hydrodynamic parameters of CyaA and pro-CyaA monomers measured by AUC. AUC velocity analysis was performed at 20°C as described in Material and Methods. Buffer: 20 mM Hepes, 150 mM NaCl, 2 mM CaCl₂, pH 7.4. Experimental details are provided in the material and methods section.

Units used for AUC experiments:

Sedimentation coefficient, S, Svedberg, 10⁻¹³ sec

Hydrodynamic radius of the protein, R_H, nm

Solvent density, ρ , g/mL

Solvent viscosity, η , poise (g.cm⁻¹.s⁻¹)

Molecular Mass, M, kDa

Partial specific volume, $\bar{\nu}$, mL/g

Supplemental references

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