Supplemental Information

SUBSTRATE SPECIFICITY OF HUMAN CARBOXYPEPTIDASE A6

Peter J. Lyons and Lloyd D. Fricker

Department of Molecular Pharmacology
Albert Einstein College of Medicine, Bronx, NY 10461

Contents:

Figure S1. Expression and purification of CPA6. (A) Conditioned media (IN) and Talon metal-affinity chromatography flow-through (FT) and eluate (E1-3) fractions were resolved by SDS-PAGE. Total protein was stained with Coomassie blue and C-terminally HA and His6 tagged CPA6 detected by western blotting using an anti-HA antibody. Approximate protein size is shown in kDa on left. (B) Metal affinity column eluates were pooled and further purified using heparin affinity chromatography. Carboxypeptidase activity was measured by incubating 10 ul of each fraction with 1 ml 0.5 mM FA-Phe-Phe for 30 minutes at 37°C. IN, input; FT, flow-through; W, wash; E1-3, elution in 50 mM Tris and 600 mM NaCl, E4-8, elution in 50 mM Tris and 800 mM NaCl; E9, elution in 50 mM Tris and 1 M NaCl. Asterisks in (A) and arrow in (B) indicate Coomassie stained bands corresponding to CPA6 protein.

Figure S1

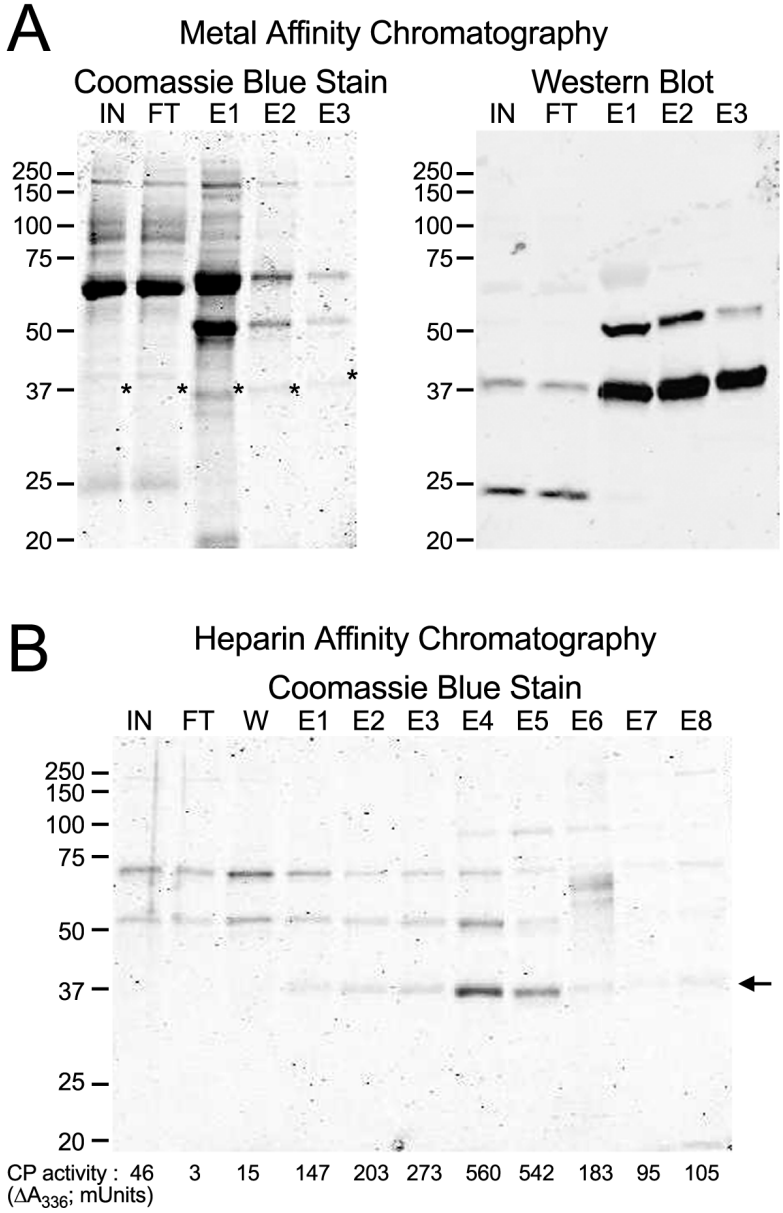

Figure S2. Binding to potato carboxypeptidase inhibitor (PCI) indicates correct folding of purified CPA6. Purified CPA6 (0.5 ml in 50 mM Tris pH 7.5, 500 mM NaCl) was passed through an equilibrated 0.5 mL column of PCI-Sepharose (IN, input; FT, flow-through). The column was washed with 3 ml of wash buffer (50 mM Tris pH 7.5, 500 mM NaCl) and 1 ml fractions collected (W1-3). The column was then eluted with 2.5 ml of 50 mM sodium phosphate pH 12.0, 500 mM NaCl, and 0.5 ml fractions collected (E1-5). Finally, the column was stripped with 1 ml of 1% SDS (E6). 15 µl of each fraction was resolved by SDS-PAGE and HA-tagged CPA6 detected by western blotting. 10 µl of each fraction was also incubated with 1 ml 0.4 mM FA-Phe-Phe in 50 mM Tris-HCl, pH 7.8, and 150 mM NaCl for 2 hrs at 22°C to determine relative activities. Enzymatic activity is shown below each lane of the western blot. nd, not detectable or < 10 mUnits, which is the limit of accurate detection.

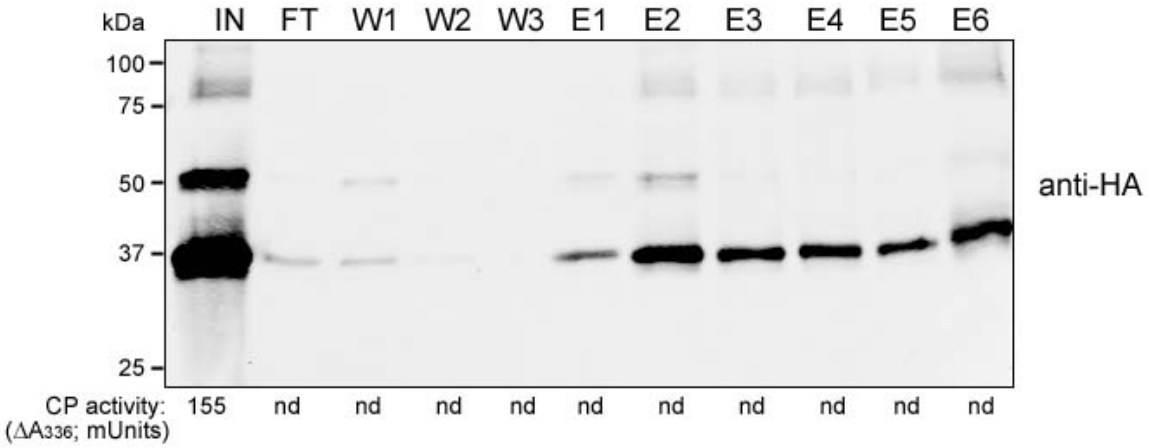

Figure S3. Effect of zinc on CPA6 catalytic activity. CPA6 cleavage of 0.4 mM FA-Phe-Phe at pH 7.8 was performed in the presence of 10 nM to 1 mM $ZnSO_4$ for the indicated times at 22°C. Low concentrations of $ZnSO_4$ (10 nM to 1 µM) had no effect on CPA6 activity, while 10 µM to 1 mM $ZnSO_4$ caused an inhibition of CPA6 activity.

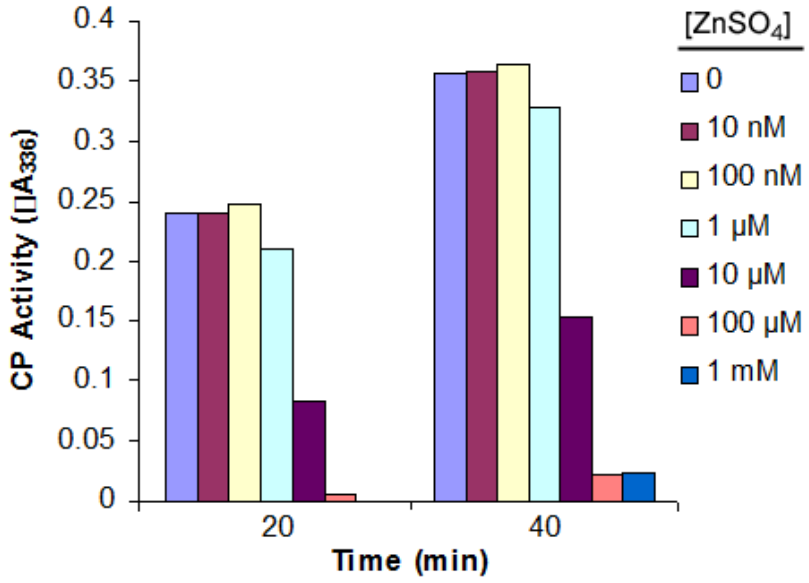

Figure S4. Purified CPA6 exhibits similar catalytic activity as ECM-bound CPA6. HEK293T cells were grown in 6-well plates and transiently transfected with a plasmid encoding CPA6-HAH6. After incubation for 48 hours, cells were removed, wells thoroughly washed, and ECM-bound CPA6 incubated with 0.4 mM FA-FF in 50 mM Tris, pH 7.8, 150 mM NaCl for two hours at 22°C while slowly shaking. Purified CPA6 (two-fold increments from 5.5 to 180 ng) was also incubated in tubes in the same manner. CP activity was measured by the change in substrate absorbance at 336 nm. Following these incubations, hot SDS-PAGE sample buffer was added to the wells to extract ECM, and to equivalent amounts of purified CPA6. These samples were resolved by SDS-PAGE and CPA6 identified by western blotting using an antibody raised against the HA epitope. As only 20% of total ECM was loaded on the gel, but all ECM incubated with substrate, enzyme activity values for ECM-bound CPA6 are shown adjusted for the amount of protein shown on gel. These results suggest that ECM-bound CPA6 exhibits generally similar activity as the purified enzyme, and that the low activity observed for purified CPA6 (relative to CPA1, 2, and 4; see Table S2) is likely to be physiologically relevant. Abbreviations: nd, not detectable or < 10 mUnits, which is the limit of accurate detection.

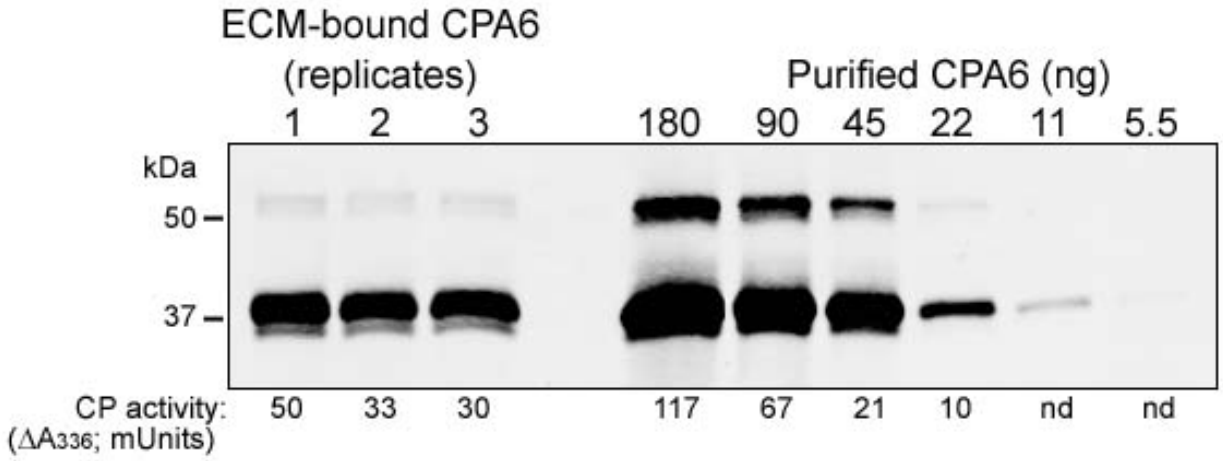

Table S1. Non-substrates of CPA6

Protein name	Peptide name	Sequence	z	T	Obs M	Theor M	ppm	Ratio, CPA4/no enzyme		
								Low CPA6	Med CPA6	High CPA6
Cerebellin 4	63-77	AANSKVAFSAVRSTN	3	2	1521.79	1521.790	0	1.13	0.79	0.99
Cerebellin 4	64-77	ANSKVAFSAVRSTN	3	2	1450.74	1450.753	-7	1.04	1.11	1.29
Cerebellin 4	66-77	SKVAFSAVRSTN	3	2	1265.66	1265.673	-12	1.17	1.20	1.14
Chromogranin B	592-597	(APQLDL)	1	1	655.35	655.35	-7	0.93	0.96	0.96
Chromogranin B	phosphorylated 357-374	GLQYRGRGpSEEDRAPRP	5	1	2179.14	2179.073	29	1.12	0.69	0.85
Chromogranin B	357-374	GLQYRGRGSEEDRAPRPR	5,6	1	2099.15	2099.073	39	0.92	0.77	1.29
Chromogranin B	313-330	PSPKESKEADVATVRLGE	4	3	1912.05	1911.990	31	1.17	0.91	0.96
Chromogranin B	588-597	SFARAPQLDL	2	1	1116.58	1116.590	-6	1.13	1.00	1.09
Elongation factor 1 beta 2	N-terminal fragment	GFGDLKTPAGLQV	2	2	1301.70	1301.698	2	0.84	0.93	1.36
Fibrinogen alpha	N-terminal fragment (after signal peptide removal)	TDTEDKGEFLSEGGGVR	3	2	1795.83	1795.822	3	1.19	0.83	0.98
Phospholipase D3	396-406	FVVPTDESQAR	2	2	1247.57	1247.615	-38	1.15	0.74	0.83
Proenkephalin	197-208	SPQLEDEAKELQ	2	2	1385.65	1385.667	-10	0.94	0.86	1.32
Prohormone convertase 1	619-628	GVEKMVNVVE	2	2	1102.54	1102.569	-25	1.04	1.02	1.12
Prohormone convertase 2	94-104 (within pro region)	IKMALQQEGFD	2	2	1278.60	1278.636	-31	1.32	1.00	1.07
Prohormone convertase 2	C-terminal fragment	SLQSILRKN	3	2	1057.62	1057.624	-2	1.23	1.14	0.86
Promelanin concentrating hormone	Neuropeptide EI	EIGDEENSAKFPI-amide	2	2	1446.69	1446.683	4	1.18	0.97	1.17
ProSAAS	Big LEN	LENPSPQAPARRLLPP	3	1	1754.98	1754.979	1	0.96	1.00	0.96
ProSAAS	Little SAAS 1-16	SLSAASAPLVETSTPL	2	1	1542.79	1542.814	-13	1.03	0.81	1.21
Prosomatostatin	Somatostatin 28-14	SANSNPAMAPRE	2	1	1243.56	1243.562	0	1.30	1.17	1.09

ProVasoactive Intestinal Peptide	111-122	ISSSISEDPVPI	2	1	1242.61	1242.634	-17	1.05	0.80	0.88
Thioredoxin 1	16-27	AAAGDKLVVVDF	2	2	1203.64	1203.650	-8	1.42	0.83	0.83
Thioredoxin 1	N-terminal fragment	VKLIESKEAFQEALAA	3	3	1745.90	1745.956	-33	1.27	1.12	0.88
Thymosin beta-10	Entire protein after removal of N-terminal Met	Ac-ADKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS	7-9	8	4933.63	4933.523	22	0.94	0.74	1.08
Thymosin beta-10	C-terminal fragment	TLPTKETIEQEKRSEIS	4	3	1987.98	1988.043	-33	1.09	0.78	0.91
Thymosin beta-4	Entire protein after removal of N-terminal Met	Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES	5-9	9	4960.49	4960.486	1	1.02	0.74	0.91
Thymosin beta-4	C-terminal fragment	PLPSKETIEQEKQAGES	3	3	1869.88	1869.932	-28	0.78	0.77	0.70
Tubulin beta	N-terminal fragment, isoforms 2-6	MREIVH(I/L)QAGQ	3	1	1280.71	1280.674	29	0.91	0.86	0.97
Vacuolar proton pump subunit G 2	C-terminal fragment	EVRPQVHPNYRVTV	4	1	1692.97	1692.913	34	0.69	0.73	1.12
VGF	489-507	NAPPEPVPPPRAAPAPTHV	3	1	1914.01	1914.010	0	1.10	1.05	1.20
Voltage-dependent anion channel protein 1 (VDAC-1)	C-terminal fragment	AGGHKLGLGLEFQA	3	2	1396.70	1396.746	-32	0.93	0.98	0.97

All peptides were identified by MS/MS sequencing except one peptide (APQLDL) for which insufficient MS/MS information was available. This peptide was tentatively identified based on matches to known peptides and predicted cleavage sites and this is indicated by parentheses surrounding the sequence. Abbreviations: z, charge; T, number of TMAB tags; Obs M, observed monoisotopic mass (after subtraction of the mass of the TMAB tags); Theor M, theoretical monoisotopic mass of the uncharged peptide without TMAB tags; ppm, difference in parts per million between observed mass and theoretical mass; ratio indicates the peak intensity observed for peptide incubated with CPA6 divided by the peak intensity for the same peptide incubated without enzyme.

Table S2. Kinetic constants for four CPA enzymes

	CPA6				CPA4				CPA1				CPA2			
Substrate	k_{cat} (1/s)	K_M (µM)	k_{cat}/K_M ($M^{-1}\cdot s^{-1}\cdot 10^{-5}$)	k_{cat}/K_M relative to FAPP	k_{cat} (1/s)	K_M (µM)	k_{cat}/K_M ($M^{-1}\cdot s^{-1}\cdot 10^{-5}$)	k_{cat}/K_M relative to FAPP	k_{cat} (1/s)	K_M (µM)	k_{cat}/K_M ($M^{-1}\cdot s^{-1}\cdot 10^{-5}$)	k_{cat}/K_M relative to FAPP	k_{cat} (1/s)	K_M (µM)	k_{cat}/K_M ($M^{-1}\cdot s^{-1}\cdot 10^{-5}$)	k_{cat}/K_M relative to FAPP
FA-Phe-Phe	9.94	266	0.37	1.00	44.3	55.6	7.97	1.00	76.6	20.4	37.6	1.00	121	36.4	33.3	1.00
FA-Phe-Trp	4.13	339	0.15	0.41	57.3	615	0.93	0.12	81.3	166	4.90	0.13	65.3	16.3	40.2	1.21
FA-Phe-Leu	4.67	386	0.19	0.51	13.4	19.4	6.93	0.87	34.0	15.7	21.7	0.58	18.6	393	0.47	0.014
FA-Phe-Ile	1.91	3070	0.0065	0.018	12.4	23.3	5.32	0.67	9.77	9.33	10.5	0.28	5.37	460	0.12	0.0035
FA-Phe-Met	4.03	786	0.066	0.18	23.9	40.0	5.81	0.73	51.8	128	4.03	0.11	NM	NM	0.040	0.0012
FA-Phe-Ala	0.40	2330	0.0017	0.0046	24.3	372	0.65	0.082	78.0	300	2.58	0.069	NM	NM	NM	NM
FA-Phe-Val	0.16	367	0.0043	0.012	19.4	57.3	3.38	0.42	12.3	8.95	14.8	0.39	NM	NM	0.030	0.0010
FA-Phe-His	0.28	723	0.0051	0.014	NM	NM	0.27	0.034	NM	NM	1.04	0.028	NM	NM	NM	NM

NM: Not measurable; below the limit of detection.
CPA1, CPA2 and CPA4 data taken from Ref. (22)

Table S3. Amino acid numbering of substrate binding residues found in preprocarboxypeptidases compared to that of active bovine CPA1.

	CPA/B Active Site Amino Acids							
Bovine CPA1 (active)	203	207	243	247	250	253	255	268
Bovine CPA1	Leu 313	Gly 317	Ile 353	Ile 357	Ala 360	Gly 363	Ile 365	Thr 378
Human CPA2	Met 311	Gly 315	Ile 351	Ile 355	Ala 358	Gly 361	Ile 363	Ala 376
Mouse CPA3	Leu 311	Gly 315	Ile 351	Ile 355	Thr 358	Ser 361	Leu 363	Ala 376
Human CPA4	Met 315	Gly 319	Thr 355	Val 359	Ala 362	Ser 365	Ile 367	Thr 380
Human CPA5	Met 330	Gly 334	Ile 370	Leu 374	Ala 377	Ile 380	Val 382	Ser 395
Human CPA6	Leu 331	Ser 335	Ala 371	Leu 375	Ser 378	Ser 381	Met 383	Ala 396
Pig CPB1	Leu 311	Ser 315	Gly 351	Ile 355	Ala 358	Gly 361	Asp 363	Thr 376
Human CPB2	Val 307	Ser 311	Gly 348	Leu 352	Ala 355	Gly 358	Asp 360	Thr 373