

Capturing cAMP-binding proteins from HepG2 cell lysate using the C8-cAMP caproKit™

Capture Compound Mass Spectrometry (CCMS) is an innovative technology to reduce the complexity of biological samples through selective isolation of targeted protein or enzyme families. Small synthetic molecules (Capture Compounds™) interrogate native proteins, even lipophilic membrane proteins. CCMS technology enables an efficient complexity reduction of the proteome and allows discovering, isolating and profiling members of functional protein families within a variety of biological samples.

Introduction

Cyclic 3',5'-adenosine monophosphate (cAMP) is an important second messenger and involved in many cellular signal transduction cascades in a wide range of organisms (1). The identification and profiling of cAMP-binding proteins is an important aspect in functional proteomics in order to understand the molecular basis of these signaling events. As a starting point to assess which proteins may be *bona fide* cAMP-binders, information can be retrieved from protein databases with respect to proteins that contain cyclic nucleotide binding domains (CNBDs). Examples for those proteins are subunits of distinct protein kinases, guanine nucleotide exchange factors, and cyclic nucleotide gated ion channels (2-4). However, further cAMP-binding proteins remain to be discovered. In order to enable this task, we designed and synthesized C8-cAMP Capture Compounds™ that are available as part of the C8-cAMP caproKit™. We here describe the application of the C8-cAMP caproKit for the capturing of cAMP-binding proteins from a lysate of human hepatocyte-derived HepG2 cells.

Materials

The C8-cAMP caproKit contains the C8-cAMP Capture Compound (C8-cAMP-CC) with 8-aminohexylamino (8-AHA-) cAMP as the selectivity group at a stock concentration of 100 μ M, free C8-cAMP as the competitor solution at a stock concentration of 4 mM, Streptavidin magnetic beads, 5x concentrated capture buffer, and 5x concentrated wash buffer as well as a preparation of purified regulatory subunit 1 of cAMP-dependent protein kinase (at a stock concentration of 1 mg/ml, SwissProt Acc. No [P10644](#)). We further used PCR-Tube strips for volumes up to 200 μ l (Thermo Fisher, cat. No. AB-1114) to prepare the samples, conduct the capturing experiments, wash and isolate the magnetic beads.

Irradiation of the samples for photo cross-linking was performed with the caproBox™, and the beads were isolated using the caproMag™. As the protein sample, HepG2 lysate at a stock concentration of 7 mg protein/ml was used. HepG2 cells were lysed by French Press in lysis buffer (6.7 mM MES, 6.7 mM NaOAc, 6.7 mM HEPES, pH 7.5, supplemented to

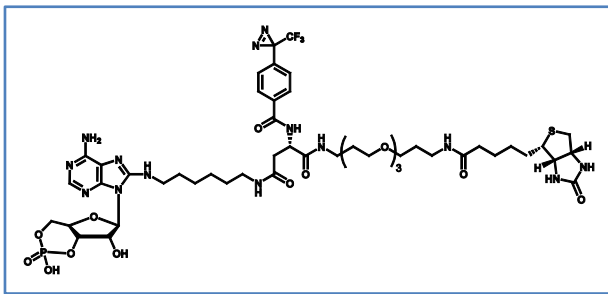


Figure 1 C8-cAMP Capture Compounds for selective isolation of cAMP/PKA/AKAP interactomes using cAMP as selectivity function.

200 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, and protease inhibitor cocktail). Debris was removed by centrifugation at 30,000 x g for 60 min at 4 °C. The supernatant was filtrated through a 0.2 μm filter and then dialysed against lysis buffer for the removal of small molecules (optional). After dialysis, centrifugation at 4000 x g was carried out to remove proteins precipitated during dialysis.

Capture experiment

The capture assays were carried out in the OnBead configuration (detailed description see: Guideline for CCMS and C8-cAMP caproKit™ Guideline OnBead) and prepared as follows: for preparation of the caproBead™, per assay 50 μl Streptavidin magnetic beads were mixed with 25 μl of the C8-cAMP-CC solution and incubated for 2 min at room temperature under vigorous shaking. The tube strip was then fitted in the caproMag, the beads collected in the tube lids, and washed twice with wash buffer and ultimately collected in the lids according to the caproMag procedure (see caproMag™ Guideline). In the meantime, the assays are prepared in the following order: Water, 5x capture buffer, and lysate were mixed by vortexing to achieve a final reaction volume of 100 μl with a final protein concentration of 3.5 mg/ml. Note that in the competition sample, prior to the addition of the lysate, free competitor (C8-cAMP) was added to the sample to a final assay concentration of 1 mM.

The prepared caproBeads were thoroughly resuspended in the sample solutions and allowed to incubate for 3 hours at 4 °C under rotation. Subsequently, the samples were very briefly centrifuged in a tabletop centrifuge with a butterfly

rotor, in order to remove any liquid from the tube lids, but short enough to avoid pelleting of the caproBeads. The samples were then irradiated for 30 min in the caproBox at 2-4 °C in intervals of 2.5 min and washed afterwards with wash buffer (see C8-cAMP caproKit Guideline OnBead for details). The beads were washed twice with HPLC grade water and incubated with 10 μl per assay of 50 mM NH₄HCO₃, 5 mM CaCl₂, and supplemented with 0.5 μg trypsin (promega, sequencing grade). This bead suspension was transferred into a new PCR tube. Tryptic digest was allowed to proceed over night at 37 °C under vigorous shaking. Beads were then fixed at the tube walls using the magnet of the caproMag, and the supernatants containing the peptides were recovered and desalted via Stage Tips™ (Proxeon, Odense, Denmark), and directly subjected to LC-MS/MS analysis.

Mass spectrometry

LC-MS/MS experiments were performed using a nanoflow HPLC system (Proxeon) coupled on-line to an ESI-LTQ-Orbitrap system. From the LC column, peptides were eluted during an 80-min linear gradient from 5 % ACN/0.1 % FA to 40 % ACN/0.1 % FA followed by additional 2 min to 100 % ACN/0.1 % FA and remaining at 100 % for another 8 min with a controlled flow rate of 300 nl/min.

MS/MS fragmentation was performed in a data-dependent mode using one survey MS scan followed by four MS/MS scans per second. Proteins were identified by automated database searching against the human UniProtKB/Swiss-Prot database using SEQUEST implemented in BioworksBrowser 3.3.1 SP1 (Thermo Fisher Scientific). Specific search parameters used in the SEQUEST analyses were 5 ppm precursor tolerance, 1 amu fragment ions tolerance, and full trypsin specificity allowing for up to two missed cleavages. Phosphorylation at serine, threonine, and tyrosine, oxidation of methionines, deamination at asparagines and glutamine, acetylation at lysine and serine, formylation at lysine, and methylation at arginine, lysine, serine, threonine and asparagine were allowed as variable modifications. No fixed modifications were used in database search. The SEQUEST peptide

identifications were required to satisfy minimum XCorr values of 2, 2.5, and 3 for singly, doubly, and triply charged peptides, a minimum ΔCn of 0.1, and a P (pep) of ≤ 0.001 . Peptides with a better score than this were accepted for analysis without further validation. The estimated false discovery rate (FDR) of peptide identifications was determined using the reversed protein database approach and was $< 0.5\%$.

Results

In two independent runs, regulatory subunits of the cAMP-dependent protein kinase were robustly identified in the capture samples while they were completely absent in the C8-cAMP competition samples. In addition, a *bona fide* interaction partner of these subunits was identified (A-Kinase anchor protein 1) as is shown in Table 1.

Table 1 Identified cAMP-binding proteins in HepG2 cells.

No.	Protein name	Uniprot/Swiss-Prot Acc. No.	No. of identified Peptides (Run1/ Run2)	Binds	MW [Da]
1	KAP2 cAMP-dependent protein kinase type RIIα	P13861	11/11	cAMP	45490
2	KAP0 cAMP-dependent protein kinase type RIα	P10644	9/6	cAMP	42955
3	KAP3 cAMP-dependent protein kinase type RIIβ	P31322	2/1	cAMP	46094
4	KAP1 cAMP-dependent protein kinase type RIβ	P31321	1/1	cAMP	43237
5	A-Kinase anchor protein 1	Q92667	5/3	PKAR	97281
6	Mannose-6-phosphate receptor-binding protein 1	O60664	2/3	unknown	47018
7	Putative nucleoside diphosphate kinase	O60361	2/2	GTP	15519
8	Tricarboxylate transport protein	P53007	3/2	unknown	33991
9	Sideroflexin-1	Q9H9B4	2/2	unknown	35596

Conclusion

The C8-cAMP caproKit is an effective tool for the isolation of cAMP binding proteins from crude protein mixtures, and the selectivity of the analysis is demonstrated by the competition experiments.

Given the relatively small amount of complex protein mixture as input material for the experiment, CCMS using the C8-cAMP caproKit is an outstandingly sensitive method for the identification and profiling of cAMP-binding proteins from cell lysates.

References:

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Ordering information:

Item Nr.	Description
1-1030-050	C8-cAMP caproKit™ 50 reactions
1-1030-010	C8-cAMP caproKit™ 10 reactions

The caproKit includes the C8-cAMP specific Capture Compound, all buffers, protein controls, C8-cAMP competitor, and Streptavidin magnetic beads.

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