

The N²-GDP caproKit™ for the profiling of GTP-binding proteins by CCMS

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

The functional isolation of proteome subsets based on small molecule-protein interactions is an increasingly popular and promising field in functional proteomics (1). Entire protein families may be profiled on the basis of their common interaction with a metabolite or small molecule inhibitor. This is enabled by novel multifunctional small molecule probes in an approach now termed chemical proteomics. One platform approach in this field are tri-functional Capture Compounds™ that contain a small molecule of interest to bind target proteins, a photo-activatable reactivity function to covalently trap bound proteins, and a sorting function to isolate Capture Compound-protein conjugates from complex biological samples for direct trypsinisation and protein identification by liquid chromatography/mass spectrometry (CCMS) (2, 3). This application note provides examples for the synthesis and application of a novel N²-GDP Capture Compound™ (Figure 1) for the functional enrichment of GTPases, a protein family that exerts key functions in signal transduction (4).

GTPases constitute a substantial subproteome of more than 150 different proteins in e.g., mammals, the majority of which can be assigned to the group of small GTPases (4). Small GTPases regulate processes such as growth control, cytoskeleton remodeling, and vesicle trafficking. Thus, functional proteomic profiling of these proteins is of great interest for the understanding of physiological and patho-physiological processes. Here, data is presented from CCMS experiments in a complex biological sample, the mitochondrial fraction from rat brain. The N²-GDP Capture Compound™ robustly and reproducibly captures a wide range of different GTPases, predominantly small GTPases, and will be a valuable tool for the proteomic profiling of this important protein family.

Materials

Assays were prepared in PCR tube strips for volumes up to 200 μ l (Thermo Fisher, cat. No. AB-1114) as reaction vessels to conduct the capturing experiments, wash and isolate the magnetic beads. Irradiation of the samples for photo cross-linking was performed using the caproBox™, and the beads were isolated using the caproMag™. As the protein

from this centrifugation. The mitochondrial pellet was resuspended in cell opening buffer (6.7 mM MES, 6.7 mM NaOAc, 6.7 mM HEPES pH 7.6, 200 mM NaCl, 10 mM β -mercaptoethanol) with protease inhibitor (Roche, Germany) and 0.5 % DDM. The suspension was rotated at 900 rpm for 1 h at room temperature, centrifuged at 10000 \times g for 15 min at 4 °C. The supernatant was used for capturing experiments.

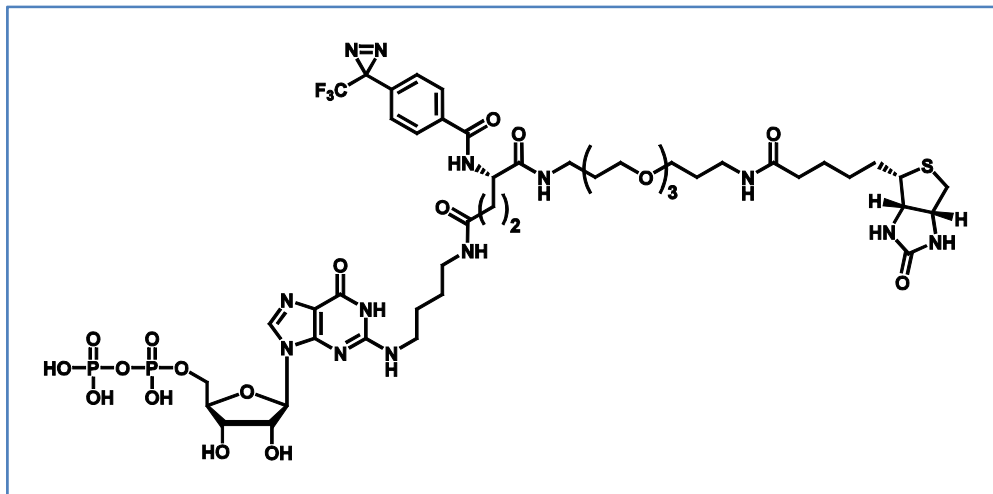


Figure 1 Structure of the N²-GDP Capture Compound™.

sample, two rat brains were homogenized by 12 strokes at 900 rpm in a motor-driven glass-teflon homogenizer in a homogenisation buffer containing 0.32 M sucrose, 5 mM HEPES/NaOH pH 7.4, supplemented with protease inhibitors. 10 volumes of homogenization buffer per gram of tissue wet weight were used. The homogenate was filtered through a nylon gauze to remove debris, and the filtrate was centrifuged for 10 min at 1000 \times g to remove nuclei. The supernatant was centrifuged for 15 min at 4 °C at 12,000 \times g to pellet organelles. The crude organelle pellet was resuspended in homogenization buffer, re-homogenized with 6 strokes at 900 rpm in a motor-driven glass-teflon homogenizer, and centrifuged for 20 min at 4 °C at 12,000 \times g. The organelle pellet was then resuspended 1.5 ml/gram of tissue wet weight buffer B (0.32 M sucrose, 5 mM Tris/HCl pH 8.1) with a plastic Pasteur pipette (pastette) and layered on top of a sucrose step gradient consisting of equal volumes of sucrose solution layers of 0.85 M sucrose, 1 M sucrose, and 1.2 M sucrose in an ultracentrifugation tube. Ultracentrifugation was carried out for 2 hours at 85,000 \times g at 4 °C. The mitochondria fraction was recovered as the pellet

Capture experiment

The N²-GDP caproKit™ differs from other available caproKits™ with respect to the formulation of the capture buffer 2, and the presence of two additional components: 1 M magnesium chloride solution and 0.1 M EDTA solution. The capture experiments were carried out in the OffBead configuration as described in the specific N²-GDP caproKit™ guideline downloadable at www.caprotec.com/support/downloads. Because GTPases are present in biological samples predominantly in a GDP-bound form, the removal of bound GDP is required to enable the N²-GDP Capture Compound™ to bind to the target proteins. This is achieved by incubation of the protein mixture in capture buffer supplemented with EDTA for 2 h at 4 °C, which triggers the release of bound GDP from the GTPases. In the next step, the N²-GDP Capture Compound™ (plus free GDP as competitor for the competition experiment) was added and allowed to incubate with the proteins for another 2 h at 4 °C. Finally, magnesium chloride solution was added to

stabilize the binding of either the Capture Compound or the competitor to the target proteins. Incubation took place for 1 h at 4 °C. Cell lysate was used at a final concentration of 5 mg/ml. Irradiation was performed with opened tube lids for 10 min using the caproBox™. Wash buffer 1 was added and the samples were vortexed thoroughly again. Next, streptavidin magnetic beads were added and the samples left rotating for 30 min at 4 °C. Capture Compound-protein conjugates were then isolated and washed using the caproMag™ procedure as described in the Guideline for caproMag™ (please refer to protocol sections at: www.caprotec.com/support/downloads). The capture samples were afterwards subjected to OnBead tryptic digestion. For this purpose, the beads after the final washing step with wash buffer 1 were washed twice with HPLC grade water, six times with 80 % (v/v) acetonitrile in water, and finally once again with water. The beads were then 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 by using the magnet of the caproMag™, and the supernatants with the peptides recovered, desalted via Stage Tips™ (Proxeon, Odense, Denmark), and then directly subjected to LC-MS/MS analysis.

Mass spectrometry

Tryptic digests were analyzed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) on an EASY-nLC™ system coupled to a LTQ-Orbitrap Velos instrument (Thermo Scientific, Bremen, Germany) through a Proxeon nanoelectrospray ion source. For chromatographic separation samples were first loaded on a reversed phase (RP) precolumn (5 cm, 100 µm, 100 Å) and separated on a RP analytical column (15 cm, 75 µm, 100 Å, NanoSeparations, Netherland) performing a 90 min linear gradient (5-35 % acetonitrile, 0.1 % formic acid).

Mass spectrometric detection was performed in the data-dependent mode allowing to automatically switch between Orbitrap-MS and LTQ-MS/MS (MS²) acquisition in a top 20 configuration at 60 K resolution for a full scan with subsequent collisionally induced dissociation (CID) fragmentation. Full scan MS spectra (from m/z 300-2000) were acquired in the Orbitrap analyzer after accumulation to a target value of 5e5 in the linear ion trap. The most intense ions (up to twenty, depending on signal intensity) with charge state ≥ 2 were sequentially isolated at a target value of 10,000 and fragmented in the linear ion trap using low energy CID with normalized collision energy of 35 %. Target ions already mass selected for CID were dynamically excluded for the duration of 63 s. The minimal signal required for MS² was 3000 counts. An activation q of 0.25 and an activation time of 10 ms were applied for MS² acquisitions.

All MS/MS data were analyzed using SEQUEST implemented in Proteome Discoverer 1.2 (Thermo Fisher Scientific) and X!Tandem (www.thegpm.org; version 2007.01.01.1, Proteome Software, Portland, OR, USA). Automated database searching against the human UniProtKB/Swiss-Prot database (release 2010_08 contains 518,350 sequence entries and 8994 rat entries) was performed with 10 ppm precursor tolerance, 1 Da fragment ions tolerance, and full trypsin specificity allowing for up to 2 missed cleavages. Methionine oxidation was used as fixed modification in the database search.

Spectra were annotated via human UniProtKB/Swiss-Prot database and analyzed using the software Scaffold (version Q+ 3_00_06, Proteome Software, Portland, OR, USA). First Proteins were selected if the peptide probability was ≥ 95 % assigned by the Peptide Prophet algorithm [5] and the protein was identified by at least 1 unique peptide. The minimum protein probability was set to 95 %. The estimated false discovery rate of peptide identifications was determined using the reversed protein database approach and was ≤ 1 %.

Results

A total of 495 proteins were identified with the above mentioned stringent search criteria using assay and control samples in triplicate experiments. Of these, 76 were rated as specifically captured due to substantial competition by free GDP. The majority of specifically captured proteins were, according to the UniProtKB annotation, known GTP-binding proteins. A small set of GTPase interaction partners was also identified. The other specifically captured proteins were annotated nucleotide binders, predominantly annotated ATP-binding proteins. 10

proteins had no obvious relation to GTP- or other nucleotide binding, and thus may represent potential new GTP binders. The categorisation of specifically captured proteins is shown in Figure 2. We further sorted the known GTP-binding proteins that were specifically captured according to their classifications into GTPase subfamilies. 33 of these proteins were GTPases of the Ras superfamily that can be divided into the Rab-, Rho-, Arf-, and Ras-families. Two G protein alpha subunits were specifically captured, and 9 GTP-binding proteins that were classified separately (Figure 3). Some of the latter were, for instance elongation factors.

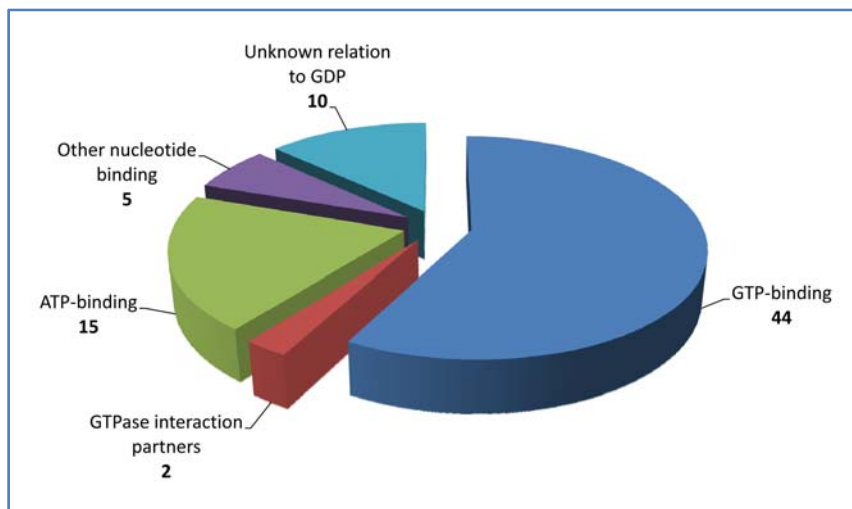


Figure 2 Specifically captured proteins. Total proteins: 495; 76 specific (15 %).

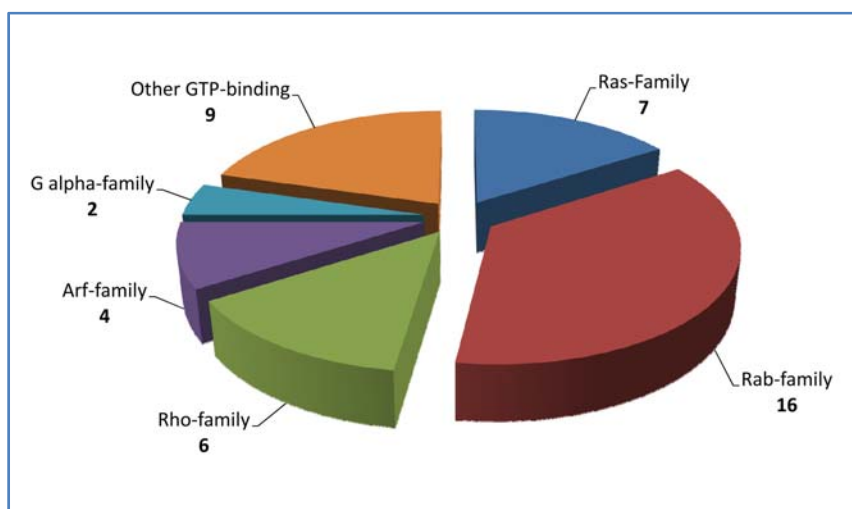


Figure 3 Classification of captured GTP-binding proteins.

The specific proteins were identified robustly and reproducibly. Selected specifically captured proteins along with the numbers of unique peptides

identified in the triplicate samples are given in Table 1.

Table 1 Selected specifically captured proteins. A: Assay, C: Competition.

Protein Name	Acc. No.	A1	A2	A3	C1	C2	C3
Elongation factor 1-alpha 2	P62632	21	26	23	3	3	3
Ras-related protein Rab-3A	P63012	13	14	15	2	2	1
Ras-related protein Rab-8A	P35280	7	14	10	1	1	1
Ras-related protein Rab-35	Q5U316	8	11	11	1	1	1
Elongation factor 2 OS=Rattus norvegicus GN=Eef2 PE=1 SV=4	P05197	18	13	20	0	0	0
Phosphoglycerate kinase 1	P16617	11	9	14	0	0	1
Guanine nucleotide-binding protein G(o) subunit alpha	P59215	9	9	8	3	3	2
ADP-ribosylation factor 1	P84079	10	12	13	1	0	0
Ras-related protein Rab-8B	P70550	6	7	7	1	1	1
Ras-related protein Rab-3C	P62824	5	6	8	1	1	1
Ras-related protein Rab-10	P35281	7	6	7	1	1	1
Ras-related protein Rab-1A	Q6NYB7	5	5	5	1	1	1
Elongation factor 1-alpha 1 SV=1	P62630	7	10	8	3	2	2
Ras-related protein Rab-14	P61107	3	4	6	1	1	1
Nucleoside diphosphate kinase A	Q05982	8	7	7	0	0	0
Ras-related protein Rab-2A	P05712	9	10	9	0	0	0
Ras-related C3 botulinum toxin substrate 1	Q6RUV5	8	7	8	0	0	0
Cell division control protein 42 homolog	Q8CFN2	7	7	7	0	0	0

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www.caprotec.com/support/downloads

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Ordering information:

Item Nr.	Description
1-1050-050	N ² -GDP caproKit™ 50 reactions
1-1050-010	N ² -GDP caproKit™ 10 reactions

The caproKit includes the N²-GDP specific Capture Compound, all buffers, protein controls, GDP competitor, and Streptavidin magnetic beads.

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