Cyclic AMP affinity purification and ESI–QTOF MS–MS identification of cytosolic glyceraldehyde 3-phosphate dehydrogenase and two nucleoside diphosphate kinase isoforms from tobacco BY-2 cells

Kris Laukens[a], Luc Roef[a], Erwin Witters[a], Herman Slegers[b], Harry Van Onckelen[a,*]

[a] *Laboratorium voor Plantenbiochemie en -fysiologie, Department of Biology, University of Antwerp (UIA), Universiteitsplein 1, B-2610 Antwerp, Belgium*

[b] *Cellular Biochemistry, Department of Biochemistry, University of Antwerp (UIA), Universiteitsplein 1, B-2610 Antwerp, Belgium*

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Abstract The soluble protein fraction of tobacco bright yellow 2 cells contained adenosine 3′,5′-cyclic monophosphate (cAMP)-binding activity, detected with both a conventional binding assay and a surface plasmon resonance biosensor. A cAMP-agarose-based affinity purification procedure yielded three proteins which were identified by mass spectrometry as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and two nucleoside diphosphate kinases (NDPKs). This is the first report describing an interaction between cAMP and these proteins in higher plants. Our findings are discussed in view of the reported role of the interaction of cAMP with GAPDH and NDPK in animals and yeast. In addition, we provide a rapid method to isolate both proteins from higher plants.

Key words: Adenosine 3′,5′-cyclic monophosphate; Cell cycle; Glyceraldehyde 3-phosphate dehydrogenase; Nucleoside diphosphate kinase; Tobacco bright yellow 2

1. Introduction

Although adenosine 3′,5′-cyclic monophosphate (cAMP) is widely known as a regulatory molecule in eukaryotes and prokaryotes, strong evidence for its occurrence and functions in higher plants is only recently emerging [1]. Previously, our group showed that cAMP levels in tobacco bright yellow 2 (TBY-2) cells are tightly connected to cell cycle progression, showing transient peaks in G1 and S phase respectively [2]. Inhibition of TBY-2 adenylyl cyclase activity with indomethacin resulted in the loss of cAMP accumulation and a cell cycle block in either G2/M or G1/S phase, depending on the time of application [2,3]. These observations are apparently similar to cell cycle features in animals, yeast and lower plants [4–6], where cAMP is known to play a key role in the cell cycle. The fact that most components of the animal cell cycle machinery are well conserved in higher plants [7] supports the idea of a comparable regulatory role of cAMP in cell cycle progression in higher plants. The question remains in which way cAMP interacts with the plant cell cycle.

In animals, yeast and lower plant cells cAMP mainly influences cell cycle progression via protein kinase A (PKA) [5,8,9]. Although cAMP-regulated kinase activity was reported in several higher plants [10–13], no plant PKA has been sequenced yet. However, molecular biological evidence showed the existence of plant protein kinases containing a high degree of sequence homology with PKA [14]. In addition, molecular biological techniques revealed the existence of cyclic nucleotide-sensitive ion channels in plants. Some voltage-gated K^+ channels [15,16] have been described of which voltage dependence appeared to be sensitive to cyclic nucleotides. More recently, characterisation of cyclic nucleotide-gated cation channels from higher plants was reported [17,18].

A few other plant proteins were shown to exert cAMP-binding activity. High affinity cAMP-binding proteins without protein kinase activity were found in various higher plant species [19–21], but the identity of these proteins remains unclear. Two plant enzymes that appeared to be inhibited by cAMP were 5′-nucleotidase and a phosphotyrosine-specific phosphatase [22,23].

In order to reveal targets for cAMP action in the plant cell cycle we have set up experiments to isolate and identify cAMP-binding proteins in TBY-2 cells. In this paper we report the purification and the mass spectrometrical identification of TBY-2 cAMP-binding proteins which were not previously shown to bind cAMP in higher plants, but have been reported as cAMP-binding proteins in animals and yeast.

2. Materials and methods

2.1. Materials and culture

[2,8-^{3}H]cAMP (1.55 TBq/mmol) was from Amersham Pharmacia Biotech (Uppsala, Sweden), amine coupling kit and CM-5 sensor chip were from BIAcore (Uppsala, Sweden). Liquid scintillation cocktail Ultima Gold was from Packard Instruments Co. (USA). Other chemicals were from Sigma (Bornem, Belgium). The TBY-2 cell culture was maintained as described [24], by weekly transfer of 1 ml of stationary culture to 50 ml of medium (30 g/ml sucrose, 200 mg/ml KH_2PO_4, 4.302 g/ml Murashige and Skoog medium (Duchefa, Haarlem, The Netherlands), 1 mg/l thiamine and 0.2 mg/l 2,4-dichlorophenoxyacetic acid). The exponential culture was obtained by transfer of

*Corresponding author. Fax: (32)-3-820 2271.
E-mail address: hvo@uia.ua.ac.be (H. Van Onckelen).

Abbreviations: AET-cAMP, 8-(2-aminoethylthio)-cAMP; BSA, bovine serum albumin; cAMP, adenosine 3′,5′-cyclic monophosphate; ESI, electrospray ionisation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MS, mass spectrometry; NAD^+, β-nicotinamide adenine dinucleotide; NDPK, nucleoside diphosphate kinase; PKA, protein kinase A; TBY-2, tobacco bright yellow 2

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17.5 ml of 7 days old stationary culture to 250 ml of medium followed by 40 h of subculture.

2.2. Soluble protein extraction

Cells from 50 ml of exponential culture were harvested by filtration on a Whatman filter paper. Soluble proteins were extracted by grinding the cells with mortar and pestle in liquid nitrogen+10 ml buffer A (50 mM glycerophosphate, 15 mM $MgCl_2$, 20 mM EDTA, 5 mM NaF, pH 7.4 unless otherwise stated) with addition of 1% (w/v) polyvinylpolypyrrolidone, 1% (v/v) plant protease inhibitor cocktail (Sigma), 500 μM Na_3VO_4, 10 μM NH_4MoO_4 and 2 mM dithiothreitol. After thawing, the extracts were cleared by centrifugation at $33\,000\times g$ for 15 min (4°C). Protein yield was typically 1.5 mg/ml, as determined with the method described by Bradford [25], using bovine serum albumin (BSA) as a standard.

2.3. Radioactive cAMP-binding assay

Quantification of cAMP-binding activity was done with a method based on radioimmunoassay [26]. 100 μl of protein extract was incubated in triplicate with 1000 Bq [2,8-^{3}H]cAMP in 100 μl phosphate-buffered saline (PBS) for 1 h at 25°C. After addition of 100 μl 0.2% (w/v) BSA in PBS, proteins were precipitated 30 min at 4°C with 900 μl saturated $(NH_4)_2SO_4$ and centrifuged at $12\,000\times g$ for 7 min. The supernatant was removed, the pellet was redissolved in 100 μl water, 1.3 ml scintillation cocktail was added, and radioactivity was counted with a Tricarb 1500 scintillation counter (Packard, USA). Values were corrected for non-specific incorporation of [2,8-^{3}H]cAMP in the pellet by performing the same assay in the presence of 50 μM non-radioactive cAMP.

2.4. Surface plasmon resonance detection of cAMP-binding

8-(2-Aminoethylthio)-cAMP (AET-cAMP) was prepared from 25 μmol 8-bromo-3′,5′-cAMP sodium salt and 25 μmol 2-aminoethanethiol as described [27] and dried, after confirmation of its identity using electrospray ionisation (ESI) tandem mass spectrometry (MS–MS). The first channel of a CM-5 sensor chip, docked in a BIAcore J biosensor system, was activated with the amine coupling kit (BIAcore), and AET-cAMP, fully redissolved in 1 ml of 10 mM sodium acetate pH 4.7, was immobilised by 50 min injection at medium flow rate. Unreacted groups were blocked with ethanolamine. The second channel of the chip was activated and immediately blocked in the same way in order to create a reference channel. Approximately 1000 resonance units of AET-cAMP were immobilised as such, roughly corresponding with 1 ng/mm^2.

The performance of the cAMP-binding measurements with this setup was verified employing polyclonal antibodies directed against cAMP [26]. During experiments, the normalised response difference between the experimental channel and the reference channel was monitored, with the value before injection as zero reference value. Extracts and elution buffer were always buffer A, pH 8.0. Temperature was set to 25°C. Flow rate during experiments was set to medium. The chip was regenerated by sequentially injecting 10 mM HCl, 10 mM NaOH and 6 M urea.

2.5. Purification of BY-2 cAMP-binding proteins

1 ml of soluble TBY-2 protein extract was applied to a column containing 200 μl of C-8 cAMP-agarose (Sigma A0144), previously equilibrated in buffer A, at 25°C. After washing with 10 ml of buffer A, bound proteins were eluted by overnight incubation in buffer A supplemented with the appropriate concentration of competitor (cAMP or β-nicotinamide adenine dinucleotide (NAD^+)), at 4°C. Eluted proteins were precipitated in 10 volumes of ice-cold acetone containing 10% (w/v) trichloroacetic acid and 0.07% (v/v) 2-mercaptoethanol, washed twice with ice-cold acetone containing 0.07% (v/v) 2-mercaptoethanol and, after drying, incubated at 95°C for 5 min in 62.5 mM Tris–HCl pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol. Fractions were analysed by 12.5% SDS–PAGE followed by silver staining [28]. Mark 12 (Invitrogen, Merelbeke, Belgium) was used as molecular weight standard mixture.

2.6. Tryptic digest and MS characterisation

Silver-stained proteins were excised from the gel and in gel digested as described by Shevchenko et al. [29]. The resulting peptide mixture of each protein was analysed by nano electrospray-quadrupole time of flight MS (QTOF II, Micromass, Manchester, UK) according to Wilm et al. [30]. Obtained peptide MS–MS spectra were analysed with Proteinlynx software and peptides were identified both by submission of MS–MS spectra to Mascot [31] and by submission of processed sequence tags to the peptide sequence tag search engine of EMBL [32].

3. Results

3.1. Soluble TBY-2 protein extract contains cAMP-binding proteins

In a first attempt to look for cAMP-binding proteins in TBY-2 cells we quantified cAMP-binding activity with a conventional assay employing radioactive cAMP. Significant binding activity was measured in the soluble protein fraction of exponentially growing TBY-2. After correction for non-specific binding typical obtained values for [^{3}H]cAMP-binding were 19 ± 5 fmol/mg protein. Additional binding activity experiments were done using surface plasmon resonance biosensor technology. BIAcore J analysis showed interaction of components in soluble TBY-2 extracts with C-8-immobilised cAMP (Fig. 1). A strong interaction was seen during injection of the extract, which was only partially washed away during elution with buffer A. More tightly bound factors rapidly dissociated following injection of 10 mM cAMP, representing potential cAMP targets.

3.2. Three cAMP-binding proteins are isolated by means of cAMP affinity chromatography

After the successful pattern of binding and elution shown with the biosensor analysis we employed a C-8-immobilised cAMP-agarose affinity chromatography to purify cAMP-binding proteins. Three proteins were bound and specifically eluted with cAMP (Fig. 2A, lane 1). They showed an apparent

Table 1
Identification of cAMP-binding proteins from TBY-2

Band	[M+H]	Sequence	Accession number[a]	Identity
40 kDa	833.5	IGINGFGR	P26521 (*Ranunculus acris*)[b]	GAPDH
	1305.6	DAPMFVVGVNEK	P09094 (*Nicotiana tabacum*)[b]	GAPDH
	1434.8	AASFNIIPSSTGAAK	P26521 (*R. acris*)[b]	GAPDH
	2130.3	GILGFTEDDVVSTDFVGDSR	P09094 (*N. tabacum*)[b]	GAPDH
	1762.9	LVSWYDNEWGYSSR	P09094 (*N. tabacum*)[b]	GAPDH
18 kDa	885.5	GDLAVVVGR	AAF08537 (*P. sativum*)[b]	NDPK
	1344.8	TFIAIKPDGVQR	AAF08537 (*P. sativum*)[b]	NDPK
15 kDa	913.6	GLVGEIIGR	Q9M7P6 (*C. annuum*)	NDPK
	979.5	GDYAIDIGR	Q9M7P6 (*C. annuum*)	NDPK
	1354.6	NVIHGSDAVESAR	Q9M7P6 (*C. annuum*)	NDPK

[a]Either SwissProt or NCBI. For clarity, only one relevant hit is shown.
[b]More database entries contained the same peptide.

molecular weight of about 40 kDa, 18 kDa and 15 kDa respectively. A control in which the purification was performed after preincubation of the extract with 10 mM cAMP did not yield any proteins (Fig. 2A, lane 2), confirming a specific interaction via cAMP.

3.3. *Isolated proteins are glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and two nucleoside diphosphate kinases (NDPKs)*

Tryptic peptides of the isolated proteins were analysed by ESI MS–MS. Table 1 shows the obtained sequences. The 40 kDa protein was identified as cytosolic GAPDH, of which the sequence is partially known in tobacco [33]. All sequenced peptides from this protein were found back in various (plant) GAPDH entries. The peptide with $[M+H]^+ = 833.5$ represents the amino-terminal part, missing in the tobacco GAPDH fragment of database entry P09094 but known in other plant GAPDH sequences. The peptide with $[M+H]^+ = 1434.8$ also differs from tobacco GAPDH entry P09094, but an identical peptide is present in most other higher plant cytosolic GAPDH entries. While the tobacco database sequence contains the peptide ATSFNIIPSSTGAAK, the corresponding TBY-2 GAPDH peptide was sequenced as AASFNIIPSSTGAAK, Thr being substituted by Ala.

The 18 kDa and 15 kDa proteins were both identified as NDPK isoforms. Both proteins have not yet been identified in tobacco. The analysed peptides from the 18 kDa band were 100% identical to *Pisum sativum* mitochondrial NDPK [34]. The sequenced peptides from the 15 kDa band appeared to be 100% identical to *Capsicum annuum* NDPK (Q9M7P6).

With the knowledge that GAPDH is a NAD^+-binding enzyme, the effect of NAD^+ on the interaction with cAMP was tested. After preincubation with 10 mM NAD^+, GAPDH was not retained on immobilised cAMP anymore (Fig. 2B, lane 1), while the NDPK isoforms were still bound. In addition, GAPDH could be eluted specifically with 10 mM NAD^+ (Fig. 2B, lane 2).

4. Discussion

cAMP-binding activity was detected in a soluble fraction of exponentially growing TBY-2 cells with both a radioactive assay and a surface plasmon resonance biosensor. The biosensor approach showed that TBY-2 proteins could be successfully captured on C-8-immobilised cAMP and subsequently eluted with cAMP. Applying this strategy with a column containing agarose-linked cAMP allowed us to isolate and identify the proteins responsible for the cAMP-binding activity. The purified proteins appeared to be GAPDH and two NDPK isoforms.

To our knowledge this is the first report of an interaction of higher plant GAPDH and NDPK with cAMP. Both proteins are reported previously as cAMP targets in yeast and animals [35–38]. The confirmation of this interaction in higher plants supports the idea that it concerns a highly conserved feature and suggests a universal relationship with cAMP. Inhibition of both GAPDH activity and NDPK activity by cAMP was shown previously, but appeared to be relatively weak [37,39], and the suggestion has been made that cAMP interaction might regulate other enzymatic activities or functions of these proteins. Indeed, isoforms of both proteins exhibit alternative

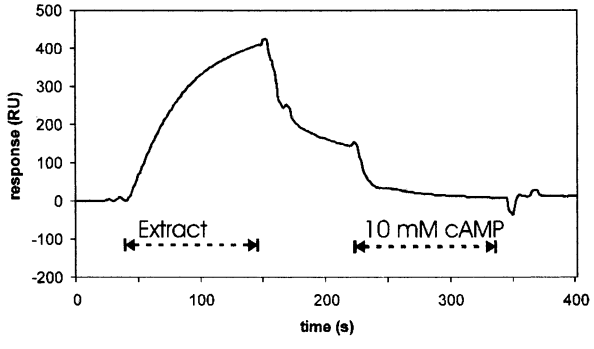

Fig. 1. BIAcore J surface plasmon resonance sensogram showing interaction between soluble tobacco BY-2 proteins and cAMP immobilised on sensor chip. Normalised response, expressed in response units (RU), is shown as function of time. Arrows assign the periods that protein extract and 10 mM cAMP were injected. Before, after and between injections the chip was eluted with buffer A (pH 8.0).

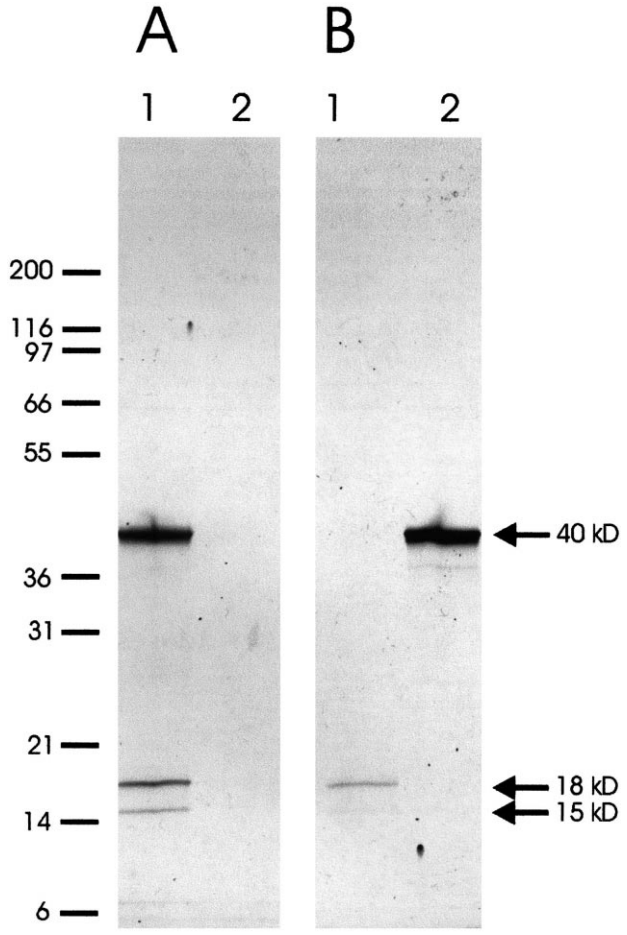

Fig. 2. 12.5% SDS–PAGE of cAMP affinity-purified TBY-2 proteins (A) and effect of NAD^+ on cAMP-binding (B). Soluble BY-2 protein extracts were applied to a cAMP-agarose column and subsequently eluted as described in Section 2. A: Fraction eluted with 10 mM cAMP (lane 1), and fraction eluted with 10 mM cAMP when extract was preincubated for 1 h with 10 mM cAMP before chromatography (lane 2). B: Fraction eluted with 10 mM cAMP when extract was preincubated for 1 h with 10 mM NAD^+ before chromatography (lane 1), and fraction eluted with 10 mM NAD^+ without preincubation (lane 2). Positions of molecular weight marker proteins (kDa) are shown on the left, estimated molecular weights of purified proteins are shown on the right.

regulatory functions in yeast and animals. GAPDH is reported to function as uracil DNA glycosylase in DNA repair [40] and binds to transfer RNA [41], while certain NDPK isoforms may function as a c-myc transcription factor [42] or phosphorylate farnesyl and geranyl pyrophosphates [43]. The effect of cAMP on these functions is not yet described. In plants little is known about alternative functions for GAPDH. Plant NDPK isoforms are involved in phytochrome signalling [44], in UV response, where they seem to act as a transcription factor [45], and in heat response, where interaction of NDPK with an unknown 86 kDa protein is involved [46]. These data point towards functions for plant NDPK isoforms in signal transduction events.

Targets for cAMP in TBY-2 should be proteins exerting a function in cell cycle progression and be affected by the reported cAMP oscillations [2]. Interestingly, both GAPDH and NDPK from animals and yeast were demonstrated previously to show cell cycle phase-dependent expression, showing an upregulation at S phase [47,48]. These findings suggest a function in animal and yeast cell cycle progression. The detection of GAPDH and NDPK isoforms in plant nuclei [45,49] could be of particular interest in the elucidation of potential roles of both proteins in plant cell cycle progression.

Interesting is also the fact that in animals interactions between GAPDH and NDPK have been demonstrated [50,51]. Interaction with GAPDH stimulates phosphotransferase activity of NDPK towards proteins [51]. Although tobacco BY-2 GAPDH and at least one of the identified NDPK isoforms are expected to reside mainly in different subcellular compartments [33,34], we cannot exclude that these proteins bind as a complex to immobilised cAMP. If GAPDH and NDPK bind cAMP as a complex, the binding site for cAMP is most likely to be found on the NDPK protein, since high concentrations of NAD^+ specifically elute GAPDH while binding of NDPK is only marginally affected. Future investigation will be focused on elucidating the true nature of the reported interaction and the characterisation of the cAMP-binding activity of GAPDH and NDPK. Furthermore, upon full sequence elucidation (e.g. with a PCR-based cloning strategy), expression and activities of GAPDH and the different NDPK isoforms during cell cycle will be investigated. The rapid one step purification procedure provided will be a valuable tool for further characterisation of both NDPK and GAPDH in higher plants.

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