

Minireview

Search for new cyclic AMP-binding proteins

S. Dremier^{a,*}, R. Kopperud^b, S.O. Doskeland^b, J.E. Dumont^a, C. Maenhaut^a^aIRIBHM, Faculty of Medicine, Free University of Brussels, 808 Route de Lennik, 1070 Brussels, Belgium^bDepartment of Anatomy and Cell Biology, University of Bergen, Jonas Lies vei 91, N-5009 Bergen, Norway

Received 25 April 2003; revised 8 May 2003; accepted 8 May 2003

First published online 26 May 2003

Edited by Richard Marais

Abstract Today, there is evidence that the cAMP-dependent kinases (PKA) are not the only intracellular receptors involved in intracellular cAMP signalling in eukaryotes. Other cAMP-binding proteins have been recently identified, including some cyclic nucleotide-gated channels and Epac (exchange protein directly activated by cAMP) proteins. All these proteins bind cAMP through conserved cyclic nucleotide monophosphate-binding domains. However, all putative cAMP-binding proteins having such domains, as revealed by computer analysis, do not necessarily bind cAMP, indicating that their presence is not a sufficient criteria to predict cAMP-binding property for a protein.

© 2003 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Cyclic AMP;
Cyclic nucleotide monophosphate-binding domain;
cAMP-binding protein

1. Cyclic AMP (cAMP)-dependent protein kinases do not account for all effects of cAMP in vertebrate cells

cAMP is a second messenger playing, in eukaryotes, a crucial role in the intracellular signal transduction of various stimuli controlling a wide variety of cellular events including function, secretion, cell shape, cytoskeletal modelling, cell migration, proliferation, differentiation, and apoptosis. Since the seventies until a few years ago, it was believed that most if not all the cAMP effects were mediated by the activation of the cAMP-dependent kinases (PKA). PKAs are present, in most cells, as two types of isoenzymes, PKAI and PKAII, which are tetramers composed of two catalytic (C) and two regulatory (RI α , β or RII α , β) subunits [1]. Each R subunit has two cAMP-binding domains (site A and site B). Upon binding of two cAMP molecules on both R subunits, the inactive tetramer is dissociated into one dimer of R subunits and two active C subunits which then phosphorylate various cytoplas-

mic and nuclear target proteins. Later, some cyclic nucleotide-gated (CNG) channels were found to be directly activated by cAMP in some specialised cells like in olfactory neurones where they play a role in the transduction of sensory signals [2–4].

In 1997, we suggested that PKA-independent mechanisms might be required in some cAMP-dependent effects in thyroid cells [5]. Indeed, the microinjection of active PKA was not sufficient to induce cell proliferation or the expression of thyroglobulin, a thyroid-specific gene, although it mimicked some other cAMP-stimulated events such as thyroperoxidase expression and typical morphological changes. However, all cAMP effects required PKA activation showing that PKA was necessary. Following our data, the involvement of PKA-independent pathways has also been suggested in other cAMP-mediated events in different cell types, for example, in the glial fibrillary acidic protein induction in glial cells [6], in the mitogen-activated protein kinases activation in melanocytes [7], in the differentiating action of FSH in granulosa cells [8], in actin depolarisation in skeletal muscle cells [9], and in the inhibition of interleukin-5 release by human T lymphocytes [10]. These conclusions were mainly based on the absence of inhibition of cAMP effects by H89, a supposedly specific PKA inhibitor. Taken together, all these reports suggested the existence and the importance of PKA-independent pathways in the transduction of many cAMP-mediated effects, and thereby called into question the dogma considering PKA as the main intracellular receptor of cAMP in mammalian cells. Based on such findings, searches for other cAMP effectors were initiated by different groups. They were based on Blast searches of proteins with a similitude to PKAs, catabolite gene activation proteins and CNG channels.

2. cAMP-binding proteins related to catabolite gene activator protein (CAP)

In 1998, two novel cAMP receptors, Epac1 and Epac2 (exchange factor directly activated by cAMP, also called cAMP-GEFI and cAMP-GEFII), were identified [11,12]. The Epac proteins are guanine nucleotide exchange factors (GEFs) which, upon cAMP binding, specifically activate the small G proteins Rap1 and Rap2 [13]. Epac1 and Epac2 have respectively one and two cAMP-binding sites which are located in the N-terminal part of the proteins. Epac1 is broadly expressed, whereas Epac2 is prominent in the brain and the adrenal glands [12].

Along with the *Escherichia coli* CAP, all the cAMP-binding

*Corresponding author. Fax: (32)-2-5554137.

E-mail addresses: sdremier@ulb.ac.be (S. Dremier), jedumont@ulb.ac.be (J.E. Dumont).

Abbreviations: cNMP, cyclic nucleotide monophosphate; PKA, cAMP-dependent protein kinase; CAP, catabolite gene activator protein; Epac, exchange protein directly activated by cAMP; CNG, cyclic nucleotide-gated; NTE, neuropathy target esterase; cAR, cAMP receptor of *Dictyostelium*

	α A	β 1	β 2	β 3	β 4	β 5	β 6	β 7	β 8	α B
CAP	LEWFLSHCHI	HKYPSKSTLI	HQGEKAETLY	YIVKCSVALI	IKDEEGK---	---EMILSY	LNQGDFTGEL	----GLFEE	QGE RSAWVRA	K-TACEVAEI SYKKFRQLIQ
Ri α -1	RSDIFDAMFS	VSFIAETVI	QCGDEGDNFY	VLDQGETDVI	VNNEWAT---	-----S	VEGGGSFGEL	-----ALIV	GT PRAATVKA	K-TNVKLWGI DRDSYRRILM
Ri β -1	RSDIFDAMFP	VTHIAETVI	QCGNEGDNFY	VVDQGEVDVI	VNGEWVT---	-----N	ISEGGSFGEL	-----ALIV	GT PRAATVKA	K-TDLKLWGI DRDSYRRILM
Ri α -2	LSQVLDAMFE	RIVKADSHVI	DQGDGDNFY	VLERGTVDIL	VTKDNGTRC-	-----VGQ	YDNRGSFGEL	-----ALMY	NT PRAATIVA	T-SEGSLWGL DRVTFRRITV
Ri β -2	MSQVLDAMFE	KLIVKDESHVI	DQGDGDNFY	VLDQGETDVI	VKCDGVGRC-	-----VGN	YDNRGSFGEL	-----ALMY	NT PRAATITA	T-SPGALWGL DRVTFRRITV
Ri α -2	RLTVADALEP	VQFEDQKIV	VQGGPDGDFI	IILEGSAAVL	QRRSENEEF-	-----VEVGR	LGPSDFEGEI	-----ALLM	NR PRAATVVA	R-GPLKCVKL DRPFRERVLG
Ri β -2	RLTVADRLPE	VQFEDSEKIV	VQGGPDGDFI	IILEGSAAVL	QRRSPNEEY-	-----VEVGR	LGPSDFEGEI	-----ALLL	NR PRAATVVA	R-GPLKCVKL DRPFRERVLG
Ri α -2	RMKIVDVIGE	KIYKDEIRII	TQGEKADSPY	IIESGEVSIL	IRSRTSKNKD	GGNQVEIAR	CHKQYFEGEL	-----ALVT	NK PRAASAYA	V-GDVKCLVM DVQAFERLLG
Ri β -2	RLKVVVDVIGT	KVYNDGEQII	AQGDSDSDFI	IVESGEVKIT	MKRKGKSEVE	E-NGAVEMPR	CSRGQYFEGEL	-----ALVT	NK PRAASAH	I-GTVKCLAM DVQAFERLLG
NTE-1	FLELCRHVMF	QRLGQDYVF	RGDPDASTY	VVDGSLLELC	LPGPDGKECV	VKEVVPGDSV	NSLLSILDVI	--GHQHPORT	VSA RAA-RDS	T-VLRLLPVEA FSAVET
NTE-2	PSLLNSRVLL	HHAKAGTIIA	RGDQDVSLH	PVLKGLHLVY	QRMIDKAED-	-----VCLFV	AQPGELVGLI	-----AVLT	GE PLIFTLRA	Q-RDCTPLRI SKSLFYETIM
NTE-3	AIDW	TAVEAGRALY	RQDRSDCTY	IULNGLRLSV	IQRSGSKKE-	-----LVGE	YGRGLIGVY	-----EALT	RQ PRATTVHA	V-RDTLAKL PESTLGHTR
Im49	NSLLTS-ARL	QLQLTAQYLF	KRGDPPCGLY	AVLEESLRI-	SAVNEQSGKE-	-----AVLSL	AELPYWFGEN	-----FLVD	GL PRTHDACA	V-GPCTLLQV PQPALLQL
EPAC	RELAVALLFE	PHSKAGTVLF	SGDQKGTSMY	IILKCSVMNV	THGKG----	-----LVTT	LHEGDFEGOL	-----ALVN	DA PRAATIIIL	REDNCHFLRV DKQDFNRRII
GEFII-1	HQICLCGYIE	NLEK-GITLF	RGDITGNWY	AVLAGSLDVK	VSRSTSHDD-	-----AVTIC	LGITGATGES	-----ILDN	AT PRAATIVT	RESS-ELLRI EQKLKFLAK
GEFII-2	RELAGVLIFE	SHAKGTVLVF	NQGEGETSMY	IILKCSVMNV	YKKG----	-----VVC	LHEGDFEGKL	-----ALVN	DA PRAASIVL	REDNCHFLRV DKEDFNRII
KIAA0313	RELCAVMVFA	VVERAGTIVL	NDGBELDSWS	VILNCSVEV-	TYPDG----	-----KAEI	LCMGNSFGVS	PTMDKEYMKG	SV M RTRVDDC	QFVCIA---- -QQDYCRIL
CNG2	LVELVLKLRP	QVSPFDDYIC	RKGDIGKEMV	IILKCSVMNV	-KLA VVADGG--VT	-----QYAL	LSAGSCFGEI	SILNIKSGSKM	GNR RTANIRS	L-GYSDFCL S

Fig. 1. Alignment of the cNMP-binding domains of new putative cAMP-binding proteins with those of the cAMP-binding proteins used as queries in Blast searches. These are *E. coli* CAP (P03020), human Ri α (P10644), Ri β (P31321), Ri α (P13861) and Ri β (P31323), human Epac1 (AF103905) and cAMP-GEFII (U78516) and human CNG-2 (Q16280). Regions of secondary structure in CAP sequence according to its crystal structure are indicated at the top. Residues of high consensus value (80%) are coloured in black, residues of low consensus value (40%) are coloured in grey.

proteins known at that time shared a common cyclic nucleotide monophosphate (cNMP)-binding domain. The structure of CAP has been crystallised. According to the crystal structure of CAP, the cNMP-binding domain consists of a stretch of 120 amino acids containing a first α -helix, an eight-stranded anti-parallel β -barrel structure followed by two other α -helices [14]. The cyclic nucleotide binding occurs in a pocket formed by the α -helix C and β -barrel, where the remaining invariant residues are located [15,16].

Based on sequence homology with the cNMP-binding domain of CNG-gated channels, a novel family of hyperpolarisation-activated cation channels (HAC1–3) expressed in brain and heart has been later identified [17]. The cAMP binding permits the channels to open more rapidly [18]. The possible existence of other cAMP-binding proteins having such a CAP-related cAMP-binding domain in eukaryotic cells is the question we have addressed in this review.

3. Other new putative cAMP-binding proteins related to CAP

To identify new cAMP-binding protein coding sequences, we performed an advanced TBLASTN search in the non-redundant DNA sequence and expressed sequence tag (EST) databases using the cNMP-binding domains of the proteins known to bind cAMP as query sequences. These proteins were the regulatory subunits Ri α , β and Ri β , α of PKA, Epac1 and 2, the olfactory channel (CNG-2) and CAP of *E. coli*. The output protein sequences unrelated to the queries were then analysed by the profile scan software available on the ExPasy web site (www.expasy.ch). Three sequences encoding proteins containing one or several cNMP-binding motifs (PS50042) were retrieved: the neuropathy target esterase (NTE, accession number: NM006702) which contains three putative cAMP-binding sites, a mouse embryo EST sequence (AI595216, Image 493605 cDNA clone) and the KIAA0313 human sequence (accession number: AB002311) provided from brain cDNA sequencing [19]. The alignment of their cNMP-binding motifs with the cAMP-binding domains of the query sequences was performed with the MultiAlign bioinformatic tool (Fig. 1). All these five putative cyclic nucleotide-binding domains fit quite well with the consensus se-

quence determined from the CAP-related proteins. Most of the conserved residues, located in the β -barrel, critical for the structure of the cAMP-binding pocket or involved in the cAMP interaction, were also found in the new putative cAMP-binding domains. In KIAA0313 we found the -G-GE-VI-G-V- and -G-FG- but not the PRAA/T motifs; in AI595216 we found -G-GD-Y-A-V-G-I- and -FGE-PRT-A-F-V- motifs; in NTE we found -G-(Q)GQ/D-Y-V-G(V)- in the three cNMP motifs of this protein; the PRAT sequence was only found in the third domain. As this motif was described to be involved in the interaction with the cyclic nucleotide, we presumed that this third cNMP-binding domain was the most likely to bind cAMP as compared with the other two.

NTE is a large transmembrane protein with a serine esterase activity which catalyses hydrolysis of lysophospholipids [20,21]. It is abundantly expressed in neurones of the brain, but also in a variety of non-neuronal tissues including intestine, placenta, testis and kidney. NTE inhibition by organophosphorus esters is responsible for the organophosphate-induced delayed neuropathy characterised by a paralysis of the lower limbs due to degeneration of long axons in the spinal cord and in peripheral nerves. In *Drosophila*, its homolog, the Swiss cheese (SWS) protein, is required for brain development. The catalytic domain of NTE is located in the C-terminal part of the protein; the N-terminal part, which contains the three cNMP-binding domains, constituting a putative regulatory domain.

The Im493605 sequence encodes a 228 residues polypeptide; we renamed it Im49. This small protein contains only one putative cNMP-binding site. Although this sequence was isolated from a mouse embryonic library, a Blast search using the Im49 clone sequence revealed only bacterial homologous sequences. It was not found in any eukaryote genome. The highest homology was found for a hypothetical protein from *Pseudomonas fluorescens* (ZP00086400.1), showing 70% identity with Im49. Furthermore, Northern blotting or reverse transcription-PCR reactions did not allow us to detect or amplify this sequence using adult or embryonic mRNA as material sources. These observations led us to conclude that Im49 was probably not coded by the mouse genome but most

probably by a bacterial organism infecting the mouse or the cDNA preparation during library construction.

KIAA0313 encodes the PDZ-GEF1 protein which was, at the same time, identified as a new Rap-specific GEF protein [22]. This protein is clearly related to the Rap-GEF family: it presents an overall similarity of 29% with Epac1, and homology raises to 50% in the cNMP-binding and GEF domains. However, unlike the Epac protein, PDZ-GEF1 was shown not to bind cAMP and its Rap-GEF activity not to be regulated by this second messenger [23]. It contains a PDZ domain, but its mechanism of regulation is not yet elucidated.

4. Demonstration of cAMP binding: methods

Following sequence analysis, the next step was to verify, by biochemical procedures, whether the putative cAMP-binding

proteins are really able to bind cAMP. A useful method, which has often been used to detect the R subunits of PKA in cell or tissue extracts, is the photolabelling with 8-azido-[32 P]cAMP. Pull down assays using cAMP-linked agarose beads (N6- or C8-linked beads from Sigma) is another simply method, which has been used by Kawazaki to demonstrate the cAMP-binding property of the Epac proteins [12]. Both can be performed using either in vitro translated proteins or proteins expressed either in BL21 bacteria such as GST-tagged proteins, or in COS cells such as myc- or His-tagged proteins. In our hand, both methods worked beautifully with the RI α subunit, but not convincingly with full length Epac. Obtaining large amounts of recombinant protein from bacteria is difficult for long transmembrane proteins, so for NTE, we decided to express only shorter fragments containing either the first cNMP-binding domain alone (NTE-d1), the second plus the

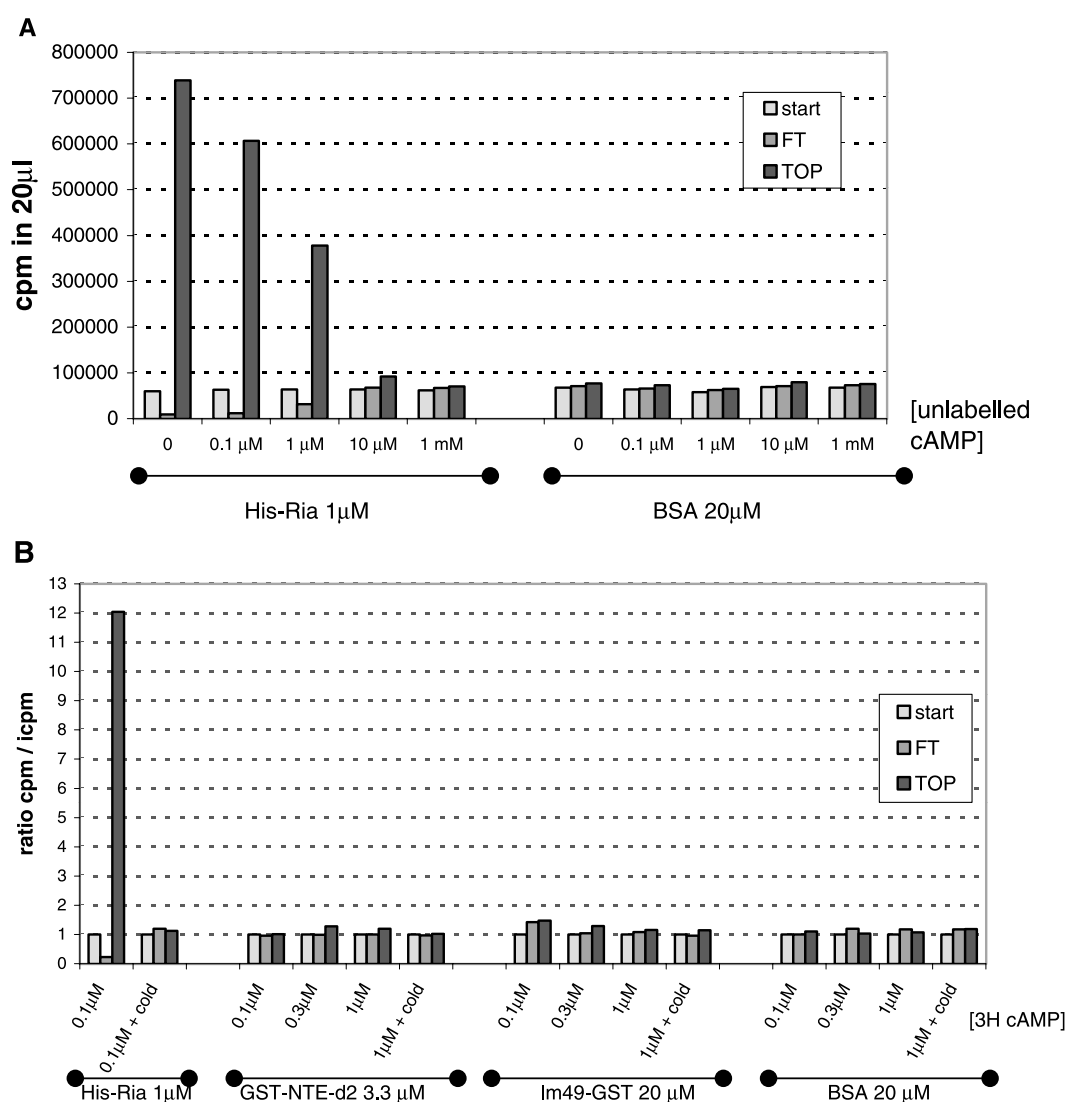


Fig. 2. cAMP-binding assay using small Microcon spin down filters (Microcon YM-10 from Amicon). The sample (300 μ l), containing [3 H]cAMP and the purified protein, was applied on the top of the filter. Filters were centrifuged so that the upper volume, in which the protein was concentrated, was reduced 10-fold. cpm content of 20 μ l was counted before the centrifugation in the initial sample (start) and after centrifugation, in the upper fraction (top) and the flow-through (FT). A: cAMP binding on purified His-tagged RI α (expressed in bacteria) using buffer containing 0.1 μ M [3 H]cAMP in the absence or with increasing amounts of unlabelled cAMP; B: binding on purified GST-tagged Im49 and GST-tagged NTE-d2 (containing the second and third cNMP-binding domains) using buffer containing 0.1, 0.3 or 1 μ M [3 H]cAMP; results were expressed in terms of the ratio of cpm counted at the end vs the initial cpm amount (icpm); competition of the binding was made with 1 mM of unlabelled cAMP (+cold).

third domains (NTE-d2), or all three domains (NTE-d3) fused to GST. EpacΔcA, deleted from its N-terminal part containing the cNMP-binding domain, and an irrelevant protein used as negative controls were negative. However, no specific cAMP binding was observed with NTE (full length or cNMP-containing domains) and Im49.

As we were unable to show binding of the 8-azido-cAMP analog to full length Epac, one of our positive controls, any conclusion could be drawn from the photolabelling experiments. The inability of Epac to bind cAMP in the pull down assays, using cAMP-agarose beads, does not agree with previous data published by Kawazaki. This negative result may be explained by the fact that the interaction of cAMP with Epac has been reported to be highly dynamic, implying a rapid dissociation rate [24]. Therefore, this suggests that the use of non-equilibrium methods may be not appropriate to detect cAMP binding on Epac. A suitable equilibrium cAMP-binding assay is the Hummel and Dreyer method based on size-exclusion chromatography (Sephadex G-25) using [³H]cAMP-containing buffer for equilibration and elution. This method worked very well in our hands with both RI subunit and Epac. Because our new putative cAMP-binding proteins NTE and Im49 might have a rapid dissociation rate, we decided to test their cAMP-binding abilities with this assay. However, our tested proteins, especially NTE, aggregated and bound irreversibly on the Sephadex columns. Several buffers were tested, but no convincing results were obtained.

We then developed a new method using small Microcon spin down filters (Microcon YM-10 from Amicon). The sample (300 μl), containing [³H]cAMP and the purified protein, is applied on top of the filter. Filters are centrifuged so that the upper volume, in which the protein is retained, is reduced 10-fold. In the case of cAMP binding to the protein, the concentration of the labelled nucleotide should increase in the upper part of the filter and decrease in the flow-through. Inversely concentrations should remain unchanged in the absence of binding. By this method both purified Epac1 and RIα subunit bound highly and specifically cAMP. The binding was abolished in the presence of unlabelled cyclic nucleotide (cAMP 1 mM) and was not observed with the bovine serum albumin used as a negative control. In the absence of unlabelled carrier, the percentage of cAMP bound to RIα was estimated to be around 81% (Fig. 2A). Consistent with the known affinity of the R subunit for cAMP, this percentage progressively diminished in the presence of increasing concentrations of unlabelled cAMP. Using this protocol, we have tested the cAMP binding on purified Im49-GST and NTE-d3-GST proteins. However, as shown in Fig. 2B, no cAMP binding to these queried proteins has been observed, even for concentrations of [³H]cAMP raising up to 1 μM (Dremier, unpublished data).

5. Significance of algorithm-predicted cNMP-binding domains

Since the discovery of CNG channels and Epac proteins and according to several studies, including our work in thyroid cells [5], reporting the involvement of PKA-independent mechanisms in various cAMP-mediated effects, it is now evident that PKAs are not the only intracellular cAMP receptors playing a critical role in the transduction of the cAMP signal in eukaryotes. Using a bioinformatics search, NTE, Im49 and

PDZ-GEF1 were identified as new putative cAMP-binding proteins having a cNMP-binding site related to CAP.

For PDZ-GEF1, which was identified simultaneously by us and several other groups, different contradictory data have been published concerning its ability to bind cAMP [25–27]. Indeed, Pham et al. reported that CNrasGEF, a protein identical to PDZ-GEF1, was a cAMP-responsive GEF for the small G protein Ras. However, a recent publication strongly suggests that PDZ-GEF1 definitely does not bind cAMP and that cAMP does not regulate its GEF activity [23]. The same conclusion has also been drawn for PDZ-GEF2, a new member of this protein family.

For NTE and Im49, neither the 8-azido-[³²P]cAMP photolabelling and cAMP-agarose pull down procedures, which worked nicely with the R subunit (but not with Epac), nor a new equilibrium cAMP-binding assay using small Microcon spin down filters, which worked with both Epac and R subunit, allowed us to demonstrate that these proteins were able to bind cAMP. Negative results could still be explained by the use of a non-appropriate method or procedure, by an incorrect folding of the protein after purification, by an absence of critical post-translational modification, or by requirement of interactions with protein partners, membranes or lipids. Nevertheless, consistent with our findings, it has recently been reported that cAMP does not regulate the in vitro esterase activity of NTE [21].

All these observations raise the question of the biochemical significance of cNMP-related binding domains. One probable explanation is that they result from biological evolution. We may suggest that their ancestral proteins were able to bind cAMP, but that this property has been lost with evolution. It is also possible that these proteins bind another nucleotide or another similar small molecule. Our observations indicate that the presence of a predicted cNMP-binding domain identified by bioinformatics tools is clearly not a sufficient criteria to predict a cAMP-binding property for a protein. By extension, we may assume that this is true for any predicted protein domain, so that such kind of data should be carefully examined before drawing conclusions about their real functional significance.

6. cAMP-binding proteins non-related to CAP

The possible existence of unknown cAR proteins remains an intriguing question.

In addition to the cAMP-binding proteins related to CAP, including R subunits of PKA, CNG channels and Epac proteins, at least two other types of cyclic nucleotide-binding proteins exist: the cyclic nucleotide phosphodiesterases (PDE, reviewed in [28]) containing a catalytic cyclic nucleotide-binding site and, for the PDE 2, 5, 6 isoforms, two additional non-catalytic cyclic nucleotide-binding sites named GAF domains (cGMP-specific and stimulated PDEs, *Anabaena* adenylate cyclases, and *E. coli* FhlA, [29]) and the plasma membrane cARs of *Dictyostelium* playing critical roles in the differentiation of this organism [30].

The observation that, in response to stimuli, cAMP is exported out of various cell types including renal cells, adipocytes, glial cells and cardiomyocytes suggests the existence of cAMP exporter proteins and even extracellular cAMP receptors in eukaryotes [31]. Identification of cAMP-binding proteins related to PDEs or to *Dictyostelium* cARs should be

attempted. Such proteins could be either identified by bioinformatics approaches, as we did with cAMP-binding proteins related to CAP, or isolated from cell or tissue membrane preparations by 8-azido-[³²P]cAMP photolabelling, as it was successfully done to isolate several cARs of *Dictyostelium* [32,33].

Acknowledgements: S.D. is Scientific Research Worker of the Fonds National de la Recherche Scientifique. The work of our lab is supported by the Ministère de la Politique Scientifique (PAI), the Fond National de la Recherche Médicale, the Télévie and the Association contre le cancer. We thank P. Glynn for the gift of the NTE cDNA and F. Zwartkruis for the gift of plasmids encoding Epac.

References

- [1] Doskeland, S.O., Maronde, E. and Gjertsen, B.T. (1993) *Biochim. Biophys. Acta* 1178, 249–258.
- [2] Dhallan, R.S., Yau, K.W., Schrader, K.A. and Reed, R.R. (1990) *Nature* 347, 184–187.
- [3] Nakamura, T. and Gold, G.H. (1987) *Nature* 325, 442–444.
- [4] Goulding, E.H., Tibbs, G.R. and Siegelbaum, S.A. (1994) *Nature* 372, 369–374.
- [5] Dremier, S., Pohl, V., Poteet-Smith, C., Roger, P.P., Corbin, J., Doskeland, S.O., Dumont, J.E. and Maenhaut, C. (1997) *Mol. Cell Biol.* 17, 6717–6726.
- [6] Anciaux, K., Van Dommelen, K., Nicolai, S., Van Mechelen, E. and Slegers, H. (1997) *J. Neurosci. Res.* 48, 324–333.
- [7] Busca, R., Abbe, P., Mantoux, F., Aberdam, E., Peyssonnaud, C., Eychene, A., Ortonne, J.P. and Ballotti, R. (2000) *EMBO J.* 19, 2900–2910.
- [8] Gonzalez-Robayna, I.J., Falender, A.E., Ochsner, S., Firestone, G.L. and Richards, J.S. (2000) *Mol. Endocrinol.* 14, 1283–1300.
- [9] Hirshman, C.A., Zhu, D., Panettieri, R.A. and Emala, C.W. (2001) *Am. J. Physiol. Cell Physiol.* 281, C1468–C1476.
- [10] Staples, K.J., Bergmann, M., Tomita, K., Houslay, M.D., McPhee, I., Barnes, P.J., Giembycz, M.A. and Newton, R. (2001) *J. Immunol.* 167, 2074–2080.
- [11] de Rooij, J., Zwartkruis, F.J., Verheijen, M.H., Cool, R.H., Nijman, S.M., Wittinghofer, A. and Bos, J.L. (1998) *Nature* 396, 474–477.
- [12] Kawasaki, H., Springett, G.M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D.E. and Graybiel, A.M. (1998) *Science* 282, 2275–2279.
- [13] de Rooij, J., Rehmann, H., van Triest, M., Cool, R.H., Wittinghofer, A. and Bos, J.L. (2000) *J. Biol. Chem.* 275, 20829–20836.
- [14] Weber, I.T. and Steitz, T.A. (1987) *J. Mol. Biol.* 198, 311–326.
- [15] Shabb, J.B. and Corbin, J.D. (1992) *J. Biol. Chem.* 267, 5723–5726.
- [16] Canaves, J.M. and Taylor, S.S. (2002) *J. Mol. Evol.* 54, 17–29.
- [17] Ludwig, A., Zong, X., Jeglitsch, M., Hofmann, F. and Biel, M. (1998) *Nature* 393, 587–591.
- [18] Santoro, B. and Tibbs, G.R. (1999) *Ann. N.Y. Acad. Sci.* 868, 741–764.
- [19] Nagase, T., Ishikawa, K., Nakajima, D., Ohira, M., Seki, N., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N. and Ohara, O. (1997) *DNA Res.* 4, 141–150.
- [20] Glynn, P. (1999) *Biochem. J.* 344, 625–631.
- [21] van Tienhoven, M., Atkins, J., Li, Y. and Glynn, P. (2002) *J. Biol. Chem.* 277, 20942–20948.
- [22] de Rooij, J., Boenink, N.M., van Triest, M., Cool, R.H., Wittinghofer, A. and Bos, J.L. (1999) *J. Biol. Chem.* 274, 38125–38130.
- [23] Kuiperij, H.B., de Rooij, J., Rehmann, H., van Triest, M., Wittinghofer, A., Bos, J.L. and Zwartkruis, F.J. (2003) *Biochim. Biophys. Acta* 1593, 141–149.
- [24] Kraemer, A., Rehmann, H.R., Cool, R.H., Theiss, C., de Rooij, J., Bos, J.L. and Wittinghofer, A. (2001) *J. Mol. Biol.* 306, 1167–1177.
- [25] Liao, Y., Kariya, K., Hu, C.D., Shibatohe, M., Goshima, M., Okada, T., Watari, Y., Gao, X., Jin, T.G., Yamawaki-Kataoka, Y. and Kataoka, T. (1999) *J. Biol. Chem.* 274, 37815–37820.
- [26] Ohtsuka, T., Hata, Y., Ide, N., Yasuda, T., Inoue, E., Inoue, T., Mizoguchi, A. and Takai, Y. (1999) *Biochem. Biophys. Res. Commun.* 265, 38–44.
- [27] Pham, N., Cheglakov, I., Koch, C.A., de Hoog, C.L., Moran, M.F. and Rotin, D. (2000) *Curr. Biol.* 10, 555–558.
- [28] Conti, M. and Jin, S.L. (1999) *Prog. Nucleic Acids Res. Mol. Biol.* 63, 1–38.
- [29] Aravind, L. and Ponting, C.P. (1997) *Trends Biochem. Sci.* 22, 458–459.
- [30] Johnson, R.L., Van Haastert, P.J., Kimmel, A.R., Saxe, C.L., Jastorff, B. and Devreotes, P.N. (1992) *J. Biol. Chem.* 267, 4600–4607.
- [31] Bankir, L., Ahloulay, M., Devreotes, P.N. and Parent, C.A. (2002) *Am. J. Physiol. Renal Physiol.* 282, F376–F392.
- [32] Theibert, A., Klein, P. and Devreotes, P.N. (1984) *J. Biol. Chem.* 259, 12318–12321.
- [33] Klein, P.S., Sun, T.J., Saxe, C.L., Kimmel, A.R., Johnson, R.L. and Devreotes, P.N. (1988) *Science* 241, 1467–1472.