Online immobilized metal affinity chromatography/mass spectrometric analysis of changes elicited by cCMP in the murine brain phosphoproteome

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An automated online immobilized metal affinity chromatography/high-performance liquid chromatography mass spectrometric (IMAC-HPLC/MS/MS) method was developed to study cytidine 3′,5′-cyclic monophosphate (cCMP)-specific protein phosphorylation, analogous to a previously successful offline IMAC method using microvolume IMAC pipette tips. The optimized method identified murine brain phosphoproteins selectively modified by challenge with cCMP, using manual interpretation of the results to confirm both phosphorylation and selectivity of response to cCMP. A number of proteins identified by this strategy have potential roles in hyperproliferation, a previously reported response to elevated levels of cCMP. Copyright © 2008 John Wiley & Sons, Ltd.

Adenosine 3′,5′-cyclic monophosphate (cAMP) has well-established functions as a biochemical second messenger, mediating the action of a wide range of mammalian hormones and neurotransmitters,1 whilst a second cyclic nucleotide, guanosine 3′,5′-cyclic monophosphate (cGMP), performs a similar but more restricted function, responding to primary messengers such as nitric oxide, natriuretic peptides and rhodopsin.2 These roles of purine cyclic nucleotides as secondary messengers posed the question as to whether pyrimidine cyclic nucleotides also exist and perform a similar role as second messengers. The natural occurrence of cCMP has now been unequivocally demonstrated by means of tandem spectrometric (MS/MS) analysis of sequentially purified tissue extracts.3 A number of factors varied biological effects of cCMP have been reported.4–6 However, the precise mechanism(s) of its action and its overall cellular function are not yet fully elucidated. The identification of the phosphorylation substrates of cCMP-responsive protein kinase is envisaged to be a key step in completely elucidating the function of cCMP in signal transduction. Bond et al.7 have previously reported Rab23 to be specifically phosphorylated in response to challenge by cCMP, phosphoproteins having been selected for using immobilized metal affinity chromatography (IMAC) ZipTip loaded with iron ions, prior to MS/MS analysis of tryptic digests. Whilst advances have been made in IMAC technology, including the use of titanium dioxide, the iron ion IMAC method that had previously proved to be successful was further developed in this study. The aim of this study is to identify more murine brain proteins which are uniquely phosphorylated in response to elevated levels of cCMP, utilizing an online, more automated and robust IMAC purification methodology applied at the peptidomic level.

Improvements in phosphopeptide enrichment by IMAC and in mass spectrometry have made it possible to identify phosphorylated proteins on a proteome-wide basis,8,9 with IMAC coupled to liquid chromatography (LC)/MS/MS a powerful method for identifying phosphorylated proteins present in complex mixtures of non-phosphorylated proteins.10,11 In this study, murine brain homogenates were incubated for a series of time periods with constant amounts of ATP together with one of three different cyclic nucleotides, cAMP, cGMP or cCMP, or ATP alone as a ‘blank’ control experiment. Normal intracellular cyclic nucleotide (cAMP) levels in brain are approximately 0.5 mM;12 hence 2 mM was chosen as an elevated level without having an adverse effect on functioning brain cells. ATP is a phosphate donor and a concentration of 5 mM was chosen such that ATP is in excess and will not be a hindering factor in the study. An online IMAC-nano-LC/MS platform for phosphopeptide profiling was prepared and validated by comparison with the IMAC ZipTip protocol shown to be successful previously7 and phosphorylated proteins in cCMP-incubated mouse brain homogenate were characterized. The identification of these phosphorylated proteins should provide new avenues for investigating the mechanism of cCMP signalling pathways and its cellular function.

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EXPERIMENTAL

Chemicals
All chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK) with the exceptions of glycerol, acetic acid, hydrochloric acid (HCl), methanol (MeOH) and acetonitrile (ACN), obtained from Fisher Scientific (Loughborough, Leicester, UK). The MALDI matrices o-cyano-4-hydroxycinnamic acid and sinapinic acid were purchased from Fluka (Sigma-Aldrich) and the MALDI calibration mixture 1 was obtained from Applied Biosystems (Warrington, UK). Formic acid (FA) was purchased from Analar (Dorset, UK), and the dialysis membrane for the dialysis of the whole brain homogenate purchased from Medicell International (London, UK). The oxygen-free nitrogen was supplied by BOC Ltd. (Guildford, Surrey, UK). Milli-Q purified water was prepared using the Elix® and Milli-Q® Ultrapure water purification system obtained from Millipore UK, Ltd. (Watford, UK).

Animals
Forty-eight 8-week-old female mice, obtained from Harlan UK Ltd. (Shaw’s Farm, Blackthorn Bicester, Oxon, UK), were sacrificed in two batches of 24 over two consecutive weeks. The sacrifice of the mice was conducted on the same day as delivery and performed rapidly by a blow to the base of the head followed by decapitation. The brains were then removed and pooled into ice-cold extraction buffer (supra vide) to avoid tissue decomposition due to post mortem changes.

Sample preparation for the extraction of proteins from mouse brain tissue
The extraction buffer was 50 mM Tris-HCl, pH 7.4 (adjusted with 6 M HCl), containing a final concentration of 78 mM diethiothreitol (DTT), 1 mM ethylenediamine tetraacetic acid disodium salt (EDTA) solution, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM pepstatin A and 20 mM leupeptin. The buffer was freshly prepared immediately before use and maintained at ice-cold temperatures during the extraction of the brain proteins. Murine brains were placed in 1:9 (w/v) of the extraction buffer immediately after removal. To ensure effective and full disruption of the cells and minimize protein denaturation due to heating, the tissue was disrupted by effective and full disruption of the cells and minimize protein denaturation due to heating, the tissue was disrupted by an Elvehjem homogenizer (Braun Melsunger, Germany) at 900 rpm for four 10-s bursts. The resulting crude tissue homogenate was aliquoted and one aliquot was utilized for protein quantitation.

A standard 2 mM solution of one cyclic nucleotide (cAMP, cGMP or cCMP) and 5 mM ATP disodium salt was prepared in ice-cold 50 mM Tris-HCl buffer (pH 7.4). A control lacking cyclic nucleotides was also prepared and 4 x 5 mL of each of these solutions were pipetted into the previously prepared mice brain homogenate aliquots. These solutions were then vortexed and incubated at 37°C for 1, 5, 15 and 30 min in a water bath to allow the transfer of phosphate moieties from the ATP donor into substrate proteins catalyzed by endogenous protein kinases. At the allocated times the incubations were transferred to an ice bath and cooled for 5 min to prevent further kinase activity. The samples were then stored at -80°C. Before further analysis, the samples were thawed and centrifuged at 16,000 g for 30 min to remove cell debris and insoluble material. The sample solution was then dialyzed against 50 mM Tris-HCl with 7 mM DTT and the protease inhibitors 1 mM EDTA and 1 mM PMSF in the cold room overnight and separated into 200 μL aliquots. Each aliquot was then freeze-dried and stored at -80°C.

Protein identification by IMAC
Trypsin digestion
Sequencing-grade modified trypsin (Promega, Madison, USA) was used for digestion of the freeze-dried brain homogenates. The trypsin solution was made by first dissolving trypsin (20 μg) in 20 μL of 50 mM acetic acid, then diluting 50 times with 40 mM NH4HCO3. The trypsin solution was added to the brain homogenate to create a trypsin/protein ratio of 1:100 after which the homogenates were incubated at 37°C overnight. The reaction was terminated by freeze-drying the samples. The samples were resuspended in 0.1% acetic acid before application to further separation and LC/MS analysis.

IMAC Ziptip
IMAC ZipTips®MC were utilized following the manufacturer’s instructions.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS
The MALDI-TOF MS analysis was conducted on a Voyager-DE STR mass spectrometer (Applied Biosciences, Warrington, UK) in positive ion detection mode, the pulsed nitrogen laser operating at 337 nm and reflector mode used for the peptide analysis. For the analyses, 0.5 μL of peptide sample was applied to the MALDI plate followed by 0.5 μL of matrix (a saturated solution of 10 mg/mL o-cyano-4-hydroxycinnamic acid in 50% 0.1% TFA/ACN). The plate was then allowed to dry at room temperature. A 400 ns delay and accelerating voltage of 25 kV were applied to the analysis and the spectra from 100 shots were accumulated.

Online IMAC-LC/MS for enrichment and identification of phosphopeptides
The online IMAC experiments were performed using an Ultimate Capillary LC system (LC Packings, Dionex, UK). A FAMOS microautosampler (Dionex) equipped with a 20 μL loop was used for injections. The online IMAC setup involved two pump-switching systems. The IMAC trap column (300 μM x 5 mm; Dionex, Camberly, UK) was installed in between the six-port injection valve and port 2 of the loading pump valve B (Fig. 1). The loading pump flow rate was 5 μL/min. The analytical column was run at a flow rate of 200 nL/min delivered by an Ultimate pump. The IMAC trap column was connected firstly to a waste line (port 3, see dashed line in valve B) and was initially flushed with 20 μL of 50 mM EDTA for 3 min, followed by two washes with 20 μL of 100 mM FeCl3 before 20 μL of sample were loaded onto the trap. Next 20 μL of 100 mM NaCl in 0.1% HAC were loaded in order to wash off any non-phosphorylated peptides. The loading pump valve B was then switched so...
that port 2 was connected to port 1 (solid line of valve B) and port 4 of valve A, while valve A was in position 1-2 (dashed line in valve A). Then 20 µL of the elution solution, 50 mM Na₂HPO₄ (pH 9.0), were loaded onto the IMAC trap, eluting phosphorylated peptides onto a C18 trap column (300 µm i.d. × 5 mm packed with 3 µm 100 Å PepMap C₁₈ stationary phase; Dionex) enabling their desalting and preconcentration. The eluted phosphopeptides were retained on the trap column for 3 min to allow sufficient time to desalt the sample. After 3 min, valve A was switched to the 1-10 position (continuous line in Fig. 1) and the peptides on the C18 trap column were back flushed onto the C18 nano-column. The Chromelon software suite (Dionex) was used for instrument control and for triggering of MS data acquisition. The peptides were resolved on an in-house prepared analytical fused-silica nano-column of 15 cm × 75 µm i.d. packed with 3 µm particles, 100 Å pore PepMap C₁₈ stationary phase (Dionex). Mobile phase A was 0.1:97.9:2 (v/v/v) FA/H₂O/ACN while mobile phase B was 0.1:1.9:98 (v/v/v) FA/H₂O/ACN. Peptides were eluted from the column with a linear gradient of 0–45% B over 110 min. Finally, the column was re-equilibrated for 30 min with the initial mobile phase A between runs. Details of valve switches of the online IMAC system are shown in Table 1. The eluted peptides were analyzed by an LCQ Deca XP ion trap mass spectrometer equipped with a nanoelectrospray ionization (ESI) source (Thermo Finnigan, Hemel Hempstead, UK). The mass spectrometer was operated in positive ionization mode with a spray voltage of 1.7 kV, a capillary temperature of 160°C and a capillary voltage of 10 V. Mass spectral data were recorded for the total 140 min run time in a data-dependent MS/MS mode which included four scan events. A full scan mass spectrum was followed by collision-induced dissociation of the three highest abundant ions selected from the full scan. The mass scan range was 475–2000 Da, with an isolation width of 2 Da and normalized collision energy of 35% (arbitrary units) for generation of product ion spectra.

### Protein sequence database searching

Bioworks database software (ThermoFinnigan, UK) was used for the correlation of trypsin-digested proteins with a differential modification of 80 Da on serine, threonine, and tyrosine. All data sets were searched using the following acknowledged constraints: ±2.5 Da mass tolerance for peptide precursor mass searching; ±0.5 Da mass tolerance for fragment ions and searched against a Mouse-protein Indexed version of NR database FASTA downloaded May 2005 and containing 26 650 entries. Given the age of this database compared to the publication date of this manuscript, the peptide sequences were subjected to manual searches via the EMBL protein similarity search facility in order to re-confirm the protein identifications derived from the peptide sequences obtained. The output files from the

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**Table 1. Details of valve switches of the online IMAC system**

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<thead>
<tr>
<th>Event</th>
<th>Valve B</th>
<th>Valve A</th>
<th>Time (min)</th>
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<tbody>
<tr>
<td>EDTA washing</td>
<td>1–10</td>
<td>1–2</td>
<td>8</td>
</tr>
<tr>
<td>Metal binding</td>
<td>1–10</td>
<td>1–2</td>
<td>8</td>
</tr>
<tr>
<td>Metal binding</td>
<td>1–10</td>
<td>1–2</td>
<td>12</td>
</tr>
<tr>
<td>Sample loading to IMAC trap</td>
<td>1–10</td>
<td>1–2</td>
<td>12</td>
</tr>
<tr>
<td>Washing off unbound peptides</td>
<td>1–10</td>
<td>1–2</td>
<td>8</td>
</tr>
<tr>
<td>Eluting from IMAC trap to C18 trap</td>
<td>1–10</td>
<td>1–2</td>
<td>15</td>
</tr>
<tr>
<td>Backflushing from C18 trap to MS</td>
<td>1–10</td>
<td>1–10</td>
<td>140</td>
</tr>
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</table>

*For valve position 1–10 and 1–2, see Fig. 1.*
correlation analysis were further studied using the XC scores, to produce a list of identified peptides and corresponding proteins. These complied with the well-accepted filter parameters; dCn >0.1, sP >150 and XCorr >1.8 (z = 1), 2.2 (z = 2) and 2.8 (z = 3) (personal communication, manufacturer). Additionally, the MS/MS spectra of identified phosphopeptide sequences were manually verified against the theoretical fragmentation patterns.

RESULTS

Evaluation of the online IMAC setup

$\beta$-Casein is a naturally occurring phosphorylated protein. According to the literature,$^{15}$ $\beta$-casein contains two tryptic phosphopeptides, one monophosphorylated peptide, FQpSEEQQQTEDELQDK (amino acid residues 48–63), and one tetraphosphorylated peptide, RELEELNVPGEI-VEpSLpEpSEESITR. In this study, the trypsin digest of $\beta$-casein was used as a standard peptide mixture in order to evaluate the IMAC phosphopeptide enrichment. The method development was based on the existing successful method developed by Bond et al.$^7$ in which IMAC ZipTips were used. MALDI-TOF spectra of standard peptides before and after passage through IMAC ZipTip are shown in Fig. 2. In this figure, after the IMAC ZipTip, the peaks with $m/z$ 1382.9, 2185.7, 2222.7, 2910.7 and 3111.7 are absent while the signal intensities, making their detection more difficult.$^{16}$ Interestingly, there is a peak at $m/z$ 1967.3 observed as being bound to the IMAC ZipTip which does not correspond to any previously reported phosphopeptide from $\beta$-casein or any anticipated cleavage of the known phosphopeptides. It may be postulated that this peptide arises due to the impurities present in the $\beta$-casein samples used (90% purity); however, to date, this ion has not been identified by our group. Despite this, the IMAC ZipTip is shown to purify the expected phosphopeptide from the peptide mixture which provides a reference spectrum for the evaluation of the performance of the proposed online clean-up setup. Hence as the IMAC ZipTip had been shown to be successful in previous reports, this process could be used as a ‘benchmark’ against which the developed technique could be compared.

$\beta$-Casein trypsin-digested peptides were loaded onto the IMAC trap column, the eluents of sample loading, washing and Na$_2$HPO$_4$ elution were collected and analyzed by MALDI-TOF MS. Figure 3(a) shows mixtures of peptides before loading onto the IMAC trap; the monophosphorylated peptide with $m/z$ 2061 is relatively weak compared to other unphosphorylated peptides (indicated by the arrow). In Fig. 3(b), the spectrum of the eluent of the sample loading step is shown and no phosphorylated peptide was observed; similarly in Fig. 3(c), the spectrum of the eluent of the washing step is shown and no phosphorylated peptide was observed; however, the spectrum exactly mimics that obtained from the IMAC ZipTip cleanup that had proved successful. The tetraphosphorylated peptide was again absent; however, the spectrum exactly mimics that obtained from the IMAC ZipTip cleanup that had proved successful. These spectra demonstrate that the online IMAC system used in this study can enrich phosphorylated peptides as efficiently as previously seen in IMAC ZipTip preparation protocols utilized for cCMP

Figure 2. MALDI-TOF mass spectra of $\beta$-casein trypsin-digested peptide mixture before and after IMAC ZipTip.
Figure 3. (a) MALDI-TOF mass spectrum of β-casein trypsin digest (monophosphopeptide m/z 2061 is shown with an arrow). (b) MALDI-TOF mass spectrum of IMAC trap column flow through following the loading of β-casein trypsin digest. (c) MALDI-TOF mass spectrum of eluent from IMAC trap column of β-casein trypsin digest as the IMAC trap column was washed with NaCl solution. (d) MALDI-TOF mass spectrum of eluent from IMAC trap column of β-casein trypsin digest as the retained peptides were eluted with Na₂HPO₄ solution from the IMAC trap column.
investigations. Whilst MALDI analysis was utilized for the validation of the purification setup, due to the speed of data acquisition and the simplicity of the peptide sample under analysis, for the study of more complex biological samples HPLC-MS/MS separation was required prior to MS analysis and so ESI-MS/MS was used in this aspect of the study. Furthermore, for validation of the online trapping system, the phosphopeptides expected are of known m/z value, whilst for the biological analysis these are not known. Hence, more informative information (rather than purely the m/z value as obtained by the MALDI analysis) is required for the study of the murine phosphopeptides and therefore tandem mass spectrometry (MS/MS) was utilized for this purpose.

Next the online IMAC setup was utilized in order to selectively purify the phosphopeptides from the murine brain digests. Phosphorylated peptides were identified by searching the mouse database using Bioworks with parameters set to detect a differential modification of 80 Da on serine, threonine, and tyrosine. The filters were applied to the database search and the assignments of phosphopeptide sequences were confirmed by manually comparing the acquired MS/MS spectra with the theoretical fragmentation pattern. On average, 40–50 phosphorylated peptides were identified in each time point of the cyclic fragmentation pattern. On average, 40–50 phosphorylated peptides were identified in each time point of the cyclic fragmentation pattern. On average, 40–50 phosphorylated peptides were identified in each time point of the cyclic fragmentation pattern. On average, 40–50 phosphorylated peptides were identified in each time point of the cyclic fragmentation pattern.

Each protein was initially determined to be unique to cCMP were therefore re-examined by studying the raw LC/MS data in order to ensure that the peptide was either unique to the cCMP incubation or that the intensity of this peptide was significantly elevated in the cCMP incubation compared to the other incubations. For example, the unique phosphopeptide from the protein serologically defined colon cancer antigen 13 was identified in the 30 min cCMP incubation. The phosphopeptide identified was RHKPGPRpTGGLYISRP via its doubly charged precursor ion at m/z 758.24 and a retention time of 89.2 min. The extracted ion chromatogram (XIC) of m/z 758.24 for the four incubations is shown in Fig. 4. There is a strong peak at retention time 89.27 min in the cCMP sample,

**Figure 4.** XIC of m/z range of 758.24 of the four cyclic nucleotide incubations as obtained with the LC/MS setup in which a 75 μm i.d. C18 column was eluted with 0–45% B for 110 min, 98%ACN with 0.1% formic acid gradient program and the effluent from the column was introduced into an LCQ Deca ion trap mass spectrometer.
but the peptide is absent from the other incubations. This peptide is characterized as unique to cCMP with high confidence, and serologically defined colon cancer antigen 13 was therefore designated as a putative cCMP-responsive protein kinase substrate.

Another example shows the exclusion of a false positive identification. A cystathionase-derived phosphopeptide was identified only in the 30 min cCMP incubation, by the Bioworks database search of the data from all four incubations. The fragmentation of the doubly charged precursor ion with m/z 962.6 at retention time 62.6 min matches the theoretical phosphopeptide in the database with the sequence of KAGDEIICMDEVYGGpTNRY. The XIC of m/z 962.6 for the four incubations is shown in Fig. 5; all four cyclic nucleotide incubations show obvious peaks with similar intensity at the specified retention time (with the highest being in the cAMP incubation). Therefore, this protein was shown to be a false identification although the peptides in cAMP, cGMP and Blank were not selected for MS/MS analysis by the mass spectrometer software. By using this screening method, the proteins that are phosphorylated specifically due to the elevated level of cCMP can be identified. Furthermore, peptides carrying a phosphate group can be identified by the neutral loss of H$_3$PO$_4$ from the precursor ions in the positive mode. In view of the suggestion that IMAC enriches phosphopeptides, but that other peptides may be co-purified, this offers the potential confirmation of the phosphorylation state of the peptide. The cCMP-specific phosphopeptides were therefore re-examined to determine whether the neutral loss of H$_3$PO$_4$ occurred and peptides without the neutral loss of H$_3$PO$_4$ again considered to be false identifications. An example of a cCMP-specific peptide that fulfis this criterion is the peptide RHKGpGpRGpTGGLVISRP (serologically defined colon cancer antigen 13) described previously. Figure 6 shows the full scan spectrum at retention time 89.27 min indicating the complexity of the sample, even after IMAC enrichment, and hence demonstrating the requirement for the focused removal of false positive results. As shown in Fig. 6(b) the peptide with m/z of 758.27 was recognized as the phosphopeptide with a sequence of RHKGpGpRGpTGGLVISRP and the most abundant product ion from the precursor ion (758.27) is the loss of the phosphate group as shown in the spectrum (Fig. 6(b), indicated by”). Also in the spectrum, 15 out of 26 expected b and y ions generated from M5/M5 of this peptide can be observed and the phosphorylation site can be assigned to threonine as the mass difference between b$_7$ and b$_6$ ions is 101 Th + 80. The phosphorylated peptides that are unique in cCMP incubation or higher in cCMP incubation at the different incubation times and their associated proteins are listed in Table 2. It can be observed that the number of phosphorylated proteins specifically arising due to treatment with CMP increases with the incubation time. This could be explained by the fact that the blank sample still contains some other cyclic nucleotides, thus there are the endogenous cCMP, cAMP and cGMP levels initially in each of the incubations. Therefore, at shorter incubation times less difference between the samples may be obvious. As time of incubation increases and the cyclic nucleotides in the brain are used up the proportional effect of the added cyclic nucleotide will be greater and be responsible for the detected phosphorylation effects. This would suggest that the later
time points represent the more cyclic nucleotide specific protein phosphorylations, whilst in earlier time points these specific modifications are competing with the low levels of protein modification caused by endogenous cyclic nucleotides. Encouragingly, given the tentative role of cCMP in hyperproliferation of cells, many of the proteins uniquely phosphorylated upon addition of cCMP are involved in the control of the cell cycle, cell development and proliferative processes, as elaborated further in the discussion.

DISCUSSION

In this study, an online IMAC-nano-HPLC/MS platform for phosphopeptide profiling was developed, validated and used to study specific phosphorylated proteins in murine brain homogenates incubated with cCMP. Phosphopeptide enrichment by immobilized metal affinity chromatography (IMAC), followed by nano-LC/MS and protein database searching, have enabled the identification of abundant phosphorylated proteins on a proteome-wide basis. Online IMAC exhibits better loading capacity than IMAC ZipTips; the samples can be processed automatically instead of employing the time-consuming ZipTip process, and the avoidance of the manual ZipTip process would be expected to provide more reproducible results by removing potential human error. The identification of proteins specifically phosphorylated in response to cCMP was an integral part of this study and this identification should provide new avenues for investigating the mechanism of cCMP signalling pathways and its precise cellular function. It has been reported that cCMP has varied biological effects, for example, the stimulation of leukaemia L-1210 cells and DNA synthesis and the inhibition of RNA synthesis. Many observations are consistent with cCMP performing a role in the regulation of cellular growth and division. Both cytidylyl cyclase and cyclic CMP-specific phosphodiesterase are regulated by endogenous proteins and by steroid hormones, with the cyclase elevated in faster growing dividing cells and the phosphodiesterase depressed. The cCMP levels are elevated in response to long acting thyroid stimulator.

Figure 6. (a) Full scan MS spectrum at retention time 89.27 min. (b) MS/MS spectrum of doubly charged peptide with sequence RHKGPGpTGGVISRP precursor ion m/z 758.24, * Neutral loss of 98 Da from precursor ions (MH22+ - 49).
increased cell proliferation rate\(^20\) (including leukaemic cells and foetal cells\(^21\)) and in regenerating cells,\(^22\) while exogenous cCMP derivatives increase the division rate of murine brain cell lines.\(^23\)

In this study, several proteins were identified as being uniquely phosphorylated due to an elevated level of cCMP, and the phosphorylation of these proteins can be linked to the biological function of cCMP. Table 2 suggests that cCMP is involved in the phosphorylation of many proteins involved in cell proliferation, consistent with observations that elevated levels of cCMP and cytidylyl cyclase activities occur in rapidly differentiating cells.

### One minute incubation phosphoproteins

A formin homology 2 domain-containing protein was unique to the cCMP-challenged phosphoproteome in the 1 min incubation samples. This is a member of a diverse family of proteins\(^24\) that have been shown to interact with Rho-family GTPases, in order to organize the cytoskeleton of the cell and also regulate gene expression.\(^25\) This organization of the cytoskeletal system is an intrinsic component of the regulation of cellular growth and division.

### Five minute incubation phosphoproteins

The MAP-kinase activating death domain isoform 8 identified as being unique to the cCMP-containing incubation is generally pro-apoptotic; however, following activation of MAPK, this isoform is pro-survival and lacks exon 16, which has the pro-apoptotic domain.\(^26\)

### Fifteen minute incubation phosphoproteins

The protein kinase identified as being phosphorylated selectively by cCMP has been shown to exhibit antitumor activity, requiring autophosphorylation and dimerization,\(^27\) and to have a role in TNF-mediated apoptosis.\(^28\) The stimulatory phosphorylation is thought to occur on a serine residue, whereas the cCMP-mediated phosphorylation is shown to occur on a tyrosine residue (as indicated in the SwissProt database) and hence may potentially play an inhibitory role. Lamin B2 is a basic structural component of nuclear lamina, which is thought to provide a framework for the nuclear envelope and may also interact with chromatin.\(^29\) The structural integrity of the lamina is strictly controlled by the cell cycle\(^30\) and increased phosphorylation of the lamins occurs before envelope disintegration and probably plays a role in regulating lamin associations. Therefore, it is possible that phosphorylation may play a role via this protein in an increase in cell cycle potentially linked to hyperproliferation.

### Thirty minute incubation phosphoproteins

A colon cancer antigen 13 is determined to be phosphorylated in response to cCMP, potentially linking cCMP action and hyperproliferative disorders such as cancer. Similarly, the mixed lineage-leukaemia translocation to 1 homolog (MLL) is an oncogene protein involved in rearrangement in leukaemia.\(^31\) The normal MLL gene plays a key role in developmental regulation of gene expression (including HOX genes) and the protein regulates cell cycle through cyclin-dependent kinase inhibitors p27 and p18.\(^32\) Low density lipoprotein receptor-related protein 1 was an

<table>
<thead>
<tr>
<th>Table 2. Phosphorylated proteins unique to murine brain incubations with exogenous cCMP, as identified by Bioworks</th>
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<tr>
<td>Incubation</td>
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<tr>
<td>1 min</td>
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<td>5 min</td>
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<td>15 min</td>
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<td>30 min</td>
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Changes elicited by cCMP in the murine brain phosphoproteome 4137
unsuspected phosphorylation product of cCMP; however, further literature searches determined that the protein can also be involved in breast tumor establishment and progression and also may function as a signalling receptor having a possible role during embryonal development. Both embryo development and breast cancer progression require hyperproliferative processes. Centromere/kinetochore protein zw10 homolog (zw10) regulates the cell cycle via its role in chromosome segregation during cell division. During the cell cycle, the zw10 protein moves from the centromere/kinetochore at prometaphase to kinetochore microtubules at metaphase, and then back to the centromere/kinetochore at anaphase, suggesting that zw10 may act at the kinetochore as part of a tension-sensing checkpoint that renders anaphase onset dependent on bipolar tension exerted across all centromeres. The deleted in azoospermia-like proteins are thought to contain RNA-binding motifs and having a possible role during embryonal development.

This finding further confirms the role of cCMP as a secondary messenger which acts via a specific kinase, which is not responsive to other cyclic nucleotide signals and so competition for fragment ions is also included. The online methodology utilized in this study was used as a more robust alternative to the previously applied microvolume pipette protocol. The analysis of phosphoproteins selectively phosphorylated due to cCMP incubation by this previous protocol identified RAB23 as a product. The absence of this protein in the study shows that the remit of this present work, the study shows that the phosphoproteome does respond selectively to cCMP incubation. This finding further confirms the role of cCMP as a secondary messenger which acts via a specific kinase, which is not responsive to other cyclic nucleotide signals and so adds to the continuing study of this relatively poorly understood cell signalling mechanism.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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REFERENCES