Mass spectrometric identification of Rab23 phosphorylation as a response to challenge by cytidine 3′,5′-cyclic monophosphate in mouse brain

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While the functions and mechanisms of action of adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) are well established and are the basis of the action of a large number of successful pharmaceuticals, the role of a third naturally occurring cyclic nucleotide, cytidine 3′,5′-cyclic monophosphate (cCMP), remains to be elucidated. Immobilized metal affinity chromatography (IMAC) was used to selectively extract proteins phosphorylated in mouse brain in response to challenge by cAMP, cGMP and cCMP, followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToFMS) and liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) of tryptic digests to identify Rab23 as the first protein reported to be phosphorylated only in response to cCMP.

The discovery of adenosine 3′,5′-cyclic monophosphate (cAMP) led to many of the current concepts of biological signalling between cells and of metabolic regulation,1 the fundamental second messenger concept being one of them.2,3 While cyclic AMP appears omnipotent in its effects upon mammalian metabolic pathways, a second naturally occurring cyclic nucleotide, guanosine 3′,5′-cyclic monophosphate (cGMP), has analogous but more restricted regulatory roles, functioning for example in the eye in visual transduction, as a mediator of atrial natriuretic peptides, and as a respondent to nitric oxide. Knowledge of these cyclic nucleotide second messenger systems has enabled their successful therapeutic manipulation by pharmacological agents ranging from β-blockers to Viagra4, with over 1000 currently prescribed drugs eliciting their effects via cyclic nucleotide-mediated processes: furthermore, understanding of cyclic nucleotide mechanisms of action led to an appreciation of the wider role of protein phosphorylation in biological regulatory processes.4

While the second messenger functions of these two purine cyclic nucleotides, cAMP and cGMP, are well established, a third naturally occurring cyclic nucleotide, cytidine 3′,5′-cyclic monophosphate (cCMP), a pyrimidine, has caused great controversy. Mass spectrometry has already played a key role in establishing the natural occurrence of cyclic CMP.

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The first evidence in 1974–1975,5,6 of the extraction, from leukaemic and regenerating liver cells, of a compound which co-chromatographed with an authentic standard, initially led to vigorous debate over the identity of the putative cCMP. Subsequently, an enzyme capable of catalyzing the synthesis of cCMP from CTP, cytidylyl cyclase, was reported,7–10 as was a phosphodiesterase capable of hydrolyzing cCMP to CMP.11–13 The argument was thus made that a third cyclic nucleotide existed in nature, together with enzymes selective for its synthesis and hydrolysis and factors capable of regulating its intracellular concentration, consistent with it possessing an analogous regulatory role to cAMP and cGMP. However, the identifications of the putative cCMP and the associated enzymes were questioned, as (a) several tissue-extracted compounds that could be separated chromatographically from authentic cCMP were found to be cCMP-immunoreactive,14–17 (b) at least one radiolabelled cytidylyl cyclase product could be separated from cCMP chromatographically,18,19 and (c) the cCMP phosphodiesterase activity was not specific but due to the action of a multifunctional enzyme.13 The debate was resolved by the unequivocal identification of cCMP in tissue extracts by mass spectrometry,20 and further by the mass spectrometric identification of cCMP together with four derivatives as products of the cytidylyl cyclase activity.21 These four derivatives, cytidine...
3',5'-cyclic monophosphate and cytidine-2'-O-glutamyl-3',5'-cyclic monophosphate, and cytidine-2'-O-aspartyl-3',5'-cyclic monophosphate, were found to cross react with anti-cCMP serum, explaining the earlier situation over a plurality of immunoreactive species: their elucidation allowed the development of specific assays for cCMP and cytidylyl cyclase. It was further established that in addition to the multifunctional phosphodiesterase, a second phosphodiesterase specific for cCMP hydrolysis occurs together with a cCMP-responsive protein kinase, the latter being initially demonstrated by quantitative mass spectrometry. Although evidence for roles in the control of cellular differentiation and proliferation has been produced (see review), the specific functions of cCMP have yet to be elucidated. The recent advances in proteomics, particularly the use of chromatography coupled to mass spectrometry, which enables selective monitoring of the response of the phosphoproteome to a specific challenge, has provided an impetus to renew the quest to elucidate the function(s) of cCMP. Here the identification of a protein, Rab23, of which the phosphorylated form is elevated in concentration in mouse brain in response to cCMP, is described. A series of incubations of mouse brain tissue with ATP as phosphate donor were carried out in the presence of cAMP, cGMP, cCMP or a non-cyclic nucleotide control. The use of immobilized metal affinity chromatography (IMAC) with immobilized Fe(III) to chelate phosphate groups linked to threonine, tyrosine or serine in proteins, followed by matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-ToFMS) and liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS), allowed the identification of a protein phosphorylated in response to cCMP but not to cAMP and cGMP. After testing a number of different protocols in trial experiments the strategy depicted in Fig. 1 was adopted. This constitutes the first report of a protein as a putative target in a chain of response to cCMP.

**EXPERIMENTAL**

**Chemicals**

All chemicals were Analar grade. Tris base was purchased from Melford Laboratories Ltd. (Ipswich, UK). Formic acid, dithiothreitol (DTT), cyclic nucleotides, ATP disodium salt, ferric chloride, ammonium hydroxide, MES (2-[N-morpholinol]ethanesulfonic acid), trifluoroacetic acid (TFA), leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF) and trypsin (E.C. 4.4.21.4, TPCK-free) from bovine pancreas were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK) and the matrices for MALDI analysis, sinapinic acid and α-cyano-4-hydroxycinnamic acid, from Fluka (Sigma). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (Loughborough, Leicester, UK). Water was prepared in-house using a Milli-Q and Elix water purification system (Millipore, UK).

**Animals**

Sixteen 8-week-old female mice were obtained from Harlan UK Ltd., Shaw’s Farm (Blackthorn Bicester, Oxon, UK) and maintained in plastic cages of dimensions 61 × 41 × 47 cm, each containing four mice, kept at 24–26°C with a 12 h light/dark cycle, in a designated holding room as per Home Office regulations. Food and water were provided to the animals ad libitum.

Sacrifice of all mice was carried out on the same day to prevent modifications in tissue content and was performed as rapidly as possible by a blow at the base of the head pursued by decapitation. The murine brains were then quickly removed and placed in ice-cold extraction buffer so as to avoid tissue decomposition due to post mortem changes.

**Sample preparation of extraction of protein from brain tissue samples**

The extraction buffer was 78 mM DTT, 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 20 μM leupeptin, 1 mM PMSF and 1 μM solution of pepstatin A, in 50 mM HEPES-EXPERIMENTAL

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**Figure 1.** Overview of the strategy used for the analysis of the effect of cCMP on the mouse brain proteome.
Tris-HCl, pH 7.4, pH adjusted with 6 M HCl. The buffer was freshly made immediately before use and maintained at ice-cold temperatures during the extraction of the mouse brain proteins.

Murine brains were placed in 1:9 (w/v) of the extraction buffer immediately after removal and then the brain tissue was cut into smaller pieces and completely disrupted by mechanical homogenization at 900 rpm in a Potter S Elvehjem homogenizer (Braun Melsunger, Germany) for four 10 s bursts in an ice bath. The resulting crude tissue homogenate was then utilized for the incubations described below.

**Incubations**

A standard solution of 2 mM of cyclic nucleotides (cAMP, cGMP or cCMP) and 5 mM ATP disodium salt was prepared in ice-cold 50 mM Tris-HCl buffer, pH 7.4. The cyclic nucleotides and the ATP were in excess so that the availability of either the source for phosphate groups and/or cyclic nucleotide agonist would not be the limiting factor in the incorporation of phosphate to proteins. Volumes of 1 mL of each of these solutions and 1 mL of the homogenate solution were pipetted into 5 mL vials in duplicate. The newly produced solutions were vortexed and incubated at 37°C for 5 min in a water bath. The incubations were transferred to an ice bath and kept for 5 min to stop any further reaction. The samples were then stored at −20°C. Next 750 μL of mouse brain homogenate treated with ATP and incubated with the cyclic nucleotide solution were centrifuged in a PK121R centrifuge (ALC International, Italy) for 10 min at 13 000 rpm (16 000 g). The supernatant was removed and dialyzed overnight against 100 mL of 50 mM Tris-HCl buffer (pH 7.4) using 12–14 kDa cut-off dialysis tubing (Medicell International Ltd., London, UK). The solution remaining in the tubing was concentrated to dryness prior to further analysis.

**Immobilized metal ion affinity chromatography**

For the concentration and isolation of the phosphoproteins of the mouse brain homogenate, IMAC ZipTips® MC (Millipore, UK) were utilized. The procedure followed was a slightly altered version of the method suggested in the Millipore protocols for IMAC.22

**Charging and equilibration of the ZipTip® MC**

The Zip Tip® MC was washed three times with 10 μL of 50% aqueous acetonitrile containing 0.1% acetic acid (wash solution I) with the wash solution dispensed to waste. The charging of the tip involved 10× aspirate and dispense cycles with 10 μL of metal solution (300 mM of ferric chloride in 10 mM HCl). The Zip Tip® MC was then washed three times with 10 μL of Milli-Q grade water (wash solution II) and three times with 10 μL of 1% acetic acid/10% acetonitrile (wash solution III), followed by the discharge of the eluent. The equilibration of the Zip Tip® MC was completed after washing the tip with 10 μL of MES buffer (binding solution) and dispensing it to waste five times.

**Binding and washing**

A volume of 10 μL of sample in MES buffer was bound to the Zip Tip® MC by full depression of the pipettor to a dead stop. A cycle of aspiration and dispensing to waste was repeated 20 times to ensure maximum binding. The Zip Tip® MC was then washed three times with binding solution (50 mM MES buffer containing 10% acetonitrile and adjusted to pH 5.5 with ammonium hydroxide), three times with wash solution I and three times with wash solution II; each time the washed eluent was placed in a separate vial to avoid contamination.

**Elution of phosphoproteins and further analysis**

A volume of 10 μL of 0.3 M ammonium hydroxide solution (elution solution) was pipetted into a 500 μL Eppendorf tube, then aspirated and dispensed through the Zip Tip® MC ten times. The eluted phosphoprotein solution was concentrated to dryness prior to further analysis.

**MALDI ToFMS**

MALDI-ToFMS analysis was performed on a Voyager-DE STR mass spectrometer (Applied Biosystems, UK) equipped with a 1-m linear ToF tube and a pulsed nitrogen laser operating at 337 nm. The instrument was operated in the positive ion detection mode and in the linear mode for protein analysis. A volume of 1 μL of sample in 70:30 0.3% TFA/acetonitrile was spotted on the MALDI plate (target) and ‘sandwiched’ between two layers of 1 μL of matrix. The matrix used for proteins was a solution of 10 mg/mL sinapinic acid in 70:30 0.3% TFA/acetonitrile.

Once the spots had dried on the target, the plate was introduced into the MALDI-ToF mass spectrometer. The ions produced were extracted with a 400 ns delay and were accelerated to 25 kV. The laser power was modified between 2600–2900 and the laser beam was fired at various areas of the sample by moving the control joystick. Signals from 100 shots were accumulated and averaged to generate each mass spectrum. The spectra were externally calibrated with calibration mixture 3 (Perseptive Biosystems USA) consisting of insulin (bovine), thioredoxin (E. coli) and apomyoglobin (equine), at the recommended manufacturer’s concentrations.

**Identification of phosphopeptides**

**Trypsin digestion**

(a) The phosphoproteins from mouse brain homogenates incubated with ATP and cyclic nucleotides, and separated by IMAC as described above, were concentrated to dryness, then reconstituted in 0.5 mL 100 mM ammonium bicarbonate, pH 8, and 20 μL of 1 mg/mL of trypsin in 100 mM ammonium bicarbonate buffer (pH 8) added. The reaction was allowed to proceed overnight at 22°C and stopped by the inactivation of the enzyme at −20°C for 30 min. The solution was then subjected to mass spectrometric analysis for the identification of the phosphopeptides.
(b) As an alternative procedure, 750 μL of mouse brain homogenates incubated with ATP and cyclic nucleotides were first treated with trypsin (1 mL of 1 mg/mL in 100 mM ammonium bicarbonate buffer, pH 8) to generate a mixture of peptides. The phosphorylated peptides were then selected by IMAC (ZipTip® C₁₈) as described above, concentrated, and subjected to mass spectrometric analysis.

Chromatography and electrospray mass spectrometry

The HPLC experiments were performed using an LC Packings Ultimate capillary LC system mounted with a UV nano flow-cell (Dionex, Amsterdam, The Netherlands). UV detection was carried out at 214 and 254 nm.

A FAMOS microautosampler (Dionex) equipped with a 20 μL loop and μL pickup settings was used for injections of 5 μL from 10 μL of sample due to the increased efficiency with this procedure. From the FAMOS loop, the sample was loaded onto a precolumn of 300 μm i.d. × 5 mm packed with PepMap C₁₈ (Dionex) stationary phase (3 μm, 100 Å) for desalting and preconcentration, via a SWITCOS pump (Dionex).

A column-switching setup was used, where the flow over the precolumn was switched between a loading pump and a gradient pump via a six-port valve after 3 min to allow sufficient desalting and the sample was back flushed onto the analytical column and separated. The Chromeleon software suite (Dionex) was used for instrument control, and also for the triggering of MS data acquisition.

The trypsin-digested samples were resolved on an analytical fused-silica nanocolumn of 15 cm × 75 μm i.d. packed with 3 μm particles, 100 Å pore C₁₈ PepMap stationary phase (Dionex). Mobile phase was delivered at 400 nL/min throughout the entire run. Mobile phase A was 2% acetonitrile + 0.06% formic acid and mobile phase B was 90% acetonitrile + 0.05% formic acid. Peptides were eluted from the column with a linear gradient of 0–60% B over 40 min. Finally, the column was re-equilibrated with the initial mobile phase A between runs.

The eluted peptides were analyzed either by an LCQ Deca ion trap mass spectrometer equipped with a nano-ESI source (Thermo Finnigan, Hemel Hempstead, UK), or by a Micromass Q-ToF2 Ultima mass spectrometer (Micromass, Waters, Wythenshaw, Manchester, UK). The LCQ Deca ion trap with operated with a spray voltage of 3 kV, a capillary temperature of 160°C, a capillary voltage of 15 V and an electron multiplier voltage of -890 V. The mass spectrometer operated in a data-dependent ‘Triple Play’ MS/MS mode, where the precursor ion was selected for a full-scan mass spectrum between 400 and 2000 Da and recorded using a maximum injection time of 200 ms and three microscans. Next a higher resolution ‘Zoom’ scan was performed in order to ascertain the charge state of the peptide from the mass difference between the protonated molecule and its C₁³⁻ isotope peak. Finally collision-induced dissociation (CID) was performed on the selected ion only once with a mass range determined from the zoom scan in order to account for possible fragments of higher mass than the precursor ion when fragmenting multiply charged peptides. An isolation width of 1.5 Da and collision energy of 35% (relative normalized collision energy) were used in the MS/MS analysis. After the MS/MS spectra had been obtained for a given peak the m/z value of the ion was added to a ±2.5 m/z unit wide dynamic exclusion list and was thereby deselected from MS/MS analysis for a period of 3 min. The QToF2 was operated in ‘V’ mode with 1.2 kV applied along the probe, a cone voltage of 30 V, with nitrogen as drying and nebulizing gas and argon as collision gas. Collision energy was varied between 20–40 V dependent upon mass and charge state of the peptide. MS was scanned between 300–1850 over 1 s, MS/MS between 50–1850 with an interscan delay of 100 ms. Data-dependent analyses were acquired for the three most abundant ions in each cycle.

To evaluate the quality of data acquired, the MS/MS spectra were correlated using the SEQUEST database, containing sequences of mouse proteins downloaded from the Havard microchemistry website. Output files from the correlation analysis were further studied using the XCorr and dCN scores, to produce a list of identified peptides and corresponding proteins.

The peptide identification and assignment of post-translational modifications/phosphorylation were performed and possible phosphorylations at serine, tyrosine and threonine residues were specified as variable modifications. All data sets were searched using the following constraints: only tryptic peptides with up to two missed cleavage sites were allowed; ±2.5 Da mass tolerance for peptide precursor mass searching; ±0.5 Da mass tolerance for the fragment ions; and the results were filtered for non-mouse peptide assignments.

RESULTS AND DISCUSSION

Fe(III) IMAC concentration of proteins from the mouse brain homogenate, followed by MALDI-ToFMS analysis, indicated phosphorylated proteins present in all the cyclic nucleotide incubations and in the non-cyclic nucleotide control. Interest was focused on any phosphoproteins which appeared in the incubates that had contained cCMP but were absent from the other incubations; such peaks were evident throughout the time course in the initial experimentation but were most prominent in the 5 min incubation, thus this time length was selected for further investigation. A tryptic phosphopeptide unique to the cCMP 5 min incubates was observed in both the IMAC-concentrated intact protein experiments and IMAC-concentrated tryptic peptide experiments in the LC/MS runs at 24 min, represented by ions at m/z of 1676.5 and 839.1 (Figs. 2(a) and 2(b)). The ion at m/z 1676.5 was shown by the study of the natural isotope pattern to be singly charged (see Fig. 2(c)) with the m/z 839.1 ion representing the doubly charged peptide. The lower resolution of the mass spectrometer in full-scan mode meant that the m/z 839.1 ion was slightly in disagreement with the fact that it arose from the m/z 1676.5 singly charged ion. However, a higher resolution zoom scan detailed the ion as being m/z 838.75 (0.01 Da difference between experimental and theoretical m/z – data not shown).
The SEQUEST database was utilized to identify the peptide, matching the MS/MS spectra produced to database sequences. Fragmentation of the m/z 1676.5 ion yielded a spectrum comparable to the theoretical b- and y-ion spectrum generated by SEQUEST (Fig. 2(d)). The SEQUEST scores from the incubates fall within the criteria necessary to identify the peptide TSVKEDLNVSEVFK (with phosphorylation most likely of the threonine residue) and to identify the parent protein as Rab23. The XCorr value obtained for the singly charged peptide was 1.91, with a dCN value of 0.19, and the ion coverage for the peptide was 26 ions detected out of 28 expected. The phosphate group is suggested to be present on the first threonine residue as this is lost to generate the y-13 ion; an unphosphorylated threonine would generate such an ion at m/z 1574, which is not indicated in the MS/MS spectrum (Fig. 2(d)). The same ion generated from the first threonine being phosphorylated would be expected to occur at m/z 1494 and an ion of reasonable relative abundance.

**Figure 2.** Identification of the peptide TSVKEDLNVSEVFK as a tryptic fragment of a phosphoprotein produced in murine brain in response to challenge by cyclic CMP. (a) Reconstructed ion chromatogram of HPLC/ESI mass spectra indicating the occurrence of the peptide with a protonated molecule at m/z 1676 in the incubations containing cyclic CMP, cyclic AMP, cyclic GMP, and the non-cyclic nucleotide control. (b) The full-scan mass spectrum derived from the analysis at 24.3 min indicating prominent ions at m/z 1676.5 and 839.1. (c) The increased resolution ‘zoom scan’ indicating a single mass difference between carbon isotope ions, thereby defining the m/z 1676.5 peak as a singly charged ion. (d) The identification by the SEQUEST algorithm of the b- and y-ions of the identified phosphopeptide from Rab23 in the acquired MS/MS spectrum. The XCorr value obtained for the singly charged peptide was 1.91, with a dCN value of 0.19.
(approx. 45%) is indicated in the MS/MS spectrum. Other Rab23-derived peptides unique to the cCMP incubation identified by the same method are SSMIQRYCK (m/z 1115.5), SSMIQR (m/z 721.5), KTGVIQFLELR (m/z 1177.8) and TKRTR (m/z 661.8); data summarized in Tables 1, 2, 3, and 4, respectively. These peptides were not phosphorylated and originated from the IMAC isolation of the phosphoprotein (see Fig. 1, right-hand side methodology pathway) followed by digestion releasing both phosphorylated and non-phosphorylated peptides. Collectively, these peptides cover 38 out of 237 amino acids, 17.36% by mass of the amino acid sequence of Rab23 (Fig. 3). A reverse database search34 in order to check for false positive hits of proteins recognized by more than one peptide (using the same probability thresholds as for the normal search) was performed and reported no protein hits.

Previous data from cCMP studies in our and other laboratories are consistent with a function in cell proliferation and growth, with the cyclic nucleotide itself found in elevated concentrations in rapidly growing cells, e.g. in leukaemic, foetal or regenerating tissue, the cytidylyl cyclase activity also being elevated in such tissues while the cCMP phosphodiesterase activity is diminished (see review27).

Table 1. Theoretical fragment b- and y-ions of SSMIQRYCK (m/z 1115.5) observed in MS/MS spectrum

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<th>Amino acid sequence</th>
<th>Number of b-ions</th>
<th>m/z of b-fragments</th>
<th>m/z of y-fragments</th>
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Charge = +1: XCorr = 1.87: dCN = 0.163: Ions covered = 88%.
Table 2. Theoretical fragment b- and y-ions of SSMIQKR (m/z 721.5) observed in MS/MS spectrum

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Charge = +1; XCorr = 1.81; dCN = 0.153; Ions covered = 83%.

Table 3. Theoretical fragment b- and y-ions of KTIGVDFLER (m/z 1177.8) observed in MS/MS spectrum

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<th>Amino Acid sequence</th>
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<tr>
<td>F</td>
<td>7</td>
<td>761.4</td>
<td>564.3</td>
<td>4</td>
</tr>
<tr>
<td>L</td>
<td>8</td>
<td>874.5</td>
<td>417.2</td>
<td>3</td>
</tr>
<tr>
<td>E</td>
<td>9</td>
<td>1003.5</td>
<td>304.1</td>
<td>2</td>
</tr>
<tr>
<td>R</td>
<td>10</td>
<td></td>
<td>175.1</td>
<td>1</td>
</tr>
</tbody>
</table>

Charge = +1; XCorr = 1.87; dCN = 0.19; Ions covered = 85%.

Table 4. Theoretical fragment b- and y-ions of TKRTR (m/z 661.8) observed in MS/MS spectrum

<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th>Number of b-ions</th>
<th>m/z of b-fragments</th>
<th>m/z of y-fragments</th>
<th>Number of y-fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>1</td>
<td>102.0</td>
<td>661.8</td>
<td>5</td>
</tr>
<tr>
<td>K</td>
<td>2</td>
<td>230.1</td>
<td>560.8</td>
<td>4</td>
</tr>
<tr>
<td>R</td>
<td>3</td>
<td>386.2</td>
<td>432.7</td>
<td>3</td>
</tr>
<tr>
<td>T</td>
<td>4</td>
<td>487.5</td>
<td>276.6</td>
<td>2</td>
</tr>
<tr>
<td>R</td>
<td>5</td>
<td>643.5</td>
<td>175.1</td>
<td>1</td>
</tr>
</tbody>
</table>

Charge = +1; XCorr = 2.04; dCN = 0.12; Ions covered = 100%.

**MLEEDMEVAIKMVVMVANGAVGKSSMIQRYCHGTITKDYKKT**

**IGYDFLERQIQYNDEYRLMLWDTAGQEEFDAITKAYRGA**

**QACVLVFSTTDRESFEAISWWREKVVAEVDIFTALVQNKIDL**

**LDDSCIKNEEAEGALKRLRFYRTSVKEDLNVESEVFYLAEL**

**KHLQKLLQQITEDPEQTHSNSNKIGVFNASVGSHLGQNSSSLN**

**GGDVINLRPNKQRTKTRTPNFSSCSVP**

**Mass (mono): 2661.6  Identifier: gi|6679589**

**Database: D:/Xcalibur/database/mouse.fasta**

**Protein coverage:**

38/237 = 16.03% by amino acid count, 4631.1/2661.6 = 17.36% by mass

**Figure 3.** The sequence of Rab23 with the peptides identified by mass spectrometry highlighted and the sequence coverage calculated.
Rab23 is a member of the Ras GTPase superfamily. Many of these GTPases have been isolated in different parts of the cell; however, the expression of Rab23 has been primarily located within the brain and is responsible for the development of the dorsal and ventral cells of the central nervous system with one of its functions the negative regulation of the sonic hedgehog signalling pathway (Shh signalling pathway). The Shh pathway is extensively activated during the formation of tumours, in the absence of down-regulation of the pathway, continual activation of the pathway causes tumour formation through the uncontrolled transcription of the target genes. In this study, Rab23 was detected only in the phosphoproteome of the mouse brain homogenate treated with elevated levels of cCMP. This phosphorylation of Rab23 will alter its activity. With Rab23 as a negative regulator of the Shh pathway, one plausible theory is that phosphorylation of Rab23 will inactivate its inhibitory effect on Shh, thereby allowing the uncontrollable activation of the Shh pathway leading to tumour formation; alternatively, the increase in cCMP concentration could be a defensive response against hyperproliferation, with the subsequent phosphorylation of Rab23 being an activation which down-regulates the Shh pathway. In both instances the action is consistent with the previously observed elevation in cCMP in hyperproliferating cells.

This identification of Rab23 as a phosphorylation response target of cCMP constitutes a vital breakthrough in confirming a function for cCMP in signal transduction in the brain, and in supporting parallels previously drawn between it and the two established cyclic nucleotide second messengers, cAMP and cGMP. The strategy we employed here has thus enabled the identification of one protein as a potential member of a cCMP-response chain; more extensive application of this phosphoproteome selection and MS analysis will enable identification of further such proteins and facilitate the elucidation of cCMP function and mechanism of action, thence hopefully allowing the development of pharmaceutical manipulation as per the two other cyclic nucleotides.

Acknowledgements

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1. Rail TW, Sutherland EW, Berthet J J. Biol. Chem. 1957; 224: 463.