Phosphorylation of a cAMP-specific phosphodiesterase (HSPDE4B2B) by mitogen-activated protein kinase

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A cAMP-specific phosphodiesterase, HSPDE4B2B, was found to be phosphorylated when expressed in Sf9 cells or yeast. Deletion of amino acids 81–151 and 529–564 had no effect on the phosphorylation of HSPDE4B2B. Mass spectrometric analysis of purified HSPDE4B2B(1–564), HSPDE4B2B(81–564) and HSPDE4B2B(152–528) showed that phosphorylation occurred predominantly on Ser⁴⁸⁷ and Ser⁴⁸⁹. The stoichiometry of phosphorylation was 1.2:1 (Ser⁴⁸⁷:Ser^{487, 489}). There was no evidence by MS for a non-phosphorylated form of HSPDE4B2B(81–564) or HSPDE4B2B(152–528) when expressed in Sf9 cells. There was no detectable phosphorylation of purified HSPDE4B2B(152–528) expressed in *Escherichia coli*. Radiolabelling experiments with ³²P revealed that phosphorylation of HSPDE4B2B(152–528) expressed in Sf9 cells was abolished when Ser⁴⁸⁷ and Ser⁴⁸⁹ were mutated to alanines.

Analysis of the amino acid sequence revealed that Ser⁴⁸⁷ and Ser⁴⁸⁹ of HSPDE4B2B conform to the consensus motifs for phosphorylation by mitogen-activated protein kinase (MAP kinase) and casein kinase II respectively. Kinetic experiments *in vitro* showed that MAP kinase-phosphorylated *E. coli* expressed and purified HSPDE4B2B(151–528) with a K_m of 63 μ M and a V_{max} of 3.0 μ mol/min per mg. In comparison, MAP kinase phosphorylated myelin basic protein with a K_m of 26.0 μ M and a V_{max} of 5.5 μ mol/min per mg under the same conditions. Using MS and mutational analysis we found that MAP kinase-phosphorylated *E. coli* expressed HSPDE4B2B(152–528) exclusively at Ser⁴⁸⁷. These results suggest that phosphodiesterases could contribute to the cross-talk between the cAMP and MAP kinase signalling pathways.

INTRODUCTION

Many hormones that alter cellular growth do so by binding to receptors that activate adenylate cyclase (reviewed in [1]). Adenylate cyclase synthesizes the second messenger cAMP, which in turn activates cAMP-dependent protein kinase (PKA). The subsequent phosphorylation of a variety of proteins by PKA leads to changes in signal transduction pathways. For example, PKA activity suppresses the mitogen-activated protein kinase (MAP kinase) signalling cascade [2,3], indicating a role for PKA in linking these two signal pathways (reviewed in [4]). In contrast with adenylate cyclase, phosphodiesterases (PDEs) degrade cAMP and inhibit the PKA pathway. Thus one role of PDEs is to control metabolic pathways by behaving as molecular switches that turn PKA off.

The PDEs are a multigene family defined by their regulatory properties and their substrate and inhibitor specificities (reviewed in [5]). The cAMP-specific PDEs (PDE4s) belong to a group of enzymes that are characterized as having a low K_m for cAMP and are selectively inhibited by rolipram. This gene family codes for at least four mammalian loci that have extensive homology with the memory and learning gene (*dnc*) of *Drosophila* [6,7]. Furthermore the expression of PDE4s in lymphocytes [7] and brain [8,9] underlies the ability of rolipram to act as an anti-inflammatory [10] or anti-depressant drug [11]. This suggests an important function for PDE4s in controlling neurological and immunological responses.

Whereas a number of PDEs have been purified and cloned, little is known about the detailed post-translational modifications for many of these enzymes. It has been shown in adipocytes and

hepatocytes that the cGMP-inhibited cAMP phosphodiesterase (type 3 PDE) and the high-affinity cAMP phosphodiesterase (type 4 PDE) are phosphorylated and activated in response to insulin respectively [12,13]. Recent evidence suggests that HPDE4A is phosphorylated in the thyroid by PKA [14]. PKA has also been shown to phosphorylate the rat PDE3.3 variant of ratPDE3/4D in a cell-free system [15]. In contrast, there is no direct evidence for phosphorylation of HSPDE4B.

Recently we expressed, purified and characterized the type-4 PDE, HSPDE4B2B(81–564) (W. J. Rocque, W. D. Holmes, D. B. Kassel, I. Patel, L. Overton, C. Hoffman, G. B. Wisely, D. Willard, R. Dougherty, O. Ittoop and M. Luther, unpublished work). Analysis by MS revealed the existence of two species of HSPDE4B2B(81–564) of different molecular masses, suggesting that this protein might be modified. The differences in molecular mass indicate that the modifications could be due to phosphorylation; we therefore investigated the phosphorylation state of HSPDE4B2B(81–564). In this paper we show that Ser⁴⁸⁷ of HSPDE4B2B is phosphorylated *in vivo* by Sf9 cells and *in vitro* by MAP kinase (p44^{mapk}). These observations suggest that HSPDE4B2B might link the cAMP and MAP kinase signal transduction pathways.

MATERIALS AND METHODS

Assay of cAMP phosphodiesterase

cAMP phosphodiesterase activity was measured as previously described [16]. As reported, these conditions produced a linear time course and used less than 10 % of the substrate.

Abbreviations used: HPDE4, human cAMP-specific PDE; LC, liquid chromatography; MAP kinase, mitogen-activated protein kinase; MS, mass spectrometry; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase.

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Synthesis and purification of oligonucleotides

The oligonucleotides used for sequencing and site-directed mutagenesis were synthesized on an Applied Biosystems automated DNA synthesizer Model 380, with phosphoramidite chemistry [17]. Individual oligonucleotides were purified by PAGE [12 % (v/v) gel] by standard procedures [18]. Site-directed mutagenesis was performed in accordance with the manufacturer's protocol (Amersham International Inc.). The other recombinant DNA techniques performed are in accordance with published protocols [19].

Cloning and expression of HSPDE4B2B(152–528) in *E. coli*

DNA sequences encoding amino acids 152–528 of HSPDE4B2B were made by inserting a *XbaI/BamHI* fragment from the HSPDE4B2B(81–564) clone (W. J. Rocque, W. D. Holmes, D. B. Kassel, I. Patel, L. Overton, C. Hoffman, G. B. Wisely, D. Willard, R. Dougherty, O. Ittoop and M. Luther, unpublished work) into a M13mp18 vector (New England Biolabs, Beverly, MA, U.S.A.). An *NdeI* restriction endonuclease site and a translation initiation codon were introduced by oligonucleotide-directed mutagenesis techniques *in vitro* (Amersham, Arlington Heights, IL, U.S.A.) with the oligonucleotide 5'-GCGTGAGAT-GCTCATATGTGTATTGTTAG. Similarly, a translation stop codon and a *BamHI* site were introduced by using the oligonucleotide 5'-TGTGCTGCTGAAAGGATCCTTAGCTGTGTCCTC. Finally, an *NdeI/BamHI* fragment from the mutant replicative form was subcloned into a pET11b vector (Invitrogen Inc.) and expressed in *E. coli* with the T7 system (Novagen Inc., Madison, WI, U.S.A.).

Mutagenesis of HSPDE4B2B in Sf9 cells

An *EcoRI/BamHI* fragment encoding residues 152–528 of HSPDE4B2B was inserted into M13mp18 (New England Biolabs) and used as a template for oligonucleotide-directed mutagenesis *in vitro* (Amersham). The serines at positions 482, 487 and 489 were changed to alanines by using the oligonucleotide 5'-AGGAAGTGGTATCAGGCCATGATACCTCAA GCTCC CGCACCACCACTGGAC. From this construct the alanine at position 487 was converted back to serine by using the oligonucleotide 5'-GCCATGATACCTCAAAGTCCCGCACCACCACTG. *EcoRI/BamHI* fragments were isolated from each of these constructs and subcloned into pVL1392 (Pharminogen Inc., San Diego, CA, U.S.A.). Recombinant clones were identified with plaque assay techniques [20]. To confirm the presence of the desired mutations, the DNA sequences were determined by using the dideoxy chain termination technique [21].

Expression and purification of HSPDE4B2B(81–564) and HSPDE4B2B(152–528)

Expression and purification of HSPDE4B2B(81–564) from Sf9 cells was performed as described elsewhere (W. J. Rocque, W. D. Holmes, D. B. Kassel, I. Patel, L. Overton, C. Hoffman, G. B. Wisely, D. Willard, R. Dougherty, O. Ittoop and M. Luther, unpublished work). A similar purification procedure was used for HSPDE4B2B(152–528) expressed in *E. coli* or Sf9 with the following modifications: 50 mM Tris/HCl, pH 7.5, was replaced with 50 mM Hepes buffer, pH 7.5. The ammonium sulphate, hydroxyapatite and gel filtration steps were eliminated. The supernatant from the low-speed centrifugation was batch-bound to fast-flow Q-Sepharose (15 mg of protein per ml of resin) for 1–3 h with gentle shaking. The resin was washed in a coarse funnel with lysis buffer and eluted with lysis buffer containing

0.5 M NaCl. The eluate was batch-bound to Cibacron Blue 3GA Type-300 resin (25 mg of protein per ml of resin) overnight at 4 °C with gentle stirring. The resin was washed and eluted with the same buffers as previously described. The eluate from the Cibacron Blue resin was loaded on to a Poros Q (15 ml) column equilibrated with 50 mM Hepes buffer, pH 7.5, containing 5 mM dithiothreitol. The column was washed with the same buffer and step-eluted with buffer containing 0.5 M NaCl. The peak was collected and stored at –80 °C for future use. MS analysis, SDS/PAGE and Edman sequencing all confirmed that the protein was more than 95 % pure.

Preparation of ³²P-labelled Sf9 cytosol

Sf9 cells (American Type Culture Collection) were grown at 28 °C in Sf900II medium (Gibco) plus 50 µg/ml gentamicin. When the cell density reached 10⁶ cells/ml the cultures were infected at a multiplicity of infection of 0.1 plaque-forming units per ml with the appropriate baculovirus constructs. At 48 h after infection the cells were centrifuged at 800 *g* for 10 min, at 4 °C. The cell pellet was suspended in 50 ml (2 × 10⁶ cells/ml) of phosphate-free growth medium (Excel 401, JRH Biosciences) containing 1.5 mCi of [³²P]P_i (Amersham) and incubated for 16 h at 27 °C. After centrifugation at 1000 *g* for 5 min, at 4 °C, the cells were suspended in PBS and recentrifuged at 1000 *g* for 5 min, at 4 °C. The pellet was suspended to 10⁸ cells/ml in PBS, pH 7.2, containing 5 mM NaF, 10 µg/ml soybean trypsin inhibitor, 0.2 mM PMSF, 0.5 µg/ml leupeptin and 0.5 µg/ml pepstatin. Digitonin, a compound that selectively disrupts the plasma membrane [16,22], was added to a final concentration of 0.02 % and the cells were incubated for 30 min at 4 °C. Non-permeabilized and semi-intact cells were removed by centrifugation at 1000 *g* for 5 min, at 4 °C. The supernatant contained more than 60 % of the original cell-associated HSPDE4B2B and lactate dehydrogenase (a cytosolic marker) activity, but less than 5 % of the original 5'-nucleotidase (a plasma membrane marker) and β-glucuronidase (a lysosomal marker) activities [16].

Phosphorylation assays

Unless stated otherwise, the phosphorylation assays contained the following in a final volume of 10 µl: 100 µM [³²P]ATP (730 c.p.m./pmol), 1.0 µM HSPDE4B2B, 50 nM MAP kinase (p44^{mapk}, Upstate Biotechnology Incorporated, Lake Placid, NY, U.S.A.), 5 mM MgCl₂, 5 mM β-glycerol phosphate, 20 mM Hepes, pH 7.3, and 1 mM dithiothreitol. For some experiments, pure HSPDE4B2B was replaced with 10 µl of Sf9 cytosol containing recombinant HSPDE4B2B. In other experiments, MAP kinase was replaced by casein kinase I or cdc2 kinase. The reactions were incubated at 30 °C for 5–60 min and stopped by the addition of 1 mg/ml BSA and 10 % (w/v) trichloroacetic acid. Trichloroacetic acid-insoluble material was collected by centrifugation at 12000 *g* for 5 min, at 4 °C. The trichloroacetic acid-precipitable radioactivity was detected in a bench-top radioisotope counter (Bioscan Inc., Washington, DC, U.S.A.). Analysis of the *K_m* and *V_{max}* values of MAP kinase for HSPDE4B2B and myelin basic protein were done under conditions that we found to produce a linear time course and to use less than 10 % of the substrate (for example 5–15 min at 30 °C, 2.5–20 µM substrate and 10–20 nM kinase).

Electrophoresis, autoradiography and PhosphorImager analysis

Electrophoresis [23] was performed with 4–20 % Tris/glycine SDS/polyacrylamide gels (Novex, San Diego, CA, U.S.A.). The proteins were immobilized on nitrocellulose [24] and detected by

exposure to X-ray film at -80°C . Quantification of the radioactive bands was performed with PhosphorImager analysis and ImageQuant in accordance with the manufacturer's specifications (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Proteolysis and purification of peptide fragments from HSPDE4B2B(81–564) and HSPDE4B2B(152–528)

Approx. 400 pmol. of HSPDE4B2B(81–564) and HSPDE4B2B(152–528) was isolated after HPLC separation on an $800\ \mu\text{m} \times 15\ \text{cm}$ Poros® R1/H capillary perfusion column (LC Packings, Inc., San Francisco, CA, U.S.A.) with a gradient of 15–65% (v/v) acetonitrile containing 0.1% trifluoroacetic acid in 5 min at a flow rate of $100\ \mu\text{l}/\text{min}$. Purified HSPDE4B2B(81–564) was subjected to Lys-C digestion *in situ* by the procedures described by Burkhart [25]. HPLC-purified HSPDE4B2B(152–528) was subjected to digestion with trypsin in solution for 12 h at 37°C with 50 mM NH_4HCO_3 , pH 8.5, and an enzyme-to-substrate ratio of 1:25. Digestion products were separated on a $320\ \mu\text{m} \times 100\ \text{cm}$ Poros® R2/H capillary column with a gradient of 1–41% (v/v) acetonitrile containing 0.1% trifluoroacetic acid in 20 min at a flow rate of $35\ \mu\text{l}/\text{min}$. The outlet of the capillary column was connected to a $75\ \text{mm} \times 30\ \text{cm}$ piece of fused silica transfer line (Polymicro Technologies, Phoenix, AZ, U.S.A.) and terminated in the electrospray ion source chamber of the mass spectrometer, as described previously [26].

MS

A triple-quadrupole mass spectrometer (PE-Sciex, Toronto, Ontario, Canada) was operated in the liquid chromatography/MS (LC/MS) mode and used to acquire all mass spectra. For LC/MS analyses of intact HPDEIVB2 and its various recombinant forms, the mass spectrometer was scanned over the mass range m/z 800–1000 in 3 s. Molecular masses were measured from the electrospray mass spectra, as described by Covey [27]. LC/MS analyses of enzyme digests were performed by scanning the mass spectrometer over the mass range m/z 400–2400 in 4 s. Sites of phosphorylation were identified by operating the mass spectrometer in the LC/MS/MS mode, as reviewed by Chait and Kent [28].

RESULTS

Phosphorylation of HSPDE4B2B in Sf9 cells

Electrospray mass spectrum analysis of baculovirus-expressed HSPDE4B2B(81–564) revealed that the protein exists as a mixture of two species, 123 and 203 Da higher in mass than predicted, with a ratio of approx. 1.2:1 (W. J. Rocque, W. D. Holmes, D. B. Kassel, I. Patel, L. Overton, C. Hoffman, G. B. Wisely, D. Willard, R. Dougherty, O. Ittoop and M. Luther, unpublished work). Digestion *in situ* with endoprotease Lys-C and subsequent analysis by LC/MS/MS identified the N-terminus as being N²-acetylated. Further, an endoprotease Lys-C fragment was observed consisting of two species whose molecular masses were 80 and 160 Da higher than that predicted for an unmodified Lys-C peptide spanning residues 442–505. The difference in mass of these two species from the predicted 442–505 endoprotease Lys-C peptide was consistent with the addition of one and two phosphate moieties respectively.

The size of this Lys-C peptide fragment precluded the identification of the sites of phosphorylation by either MS or gas-phase Edman sequencing. Consequently the Lys-C peptide was isolated by reversed-phase HPLC and subjected to digestion with trypsin in solution to produce a smaller fragment spanning the region(s)

Figure 1 Capillary perfusion column LC/MS/MS spectrum of HSPDE4B2B(81–564) phosphopeptide generated after digestion with endoprotease Lys-C and trypsin

The 7.6 kDa Lys-C fragment of HSPDE4B2B(81–564) containing the sites of phosphorylation from Sf9 cells was digested with trypsin. The tryptic digest was separated on a reversed-phase perfusion column (0.8 mm internal diam. $\times$ 150 mm) and analysed by LC/MS. Phosphotryptic peptides were identified on the basis of a mass difference of 80 Da between the predicted and observed molecular masses. A subsequent LC/MS/MS analysis was performed on the two identified phosphotryptic peptides. The spectra for the monophosphorylated and diphosphorylated tryptic peptides of HPDEIVB2 are shown in (A) and (B) respectively. For the monophosphorylated tryptic peptide (A), Ser⁴⁸⁷ (*) was identified as the site of phosphorylation, on the basis of the mass difference between the y_{10} and y_{11} ions. For the diphosphorylated tryptic peptides (B), Ser⁴⁸⁷ and Ser⁴⁸⁹ (*) were identified as the sites of phosphorylation, on the basis of the mass differences between the y_8 and y_9 ions and the y_{10} and y_{11} ions respectively. The asterisks on the graphs represent the loss of a phosphate ion from the y_{11} peak.

of phosphorylation. The resultant products of this digestion were analysed by capillary LC/MS. Phosphorylation of HPDEIVB2 was localized to two tryptic fragments whose molecular masses were determined by LC/MS as 2469 and 2549 Da respectively. LC/MS/MS analyses were performed on these two peptides to identify the sites of phosphorylation. The LC/MS/MS spectra for phosphotryptic peptide 1 (2469 Da) enabled the site of phosphorylation to be localized to Ser⁴⁸⁷ on the basis of the mass difference between the fragment ions, designated y_{10}/y_{11} in Figure 1(A). The LC/MS/MS spectra for phosphotryptic peptide 2 (2549 Da) enabled the sites of phosphorylation to be localized to Ser⁴⁸⁷ and Ser⁴⁸⁹ on the basis of the mass difference between the fragment ions, designated y_8/y_9 and y_{10}/y_{11} respectively in Figure 1(B). For a comprehensive review of peptide fragment ion nomenclature, refer to Biemann [29]. Similar results were obtained on analysis of HPDEIVB2(1–564) purified from a yeast expression system (results not shown).

To confirm that the post-translational modifications of HSPDE4B2B(81–564) were due to phosphorylation at Ser⁴⁸⁷ and Ser⁴⁸⁹, serines 482, 487 and 489 were mutated to alanines and the protein was expressed in baculovirus and purified as described in the Materials and methods section. Ser⁴⁸² was mutated to alanine because of its close proximity in linear sequence to Ser⁴⁸⁷ and Ser⁴⁸⁹ and the possibility that this site might be modified. LC/MS analysis of the purified protein showed that the molecular mass was the same as predicted for non-phosphorylated HSPDE4B2B(81–564) (taking into account that the N-terminus was acetylated and the serines were now alanines; see Table 1).

Table 1 List of Predicted molecular masses and molecular masses determined by LC/ESI*MS

Protein construct	Calculated molecular mass (Da)	Observed molecular mass (Da)	Difference in molecular mass (Da)	Modification
1–564, wild-type, yeast	64 352	64 434, 64 512	80, 160	Mono- and di-phosphorylated
81–564, wild-type, Sf9	55 453	55 575, 55 655	123, 203	Acetylated, mono- and di-phosphorylated
81–564, SΔ A triple mutant	55 405	55 447	42	Acetylated
152–528, wild-type, Sf9	43 332	43 454, 43 534	123, 203	Acetylated, mono- and di-phosphorylated
152–528, SΔ A triple mutant	43 284	43 326	42	Acetylated
152–528, wild-type, <i>E. coli</i>	43 332	43 330	–	–

* ESI, electrospray ionization

Figure 2 Site-directed mutagenesis shows that Sf9 cells phosphorylate HSPDE4B2B(152–528) at Ser⁴⁸⁷

Sf9 cells were transfected with baculovirus containing DNA that codes for amino acids 152–528 of HSPDE4B2B (**A**, lane 1; **B**, white bars), 152–528 of HSPDE4B2B with alanines at residues 482 and 489 (**A**, lane 2; **B**, grey bars) and 152–528 of HSPDE4B2B with alanines at residues 482, 487 and 489 (**A**, lane 3; **B**, black bars). The cells were labelled with [³²P]P_i for the last 16 h of a 48 h culture period at 27 °C. The reactions were stopped by adding 10% (w/v) trichloroacetic acid. Trichloroacetic acid-insoluble material was separated by SDS/PAGE, immobilized on nitrocellulose and the blot was exposed to X-ray film at –80 °C (**A**). PhosphorImager analysis (**B**) was used to quantify the amount of radioactivity in each lane. The amounts of radioactivity incorporated into the bands labelled PDE-IV and the two endogenous protein controls (labelled C1 and C2) are expressed as a percentage of the total radioactivity found within a lane.

To perform phosphorylation experiments *in vitro* on HSPDE4B2B(81–564), we expressed this protein in *E. coli* to generate a non-phosphorylated substrate. However, HSPDE4B2B(81–564) was found within inclusion bodies in *E. coli*, precluding a thorough characterization of the protein. Thus truncated forms corresponding to amino acids 152–528 of HSPDE4B2B were introduced into *E. coli* and Sf9 cells. These truncated forms were soluble, retained PDE activity, and retained sensitivity to rolipram (see below). Moreover MS analysis indicated that Sf9-expressed HSPDE4B2B(152–528) existed as a mixture of mono- and di-phosphorylated isoforms with a

stoichiometry of approx. 1.2:1 (results not shown). These observations indicated that amino acids 81–151 and 529–564 were not needed for catalytic activity or phosphorylation of HSPDE4B2B.

The identity of the site(s) phosphorylated in HSPDE4B2B(152–528) was determined by constructing various Ser to Ala point mutations and expressing the proteins in Sf9 cells. After the cells were radiolabelled with [³²P]P_i, equal amounts of PDE4 were separated by SDS/PAGE. Radiolabelled proteins were detected by autoradiography (Figure 2A) and PhosphorImager analysis (Figure 2B). Western blot analysis with a monoclonal antibody to HSPDE4B2B was used to confirm the identity of the bands labelled PDE4 in Figure 2 (results not shown). When serines 482 and 489 were mutated to alanines (lane 2), there was no significant decrease in phosphorylation compared with the construct lacking these point mutations (lane 1). However, phosphorylation of HSPDE4B2B(152–528) decreased markedly when serines 482, 487 and 489 were mutated to alanines (lane 3). These mutations did not affect phosphorylation of endogenous Sf9 proteins (labelled C1 and C2).

Although phosphorylation at Ser⁴⁸⁷ was readily detectable by PhosphorImager analysis, phosphorylation at Ser⁴⁸⁹ was not easily discernible with this method (Figure 2). LC/MS, an extremely accurate method for detecting small changes in post-translational modification, was used to determine that the ratio of monophosphorylated (Ser⁴⁸⁷) to diphosphorylated (Ser^{487,489}) HSPDE4B2B was 1.2:1. This translates to a ratio of 2.2:1 when comparing Ser⁴⁸⁷ with Ser⁴⁸⁹. In other words, 69% of the sites were phosphorylated at Ser⁴⁸⁷ and 31% of the sites were phosphorylated at Ser⁴⁸⁹. It is accepted that PhosphorImager analysis is not nearly as reliable or accurate as LC/MS at detecting these small changes (31% reduction) in phosphorylation. The LC/MS spectra of the Ser to Ala 482, 487 and 489 HSPDE4B2B(152–528) revealed the observed molecular mass was the same as predicted for non-phosphorylated HSPDE4B2B(152–528) (taking into account that the N-terminus is acetylated and serines 482, 487 and 489 were mutated to alanines; see Table 1). Taken together, these results confirmed that Ser⁴⁸⁷ and Ser⁴⁸⁹ of HSPDE4B2B(152–528) were phosphorylated by Sf9 cells and amino acids 81–151 and 529–564 were not needed for phosphorylation to occur.

Phosphorylation of HSPDE4B2B by MAP kinase

Several serine kinases (e.g. casein kinase I, cdc2 kinase and MAP kinase) have requirements for proline residues near the phosphorylation site [30]. HSPDE4B2B has a serine–proline rich sequence (SMIPQSPSP) between amino acids 482–491. This

Figure 3 UV chromatogram of HSPDE4B2B(152–528) tryptic digest

Purified HPDEIVB2(152–528) was digested with trypsin and analysed by LC/MS (A) before and (B) after incubation with MAP kinase. The numbers above the peaks represent the numbers of protons associated with each peak.

sequence contains the PXSP recognition motif for MAP kinase suggesting Ser⁴⁸⁷ may be a site for MAP kinase. Thus we explored the possibility that HSPDE4B2B(152–528) serves as a substrate for MAP kinase.

HSPDE4B2B(152–528) was purified from an *E. coli* expression system and phosphorylated *in vitro* with MAP kinase before analysis by electrospray MS. The molecular mass of the protein before incubation with MAP kinase was found to be 43330 ± 4.7 Da; within the experimental error for the calculated mass, the calculated molecular mass is 43332 Da. After incubation with MAP kinase, the mass of the protein shifted by approx. 80 Da and was found to be 43407 ± 5.5 Da, consistent with incorporation of one phosphate moiety. The region that contains this phosphorylation site was further defined by treating HSPDE4B2B(152–528) with MAP kinase (or not, as control) and digesting with trypsin. Tryptic peptides were subsequently analysed by LC/MS and LC/MS/MS. The UV chromatograms for the digests treated with MAP kinase (Figure 3B) revealed the appearance of a phosphopeptide that was lacking in non-MAP kinase-treated HSPDE4B2B(152–528) (Figure 3A). The phosphotryptic peptide was further characterized (Figure 4) by the technique of negative-ion orifice potential stepping [31,32]. Figure 4(A) shows the total ion current chromatogram, Figure 4(B) the selected mass chromatogram for the designate PO_3^- anion (m/z 79), and Figure 4(C) the negative-ion electrospray mass spectrum corresponding for the peptide eluting at 24.7 min. The mass of this peptide was calculated as 2368 Da, 80 Da higher than the non-phosphorylated tryptic peptide. Analysis of the trypsin and MAP kinase sites in the HSPDE4B2B(152–528) sequence that would generate a peptide of this size suggests that this peptide corresponds to amino acids 478–497.

The site of phosphorylation of MAP kinase-treated *E. coli* HSPDE4B2B(152–528) was assessed by both MS and Edman sequencing. The signal-to-noise ratio of the LC/MS/MS spectra and the Edman sequencing data for this tryptic peptide were relatively poor and made conclusive identification of the site of phosphorylation difficult. Consequently a second aliquot of MAP kinase-treated HSPDE4B2B(152–528) was digested with endoprotease Asp-N to produce a smaller peptide fragment that was more amenable to sequencing by LC/ESI/MS/MS (where

Figure 4 Identification of a tryptic peptide phosphorylated by MAP kinase

MAP kinase-treated HPDEIVB2 was digested with trypsin and analysed by LC/MS. By analysing the digest in the negative-ion mode, it was possible to monitor specifically for the phosphate anion and identify the phosphotryptic peptide. (A) Negative-ion LC/MS chromatogram of the tryptic digest of MAP kinase-treated HPDEIVB2 tryptic digest. (B) The tryptic peptide observed at 24.7 min is indeed phosphorylated, as evidenced by the increase in the ion current associated with the PO_3^- ion. (C) Negative-ion mass spectrum of the phosphotryptic peptide observed at 24.7 min. The phosphate anion is observed at m/z 79. TFA, trifluoroacetic acid.

Figure 5 Identification of Ser⁴⁸⁷ as a site for MAP kinase phosphorylation in HSPDE4B2B(152–528)

The Asp-N digested peptide, identified from negative-ion LC/MS analysis, was subjected to a positive-ion LC/MS/MS analysis to identify the site of phosphorylation. The phosphorylation site (*) was readily localized to Ser⁴⁸⁷, on the basis of the mass difference between the y_5 and y_6 ions and the presence of a $y_6\text{-H}_3\text{PO}_4$ ion.

ESI is electrospray ionization). Figure 5 shows the LC/MS/MS spectrum of the endoprotease Asp-N phosphopeptide. Fragmentation sequence information permitted unambiguous identification

Figure 6 MAP kinase phosphorylates HSPDE4B2B(152–528) at Ser⁴⁸⁷

Sf9 cells were transfected with baculovirus containing DNA that codes for residues 152–528 of HSPDE4B2B (**A**, lane 1; **B**, white bars), 152–528 of HSPDE4B2B with alanines at residues 482 and 489 (**A**, lane 2; **B**, grey bars) and 152–528 of HSPDE4B2B with alanines at residues 482, 487 and 489 (**A**, lane 3; **B**, black bars). HSPDE4B2B was solubilized with digitonin and labelled with MAP kinase as described in the Materials and methods section. (**A**) Autoradiograph of ³²P-labelled proteins resolved by SDS/PAGE and immobilized on nitrocellulose. PhosphorImager analysis (**B**) was used to quantify the amount of radioactivity in each lane. The amounts of radioactivity incorporated into the bands labelled PDE-IV and the two endogenous protein controls (labelled C1 and C2) are expressed as a percentage of the total radioactivity found within a lane.

Table 2 Phosphorylation of PDE-IV *in vitro* by MAP kinase, cdc2 kinase and casein kinase I

The indicated HSPDE4B2B constructs were purified from *E. coli* and baculovirus expression systems and phosphorylated as described in the Materials and methods section with MAP kinase, cdc2 kinase or casein kinase I (activities shown in units of mol of phosphate per mol of protein). Phosphorylation was stopped by the addition of 1 mg/ml BSA and 10% (w/v) trichloroacetic acid. Trichloroacetic acid-insoluble material was collected by centrifugation and assayed with a bench-top radioisotope counter.

Expression system; PDE-IV construct	MAP kinase	Casein kinase I	cdc2 kinase
<i>E. coli</i> ; 152–528	0.9	0.0	0.17
Sf9; 80–564	0.14	0.024	0.17
Sf9; 152–528	0.13	0.002	0.12
Sf9; 152–528 Ala ⁴⁸² , Ala ⁴⁸⁷ , Ala ⁴⁸⁹	0.0	0.003	0.06

of the site of phosphorylation site as Ser⁴⁸⁷ (based most notably on the γ_6 , γ_6 -H₃PO₄ and β_{13} -H₃PO₄ ions). The site that MAP kinase phosphorylates in HSPDE4B2B was confirmed by mutating serines 482, 487 and 489 to alanines in HSPDE4B2B(152–528). MAP kinase and ³²P-ATP were used to radiolabel Sf9 cell extracts containing the HSPDE4B2B constructs. Equal amounts of PDE4 were separated by SDS/PAGE and analysed by autoradiography (Figure 6A) and PhosphorImager analysis (Figure 6B). Western blot analysis was used to confirm the identity of the bands labelled as PDE4 in Figure 6 (results not shown). However, when serines 482 and 489 were mutated to alanines (lane 2) there was no decrease in

Figure 7 HSPDE4B2B phosphorylation by MAP kinase

The autoradiograph shows MAP kinase-phosphorylated HSPDE4B2B(81–564) from Sf9 cells (lane 1), HSPDE4B2B(152–528) from Sf9 cells (lane 2), HSPDE4B2B(152–528) from *E. coli* (lane 3) and HSPDE4B2B(152–528) with alanines at residues 482, 487 and 489 from Sf9 cells (lane 4).

phosphorylation of HSPDE4B2B(152–528) compared with the construct lacking these point mutations (lane 1). However, when serines 482, 487 and 489 were mutated to alanines (lane 3), phosphorylation of HSPDE4B2B(152–528) was decreased. These mutations did not affect phosphorylation of the endogenous Sf9 proteins (labelled C1 and C2). These results confirm that Ser⁴⁸⁷ serves as a major site for phosphorylation by MAP kinase. The appearance of the lower band in Figure 6 and the change in its intensity in lanes 1 and 2 may be the result of proteolysis of HSPDE4B2B(152–528) and HSPDE4B2B(152–528)(Ala⁴⁸², Ala⁴⁸⁹). As a control, the specificity of phosphorylation of HSPDE4B2B by various proline-directed serine kinases (e.g. MAP kinase, cdc2 kinase and casein kinase I) was compared. Several different HSPDE4B2B constructs were purified from *E. coli* and baculovirus expression systems and phosphorylated as described in the Materials and methods with MAP kinase, cdc2 kinase, or casein kinase I. Phosphorylation was detected by radioisotope counting for all the samples (Table 2) and by autoradiography for the MAP kinase-treated samples (Figure 7). The results in Table 2 show that MAP kinase was better than cdc2 kinase or casein kinase I at phosphorylating *E. coli*-expressed HSPDE4B2B(152–528). When HSPDE4B2B(152–528) was expressed in Sf9 cells, phosphorylation by MAP kinase decreased by 85 % (Figure 7 and Table 2). A similar decrease was not observed for cdc2 kinase or casein kinase I. Furthermore the removal of amino acids 81–151 and 529–564 decreased phosphorylation by cdc2 kinase and casein kinase I, but not phosphorylation by MAP kinase (Figure 7 and Table 2). Thus Sf9 cells phosphorylated HSPDE4B2B(152–528) at a site that was recognized by MAP kinase but not by cdc2 kinase or casein kinase I. As expected, mutating serines 482, 487, and 489 to alanines abolished MAP kinase phosphorylation of HSPDE4B2B(152–528) (Figure 7 and Table 2).

Kinetic properties of MAP kinase and HSPDE4B2B

The kinetics of MAP kinase phosphorylation of HSPDE4B2B and myelin basic protein, a well-characterized substrate for MAP kinase [33], were compared by using double-reciprocal plots. The K_m of MAP kinase for *E. coli*-expressed purified HSPDE4B2B(152–528) was calculated to be $63 \pm 5 \mu\text{M}$. In comparison, the K_m for myelin basic protein was calculated as $26 \pm 3 \mu\text{M}$. This is in agreement with the published K_m value ($25 \mu\text{M}$) of MAP kinase for myelin basic protein [34]. The V_{max} values for HSPDE4B2B and myelin basic protein were calculated

as 3.0 ± 1.1 and 5.5 ± 2.1 $\mu\text{mol/min}$ per mg respectively (the calculations were from triplicate assays of three independent experiments).

The maximum amount of phosphate that can be incorporated into HSPDE4B2B(152–528) was determined by incubating purified *E. coli*- and Sf9-expressed HSPDE4B2B(152–528) (1 μM) with [^{32}P]ATP (100 μM) and MAP kinase (50 nM) for various times. Analysis of the trichloroacetic acid-precipitable counts revealed that the initial rate of phosphorylation slowed markedly after 40 min of incubation at 30 °C. On the basis of one MAP kinase site per molecule of HSPDE4B2B(152–528), we estimate that 50% and 81% of the *E. coli*-expressed HSPDE4B2B(152–528) were phosphorylated after 10 and 40 min respectively. Under similar conditions MAP kinase did not phosphorylate Sf9-expressed HSPDE4B2B(152–528) with serines or alanines at positions 482, 487 and 489.

The effects of phosphorylation on PDE activity were determined after incubating HSPDE4B2B(152–528) from *E. coli* under conditions that led to more than 80% phosphorylation. The kinetics of cAMP hydrolysis by phosphorylated and non-phosphorylated HSPDE4B2B(152–528) was compared by using double-reciprocal plots. Phosphorylation of HSPDE4B2B(152–528) by MAP kinase did not affect the K_m (68.7 μM) or V_{max} (6.4 $\mu\text{mol/min}$ per mg) for cAMP. Similarly phosphorylation did not affect the IC_{50} (350 nM) of rolipram on HSPDE4B2B(152–528) phosphodiesterase activity. It should be noted that these results may not reflect the effects of phosphorylation on the full-length molecule.

To determine whether phosphorylation was important in the overall secondary structure of the HSPDE4B2B proteins isolated from Sf9, CD and fluorescence studies were performed. CD studies have previously been performed with Sf9-expressed HSPDE4B2B(81–564) (W. J. Rocque, W. D. Holmes, D. B. Kassel, I. Patel, L. Overton, C. Hoffman, G. B. Wisely, D. Willard, R. Dougherty, O. Ittoop and M. Luther, unpublished work). CD scans revealed a large amount of α -helix in the HSPDE4B2B(152–528) and HSPDE4B2B(152–528)(Ala^{482, 487, 489}) constructs isolated from Sf9 cells with only minor differences in ellipticity intensities. The CD scans of HSPDE4B2B(81–564) and HSPDE4B2B(81–564)(Ala^{482, 487, 489}) from Sf9 cells were also similar. Fluorescence denaturation studies with urea or guanidinium chloride showed a similar pattern of unfolding for the two constructs. Furthermore HSPDE4B2B(152–528) had a melting temperature of 40.5 °C, whereas the HSPDE4B2B(152–528)(Ala^{482, 487, 489}) protein melted at 39.3 °C. Taken together, these observations suggest that phosphorylation plays a minimal role in the overall secondary structure of HSPDE4B2B in Sf9 cells.

DISCUSSION

Recently several groups have shown that cAMP inhibits and Ras activates the MAP kinase cascade for transmitting growth signals [2,3,35–37]. It has been suggested that cAMP's inhibitory effect is a result of PKA activation [2,3,25,38]. PKA is proposed to phosphorylate and block the activity of Raf-1, a protein required for signalling from Ras to MAP kinase [26,27]. Thus PKA might link both the cAMP and Ras signalling pathways (reviewed in [4]). In this study we demonstrate that a cAMP-specific PDE (HSPDE4B2B) serves as a substrate for MAP kinase. This suggests that the phosphorylation of HSPDE4B2B by MAP kinase might serve as a second link for the cAMP and Ras signalling pathways.

Three lines of evidence show that HSPDE4B2B(81–564) and HSPDE4B2B(152–528) was phosphorylated in Sf9 cells. First,

MS analysis of the purified proteins from transfected Sf9 cells demonstrated that nearly 1.5 mol of phosphate were incorporated per mol of HSPDE4B2B(81–564). This observation suggests that Sf9 cells phosphorylate more than one site in HSPDE4B2B(81–564). Secondly, radiolabelling with [^{32}P]P_i indicated that HSPDE4B2B(152–528) represented nearly 20% of the total phosphorylated protein within the transfected cells. This could in part be a result of overexpression, which might allow HSPDE4B2B(152–528) to serve as a better kinase substrate owing to mass action. Thirdly, HSPDE4B2B(152–528) purified from *E. coli* served as a better substrate *in vitro* for MAP kinase than the Sf9-expressed protein. One reason for this is that *E. coli* does not phosphorylate HSPDE4B2B(152–528) (Table 1). In contrast, Sf9 should phosphorylate HSPDE4B2B(152–528) and cause it to become a poor substrate for subsequent phosphorylation *in vitro* by MAP kinase.

Although Sf9 cells are commonly used as models for studying phosphorylation in eukaryotic cells, it should be noted that direct evidence is lacking for HSPDE4B2B phosphorylation in the relevant human tissues (such as monocytes). Because of the low expression level of PDE4B2B in monocytic cells, we were unable to detect phosphorylation of PDE4 from these cells. However, we have observed that *E. coli*-expressed HSPDE4B2B(1–564) has increased mobility in SDS/polyacrylamide gels compared with HSPDE4B2B from HL-60 and THP-1 cells or when compared with HSPDE4B2B(1–564) overexpressed in yeast. This difference in mobility is consistent with phosphorylation of HSPDE4B2B in monocytic cell lines and yeast. In support of these observations, Western blot analysis has revealed an increased mobility in SDS/polyacrylamide gels of HSPDE4B2B(81–564)(Ala^{482, 487, 489}) compared with HSPDE4B2B(81–564)(Ser^{482, 487, 489}). We have also confirmed that HSPDE4B2B(1–564) is phosphorylated when overexpressed in yeast (see Table 1). However, HSPDE4B2B(1–564) expressed in yeast is unstable and susceptible to proteolysis.

The mass spectra of phosphopeptides generated by enzymatic cleavage with trypsin revealed that Ser⁴⁸⁷ is a major site for phosphorylation whereas Ser⁴⁸⁹ is a minor site for phosphorylation of HSPDE4B2B(81–564) expressed in Sf9 cells. Similar observations have been made for HSPDE4B2B(1–564) expressed in yeast (D. Kassel, unpublished work). Whereas the results in this paper show that Ser⁴⁸⁷ serves as a site for MAP kinase, the kinase that phosphorylates Ser⁴⁸⁹ remains poorly characterized. Examination of the sequence near Ser⁴⁸⁹ reveals that it might conform to the consensus sequence for phosphorylation by casein kinase II. Consistent with this observation, preliminary evidence suggests that HSPDE4B2B(81–564) serves as a substrate *in vitro* for casein kinase II (J. M. Lenhard, unpublished work). It is worth noting that monophosphorylation of Ser⁴⁸⁹ was not observed. This suggests that phosphorylation at Ser⁴⁸⁷ is a prerequisite for phosphorylation of Ser⁴⁸⁹.

MS and mutational analysis showed that Ser⁴⁸⁷ of HSPDE4B2B(152–528) served as the only site for MAP kinase phosphorylation. Phosphorylation experiments that were performed with excess MAP kinase and ATP confirmed that not more than one phosphate was incorporated per molecule of HSPDE4B2B(152–528). Consistent with these observations, PQS⁴⁸⁷P is the only sequence in HSPDE4B2B that conforms to the optimal primary sequence for phosphorylation by MAP kinase [39]. These observations suggest that Ser⁴⁸⁷ may serve as a unique site for phosphorylation of HSPDE4B2B by MAP kinase.

Analysis of the kinase specificity for Ser⁴⁸⁷ indicates that other proline-directed kinases (e.g. cdc2 kinase and casein kinase I)

lack the specificity that was observed for MAP kinase. Furthermore a comparison of HSPDE4B2B(152–528) and myelin basic protein, a well-characterized substrate for MAP kinase [30], as substrates for MAP kinase revealed that they had similar $V_{\max}$ and K_m values. Taken together, these observations suggest that Ser⁴⁸⁷ of HSPDE4B2B might serve as an authentic substrate for MAP kinase phosphorylation.

Results from CD and fluorescence studies indicate that the overall secondary conformation of the HSPDE4B2B(81–564) and HSPDE4B2B(81–564)(Ala^{482, 487, 489}) from Sf9 cells are similar. Furthermore phosphorylation by MAP kinase *in vitro* had no pronounced effect on the catalytic activity or the inhibition by rolipram of the catalytic activity of HSPDE4B2B(152–528) purified from *E. coli*. A lack of differences in the kinetic, binding and inhibitory properties was also observed on comparison of HSPDE4B2B(81–564) and HSPDE4B2B(81–564)(Ala^{482, 487, 489}) (W. J. Rocque, W. D. Holmes, D. B. Kassel, I. Patel, L. Overton, C. Hoffman, G. B. Wisely, D. Willard, R. Dougherty, O. Ittoop and M. Luther, unpublished work). Other studies (W. J. Rocque, W. D. Holmes, I. Patel, R. Dougherty, O. Ittoop, L. Overton, C. Hoffman, G. B. Wisely, D. Willard and M. Luther, unpublished work) identified a catalytic domain (amino acids 152–528) that had the same K_{cat} and K_m as full-length HSPDE4B2B. Taken together, these observations suggest that a change in activity on phosphorylation was not masked by deletion of the amino acids outside the catalytic domain. It is interesting to note that we have observed that the non-phosphorylated form of HSPDE4B2B is more susceptible to proteolysis than the phosphorylated form (W. Rocque, unpublished work), suggesting a role for phosphorylation in the stability of HSPDE4B2B. Alternatively, MAP kinase might regulate an unknown function coded for by HSPDE4B2B. One speculation is that phosphorylation regulates interaction of HSPDE4B2B with other proteins. Such protein–protein interactions could control HSPDE4B2B localization or activity. Alternatively, interaction of HSPDE4B2B with other proteins might alter the function of one or more unknown downstream effectors. Ser⁴⁸⁷ and Ser⁴⁸⁹ are interesting in that these phosphorylation sites fall between a highly conserved region and the variable C-terminus of PDEs. Although the conserved region contains the catalytic site of PDEs, the C-terminus serves an unknown function. Thus defining a function for phosphorylation and the C-terminus is warranted in future studies.

Others have shown that HPDE4A [14] and rat PDE3/4D [15] are phosphorylated by PKA. Moreover, insulin has been shown to stimulate the phosphorylation and activation of type 3 PDE in adipocytes [12], hepatocytes [13] and platelets [40]. The results presented here are the first direct evidence that HSPDE4B2B is phosphorylated by MAP kinase. It is interesting to note that insulin and a variety of other growth factors stimulate MAP kinase [41,42]. It has been proposed that PKA might link both the cAMP and MAP kinase signalling pathways by phosphorylation and inhibition of Raf-1 [4,26,27]. As HSPDE4B2B serves as a substrate for MAP kinase, we suggest that the phosphorylation of HSPDE4B2B by MAP kinase could serve as a second link for the cAMP and Ras signalling pathways. Thus growth-factor activation of MAP kinase might be an important pathway regulating HSPDE4B2B.

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