Phosphorylation of septin 3 on Ser-91 by cGMP-dependent protein kinase-I in nerve terminals

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The septins are a family of GTPase enzymes required for cyto-kinesis and play a role in exocytosis. Among the ten vertebrate septins, Sept5 (CDCrel-1) and Sept3 (G-septin) are primarily concentrated in the brain, wherein Sept3 is a substrate for PKG-I (cGMP-dependent protein kinase-I) in nerve terminals. There are two motifs for potential PKG-I phosphorylation in Sept3, Thr-55 and Ser-91, but phosphoamino acid analysis revealed that the primary site is a serine. Derivatization of phosphoserine to *S*-propyl-cysteine followed by N-terminal sequence analysis revealed Ser-91 as a major phosphorylation site. Tandem MS revealed a single phosphorylation site at Ser-91. Substitution of Ser-91 with Ala in a synthetic peptide abolished phosphorylation. Mutation

of Ser-91 to Ala in recombinant Sept3 also abolished PKG phosphorylation, confirming that Ser-91 is the major site *in vitro*. Antibodies raised against a peptide containing phospho-Ser-91 detected phospho-Sept3 only in the cytosol of nerve terminals, whereas Sept3 was located in a peripheral membrane extract. Therefore Sept3 is phosphorylated on Ser-91 in nerve terminals and its phosphorylation may contribute to the regulation of its subcellular localization in neurons.

Key words: cGMP, protein phosphorylation, cGMP-dependent protein kinase (PKG), Sept3, septins, synaptosomes.

INTRODUCTION

Nitric oxide (NO) and natriuretic peptide hormones play key roles in a variety of neuronal functions, including modulating neurotransmission, neuronal differentiation and gene expression, learning and memory, brain seizure activity, neurotoxicity and apoptosis [1]. They exert converging actions by elevating intracellular cGMP through the activation of soluble and particulate guanylate cyclases [2]. The mechanisms whereby cGMP modulates neuronal functions are not always known, but most cGMP signalling leads to the activation of two Ser/Thr protein kinases: PKG-I (cGMP-dependent protein kinase-I) and -II [3]. PKG plays a key role in a number of biological functions, including smooth-muscle contraction and relaxation, cardiac and skeletal-muscle contractility, intestinal motility and ion transport in the gut [4]. In the nervous system, PKG is important in LTP (long-term potentiation) [5], LTD (long-term depression) [6,7], pain processing in spinal cord [8] and axon pathfinding in neural development [9]. Nerve terminals are the major sites of synaptic vesicle exocytosis and endocytosis. Strikingly, the cGMP and PKG signalling system plays multiple roles in the modulation of synaptic vesicle exocytosis and endocytosis in nerve terminals [10]. The activation of cGMP and PKG disrupts the release of synaptic vesicles from the reserve pool to the readily releasable pool, thereby greatly reducing the release of glutamate from cortical or hippocampal synaptosomes or slices [6,11]. PKG also regulates nerve terminal transport of glucose and glutamate [12]. In addition to the regulation of glutamate exocytosis, cGMP and PKG mediate the presynaptic component of both LTD [7] and LTP [5,6] in hippocampal neurons.

Sept3 (originally called G-septin) is a member of a family of highly conserved 40–60 kDa GTPase enzymes called septins. Many septins assemble as intracellular filaments involved in the organization of submembranous structures, neuronal polarity and vesicle trafficking [13–15]. The amino acid sequences of most of

the known septins contain a P loop nucleotide-binding consensus sequence for GTP binding near the N-terminus, and most are predicted to have a coiled-coil domain at the C-terminus. A polybasic motif is conserved near the N-terminus and close to the first GTP-binding motif of most septins. It is important for Sept4 (also known as H5) attachment to the plasma membrane [16].

Septins play multiple cellular roles. First, they play an essential role in cytokinesis in yeast [14], *Drosophila* [17] and mammals [18,19]. Groups of different septins assemble as filaments, and different filament structures have been also found in yeast, *Drosophila* and in mammalian cells [14,15]. A number of septins are found in post-mitotic neurons. Groups of septins associate with the exocyst complex, which is involved in vesicle targeting or tethering [18]. The brain-specific Sept5 and the more widely expressed Sept2 (Nedd5) proteins co-immunoprecipitate with syntaxin, a SNARE protein predominantly present on the plasma membrane that is essential for exocytosis [13]. Targeted disruption of the mouse Sept5 gene produces no major phenotype, suggesting a functional redundancy with other septins [20]. Platelets from Sept5^{−/−} mice are sensitized to collagen-induced activation and serotonin secretion [13]. Sept4, but not five other septins, is found in the α -synuclein-positive cytoplasmic inclusions of Parkinson's disease, dementia with Lewy bodies and multiple system atrophy [21]. Sept5 interacts with Parkin, an E3 ubiquitin-protein ligase implicated in autosomal recessive familial Parkinson's disease, promoting Sept5 degradation [22]. Sept5 overexpression in the brain induces selective dopamine neurodegeneration and inhibits dopamine secretion [23]. Three septins have been associated with acute myeloid leukaemia [Sept5, Sept6 (septin 6) and Sept9 (MSF, also called E-septin or Ov/Br)] by fusion with the MLL gene [24,25]. Four septins, Sept2, Sept4, Sept1 (Diff6) and Sept7 (cdc10), are found in neurofibrillary tangles in post-mortem human brain from patients affected by Alzheimer's disease [26], suggesting that septins might have a function in the aetiology of neuronal disease.

Abbreviations used: GFP, green fluorescent protein; MALDI–QqTOF-MS, matrix-assisted laser-desorption ionization with hybrid quadrupole time-of-flight mass spectrometry; 8-pCPT-cGMP, 8-*p*-chloro-phenylthio-cGMP; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase.

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Sept3 and Sept5 are regulated by phosphorylation. Sept3 is phosphorylated by PKG-I *in vitro*, and its *in vivo* phosphorylation is elevated by cGMP analogues in nerve terminals [27]. Cloning of Sept3 revealed that it contains the predicted motifs for PKG phosphorylation [27]. The aims of this study were to identify the phosphorylation sites in Sept3. In the present study, we demonstrate that Ser-91 of Sept3 is the major phosphorylation site of PKG both *in vitro* and *in vivo*. PKG phosphorylation of Sept3 at Ser-91 may regulate its translocation from the peripheral membrane to cytosol in intact nerve terminals, suggesting that phosphorylation regulates its subcellular localization.

MATERIALS AND METHODS

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol) was from Amersham. Peptides containing the potential phosphorylation sites were synthesized (90 % purity) by Auspep (Melbourne, Australia): peptide A – Sept3₅₀₋₆₁, QMRKKT**MT**KGFD; peptide B – Sept3₈₆₋₉₈, VSRKA-**SS**WNREEK; peptide B1, VSRKAAS**WN**REEK; and peptide B2, VSRKA**S**AWNREEK. Underlined residues are potential phosphorylation sites; bold residues are where sequence alterations were generated *in vitro* by peptide synthesis.

Protein purification and expression

Sept3 was purified from rat brain, and His₆-tagged Sept3 (rat sequence) was expressed in *Escherichia coli* and purified on Ni²⁺-nitrilotriacetate resin column (Qiagen) as described previously [27]. PKG-I was purified from bovine lung [27]. The catalytic subunit of PKA (cAMP-dependent protein kinase) was expressed in *E. coli* [28].

Protein phosphorylation

Protein phosphorylation was performed in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 5 min [27,29]. Phosphoproteins were detected by gel electrophoresis and autoradiography [27]. Phosphoamino acid analysis of ³²P-labelled proteins excised from polyacrylamide gels, protein kinase activity and enzyme kinetics were determined as described previously [29]. After phosphorylation, dephosphorylation was achieved by the addition of 20 units of alkaline phosphatase (cat. no. 1097075; Roche, Lewes, East Sussex, U.K.)/reaction and incubation for 1 h at 30 °C.

Protein kinase activity was determined in the presence of 30 mM Tris/HCl (pH 7.4), 1 mM EGTA, 200 μM ATP, 2 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 10 mM MgSO₄ in 40 μl final reaction volumes. Incubations were for 5 min at 30 °C using the synthetic peptide substrates PL₈₋₂₁ [30], Sept3₅₀₋₆₁ or Sept3₈₆₋₉₈ at 0.1 mg/ml. Reactions were initiated by the addition of 40 ng of PKG or 20 ng of the catalytic subunit of PKA. The amounts of PKG and PKA required to phosphorylate PL₈₋₂₁ to the same level were determined from previous experiments since this substrate has the same V_{max} for both protein kinases [30]. For stoichiometric phosphorylation of Sept3₈₆₋₉₈ (or the same peptide with Ser → Ala substitutions), the assay was performed in the presence of 5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 1 mM ATP. Sept3₈₆₋₉₈ was used over a broad concentration range (0.003–0.3 mg/ml) for determination of substrate kinetics. Reactions were terminated by the addition of 75 mM phosphoric acid, and aliquots were spotted on to Whatman P81 paper, washed three times for 10 min each in 75 mM phosphoric acid, dried and counted for radioactivity by liquid-scintillation techniques [31]. Kinetic constants, K_m and V_{max} , were determined with the PC program Enzfitter (written by Robin Leatherbarrow, from Sigma).

Derivatization of phosphoserine

For direct sequencing of phosphoserine phosphorylation sites, recombinant Sept3 was phosphorylated by PKG for 60 min in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the phosphoprotein was resolved on SDS/PAGE and excised from the wet gel. After tryptic digestion, the fragments were separated by HPLC and a single radiolabelled peptide was isolated. Since we had determined that the only phosphorylated amino acids in Sept3 were phosphoserine (see the Results section), phosphoserine was derivatized to *S*-propylcysteine and the peptides were sequenced as described previously [29].

MS analysis

Recombinant Sept3 was phosphorylated by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 10 min in the presence of PKG. The phosphoprotein was resolved by SDS/PAGE and excised from the wet gel. After tryptic digestion, the fragments were separated by HPLC and collected in fractions that were later analysed for radioactivity as described previously [29]. A single, highly radioactive fraction was concentrated to 5 μl in a vacuum centrifuge (ALPHA-IR, Christ, Germany). This was combined with 10 μl of 2 % (v/v) formic acid solution and loaded on to a microcolumn [32] manufactured by packing a pipette tip (GELoader, Eppendorf, Germany) with activated charcoal (Sigma) [33]. The microcolumn was washed with 10 μl of 2 % (v/v) formic acid solution and the bound material was eluted on to a MALDI (matrix-assisted laser-desorption/ionization) plate in 0.3 % (v/v) trifluoroacetic acid, 60 % (v/v) acetonitrile solution containing 10 mg/ml α -cyano-4-hydroxycinnamic acid. The sample was analysed by MALDI-QqTOF-MS (MALDI with hybrid quadrupole time-of-flight mass spectrometry) using a QSTAR XL with an oMALDI2 ion source (MDS Sciex, Sciex, Thornhill, Ont., Canada). For the analysis of the Sept3 phosphopeptide, 600 spectra were averaged and nitrogen gas was used for fragmentation at a collision energy of 57 V.

Antibodies and immunoblot analysis

Anti-Sept3 antibodies were raised in sheep against two different synthetic peptides Sept3₉₇₋₁₁₂ and Sept3₂₄₄₋₂₅₉ as described in [27]. Anti-phospho-Sept3 antibodies were also raised in sheep against the peptide Sept3₈₉₋₉₆ Cys-K**ASS**WNRE, which was synthesized with Ser-91 as a phosphoserine (Mimotopes, Vic., Australia). This was coupled with a diphtheria toxoid through an additional N-terminal Cys and used to immunize sheep. Immunoblotting was performed with horseradish peroxidase-coupled secondary antibodies and chemiluminescent detection (Pierce West Pico kit). Phosphoproteins were denatured in a reducing SDS sample buffer and resolved on 12 % polyacrylamide mini-gels. The proteins were transferred on to a nitrocellulose transfer membrane (0.45 μm ; Schleicher and Schuell, Dassel, Germany) as described previously [34]. The membrane was blocked with 5 % skim milk in PBS (pH 7.4) overnight and washed in Tris-buffered saline (pH 7.4) containing 0.1 % Tween 20 (TBST), then incubated with the first antibody for 2 h and with the second antibody for 1 h in TBST containing 0.5 % polyvinylpyrrolidone 40 and washed with TBST buffer.

Site-directed mutagenesis on Ser-91 and Ser-92 in Sept3

Wild-type Sept3 was cloned into pEGFP-C1 vector (ClonTech, Basingstoke, U.K.). Site-directed mutagenesis was performed to mutate Ser-91 to Ala (S⁹¹ → A) or Ser-92 to Ala (S⁹² → A) using the QuickChange Kit (Stratagene) following the manufacturer's recommendations. GFP (green fluorescent protein)-tagged Sept3 DNA was used as template for the following two pairs of oligonucleotides:

Figure 1 PKG-I phosphorylation site in Sept3

(A) Phosphorylation of Sept3 by PKG. Recombinant His₆-tagged Sept3 was phosphorylated in duplicate with [γ -³²P]ATP without any additions (lane 1), in the presence of PKG, cGMP and inhibitor protein for PKA (lane 2) and visualized by autoradiography. The results are representative of at least five experiments. (B) Phosphoamino acid analysis of Sept3. His₆-tagged Sept3 was phosphorylated by PKG, separated on a gel, excised and subjected to phosphoamino acid analysis. An autoradiograph is presented. The migration position of phosphorylated serine, threonine or tyrosine standards is indicated on the left. (C) Conversion of phosphoserine into *S*-propylcysteine reveals the phosphorylation site in Sept3. Recombinant Sept3 was phosphorylated for 60 min in the presence of [γ -³²P]ATP, cGMP and PKG-I. Phosphoproteins were resolved on a 7.5–15% gradient acrylamide gel. The Coomassie Blue-stained Sept3 band was excised from the wet gel and digested with trypsin. A single radiolabelled peptide was isolated by HPLC and derivatized to *S*-propylcysteine. The resultant peptide was N-terminally sequenced by the Edman method to detect PTH-*S*-propylcysteine. Each cycle of the sequencing released one amino acid (panels a–f); (a) Lys, (b) Ala, (c) *S*-propylcysteine (PrC), (d) *S*-propylcysteine (PrC), (e) W and (f) N respectively. S indicates the normal serine elution position, which was unchanged in this experiment. mAU, milli-absorbance units.

S⁹¹ → A (a) (5'-GTGAGCCGGAAGGCCGCCAGCTGGAA-CCGG-3') and (b) (5'-CCGGTTCCAGCTGGCGGCCCTCCG-GCTCAC-3'); S⁹² → A (a) (5'-GTGAGCCGGAAGGCCTCC-GCCTGGAACCGG) and (b) (5'-CCGGTTCCAGGCGGAGGC-CTTCCGGCTCAC-3').

Cell culture, transient transfection and immunoprecipitation

COS7 cells, plated at 2×10^6 cells/dish 1 day before transfection, were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum at 37 °C in a 5% CO₂ incubator. Cells were transiently transfected with 0.5 μ g of DNA using FugeneTM 6 according to the manufacturer's instructions (Roche), and cultured for an additional 24 h. Transfected cells were then washed in cold PBS and resuspended by scraping in cold cell lysis buffer containing 10 mM Tris buffer (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10 μ g/ml leupeptin, protease inhibitor cocktail tablets (Roche) and 1 mM PMSF. Cells were lysed at 4 °C by vortex-mixing and passing through a 25-gauge needle three times. Cell extracts were centrifuged at 20000 *g* at 4 °C for 30 min. The immunoprecipitation was performed as described in [29] with some modifications. In brief, Protein G-Sepharose (Roche) was washed and equilibrated with cell lysis buffer and then incubated with anti-GFP polyclonal antibodies (ClonTech) for 1 h. The Sepharose was washed three times with cell lysis buffer and then incubated with the cell extracts for an additional 2 h. The aliquots were subjected to phosphorylation or immunoblot analysis.

Phosphorylation in intact synaptosomes

Rat brain P2 synaptosomes were prepared [35], washed once with 350 mM NaCl to remove extracellular peripheral membrane protein contaminants, then twice with PBS (pH 7.4). The synapto-

somes were resuspended in pre-warmed Hepes-buffered Krebs solution, containing 20 mM Hepes (pH 7.4), 118 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO₄, 0.1 mM Ca²⁺ and 10 mM D-glucose and incubated for 15 min at 37 °C. The synaptosomes were then incubated without additions or with membrane-permeant cyclic nucleotide analogue 8-*p*-chloro-phenylthio-cGMP (8-*p*CPT-cGMP, 500 μ M) for 15 min at 37 °C. After stimulation, synaptosomes were collected by centrifugation and lysed in 5 mM Tris/HCl (pH 7.4), containing both protease inhibitor tablets (Roche) and phosphatase inhibitor tablets (Calbiochem). After centrifugation at 20000 *g* for 15 min to collect cytosol, the particulate fractions were extracted with 250 mM NaCl in the same buffer for 15 min and re-centrifuged to collect the peripheral membrane extract.

RESULTS

Purified rat brain Sept3 was reported as an excellent substrate for PKG-I [27]. There are two predicted PKG phosphorylation site motifs in Sept3, namely Thr-55 and Ser-91. To identify the phosphorylation site for PKG, His₆-tagged-Sept3 was phosphorylated in the presence of PKG-I (Figure 1A) and subjected to phosphoamino acid analysis. PKG phosphorylation was exclusively on serine (Figure 1B), ruling out Thr-55. To identify the phosphorylation site, recombinant Sept3 was phosphorylated by PKG for 60 min and phosphoserines were derivatized to *S*-propylcysteines [29]. In this procedure, Ba²⁺-catalysed β -elimination of phosphoserine generates an α - β unsaturated dehydroalanine, which is converted into its thioether derivative, *S*-propylcysteine, by addition of an alkanethiol. The samples were then subjected to N-terminal sequence analysis to detect phenylthiohydantoin-*S*-propylcysteine. After tryptic digestion and HPLC, a single

Figure 2 Phosphorylation of Sept3 by PKG on Ser-91

(A) Recombinant Sept3 was phosphorylated by PKG for 10 min. The Coomassie Blue-stained Sept3 band was excised from the wet gel and digested with trypsin. The peptide mixture was subjected to HPLC and collected in fractions. On the primary axis, the counts per minute of Cerenkov radiation was measured for each fraction. On the secondary axis, the concentration of phase B for each fraction eluted by reversed-phase HPLC is shown. (B) The product ion spectrum from the fragmentation of m/z 928.44. The peak at m/z 830.43 has been truncated to one-third of its height to improve legibility. The y ion series describes a sequence of standard amino acid residues up to y_5 , where β -elimination of phosphoric acid (loss of 98 units by conversion of phosphoserine into dehydroalanine) is favoured during fragmentation. The β -elimination of y ion series ($y_n - 98$) continues to complete the sequence. The position of the phosphoserine is unambiguously determined by the change from y ions to $y - 98$ ions (from the C-terminal end of the peptide). The spectrum is exclusively consistent with the Sept3₈₉₋₉₅ phosphopeptide KASSWNR.

radiolabelled peptide was isolated from PKG phosphorylated Sept3. This peptide was derivatized to *S*-propylcysteine and sequenced. Each cycle of the sequencing released one amino acid (Figure 1C). The sequence obtained was KASSWN, with *S*-propylcysteine (PrC) being released instead of Ser at the third and fourth cycles. This reveals that Ser-91 is phosphorylated by PKG; however, it is unclear whether Ser-92 was phosphorylated or represented carry-over from the previous fraction in the HPLC. No evidence for phosphorylation of other sites was obtained.

Since it was not clear whether Ser-92 as well as Ser-91 were phosphorylated, tandem MS was employed to resolve the question. His₆-tagged Sept3 was phosphorylated by PKG in the presence of [γ -³²P]ATP for 10 min. The shorter time of phosphorylation was chosen to reduce any potential artifact arising from long *in vitro* incubations. Proteins were trypsin-digested and radiolabelled peptides were separated with HPLC (Figure 2A). Most of the radioactive peptides were collected in a single fraction. This fraction was analysed using MALDI-QqTOF-MS. A full scan of the sample revealed a number of m/z peaks (results not shown). A peak was present at m/z 928.44 which corresponded to the theoretical monoisotopic m/z of the phosphorylated peptide KASSWNR (Sept3₈₉₋₉₅, m/z 928.40). There was also a peak at 98 units less (m/z 830.43), indicating that β -elimination of

phosphoric acid might be occurring [36]. No other m/z peaks from this spectrum matched theoretical phosphopeptides from the Sept3 sequence [27]. The parent ion at m/z 928.44 was selected for fragmentation to obtain sequence information (Figure 2B). The spectrum revealed the sequence KASSWNR, where only Ser-91 is phosphorylated. The mass spectrum also contained a series of y ions [37], $y_7 - 98$ to $y_5 - 98$, which was consistent with β -elimination of phosphoric acid until the first serine, but is not part of the non-phosphorylated series (y_4 to y_1). This unambiguously shows that the position of the phosphorylation site is Ser-91. There was no evidence in the mass spectrum that Ser-92 was phosphorylated.

To confirm these results using unrelated methods, two peptides were synthesized based on the phosphorylation site sequences from Sept3. Peptide A contained Thr-55 (Sept3₅₀₋₆₁, QMRK-KTMTKTGFD) and peptide B contained Ser-91 and Ser-92 (Sept3₈₆₋₉₈, VSRKASSWNREEK, Figure 3A). Peptide A was not phosphorylated by either PKG or PKA (Figure 3B). Peptide B was highly phosphorylated by PKG and to a much lesser extent by PKA (Figure 3B). This supports the sequencing results that the phosphorylation site for PKG is contained within peptide B, but does not reveal whether Ser-91, -92 or both were phosphorylated. Two substitutions were made in peptide B: peptide

Figure 3 The PKG phosphorylation site(s) in Sept3 peptides

(A) Four synthetic peptides used to determine the phosphorylation site in Sept3. (B) Peptides A and B were used as substrates in an assay for PKG and PKA. The peptides were incubated with inhibitor protein for PKA/cGMP/PKG/[γ - 32 P]ATP or the catalytic subunit of PKA/[γ - 32 P]ATP for 10 min at 30 °C. Aliquots of each were then pipetted on to Whatman P81 ion-exchange paper and free 32 P was washed off with diluted phosphoric acid. Protein kinase activity was determined by scintillation counting of the papers (units are c.p.m. $\times 10^4$). (C) The modified B series of peptides were used as substrates in a PKG assay as described in (B), except that the incubation was for standard (10 min) or prolonged (30 min) times. (D) Time course of phosphorylation of peptide B ($\square$) and B2 ($\triangle$). Values (mol of P_i/mol of peptide) are the means for triplicate experiments and error bars indicate S.E.M.

Table 1 Kinetics of phosphorylation of Sept3 peptide B and PL peptide substrates by PKG and PKA

Substrate	K_m (μ M)		V_{max} (μ mol $\cdot$ mg $^{-1}$ $\cdot$ min $^{-1}$)		V_{max}/K_m		Specificity index (PKG/PKA)
	PKG	PKA	PKG	PKA	PKG	PKA	
Sept3 ₈₆₋₉₈	86.5 $\pm$ 8.17	683.4 $\pm$ 103	5.44 $\pm$ 0.14	3.94 $\pm$ 0.37	0.063	0.0058	10.9
PL ₈₋₂₁	23.5 $\pm$ 2.64	13.8 $\pm$ 2.6	5.29 $\pm$ 0.15	3.65 $\pm$ 0.16	0.225	0.26	0.85

B1 contains S⁹¹ $\rightarrow$ A (VSRKAASWNREEK) and peptide B2 has S⁹² $\rightarrow$ A (VSRKASAWNREEK). Peptide B1 was not phosphorylated by PKG, even after extending the incubation to 30 min, confirming the Ser-91 site, whereas peptide B2 surprisingly showed increased phosphorylation (Figure 3C). A time course of phosphorylation of peptides B and B2 showed that the stoichiometry of phosphorylation of B was 1 mol/mol, whereas that of B2 was increased to 2 (Figure 3D). Since B2 has only two serine residues remaining, this shows that mutation of S⁹² $\rightarrow$ A alters the peptide such that Ser-87 now becomes a PKG substrate when it previously was not. These results provide strong evidence that Ser-91 represents the major, if not the sole, PKG phosphorylation site in Sept3.

Kinetics of Sept3 phosphorylation were determined with peptide B. The kinetics (K_m and V_{max}) of phosphorylation by PKG and PKA was compared with that of one of the best PKG peptide substrates, PL₈₋₂₁, which exhibits similar kinetics for PKG and PKA [30]. PL₈₋₂₁ showed a similar affinity (K_m) for PKG and PKA and similar high rates (V_{max}) of phosphorylation (Table 1). The specificity index provides the V_{max}/K_m ratio for each kinase and is a measure of the relative specificity of a substrate for two protein kinases [29]. The specificity index for PL₈₋₂₁ was close to 1, as reported previously [29,30], indicating that it is almost equally

efficiently phosphorylated by PKG and PKA. Peptide B had a 4-fold lower affinity for PKG but the same maximal rate of phosphorylation. PKA phosphorylated peptide B at a fast rate, but with significantly reduced affinity. The specificity index revealed a 10.9-fold specificity for PKG relative to PKA.

To confirm that Ser-91 is a major phosphorylation site in Sept3 independently, polyclonal antibodies were raised in sheep against a synthetic peptide encompassing the phosphorylation site and which included a phosphorylated Ser-91 [Sept3₈₉₋₉₆ KAS(p)-SWNRE]. The anti-phospho-Ser-91 antibodies preferentially detected PKG phosphorylated Sept3 (Figure 4A, lane 2; Figure 4B, lanes 3 and 4). Alkaline phosphatase treatment of phosphorylated Sept3 abolished immunoreactivity (Figure 4B, lanes 5 and 6). When the previously reported antibodies to Sept3 [27] were used as controls, surprisingly, they preferentially detected dephospho-Sept3 (Figure 4C). This suggests that the previous Sept3 sheep antibodies are somewhat conformation-dependent and that phosphorylation induces a conformational change in Sept3.

Finally, the PKG phosphorylation site in Sept3 was confirmed with wild-type and mutant Sept3 expressed in mammalian COS7 cells. Ser-91 or Ser-92 were mutated to Ala (Sept3-S91A or Sept3-S92A) in the full-length protein and the constructs were expressed in COS7 cells as GFP-tagged fusion proteins. Each

Figure 4 Characterization of anti-phospho-Sept3 antibodies

(A) Phosphorylation-site-specific antibodies. Recombinant Sept3 (50 ng) was mock-phosphorylated (lane 1) or phosphorylated with PKG (lane 2), run on a 12 % acrylamide gel and transferred on to a nitrocellulose membrane. (B) Specificity of PKG phosphorylation on Ser-91 in Sept3. The recombinant Sept3 on Ni^{2+} -nitrilotriacetate beads was phosphorylated in duplicate with unlabelled ATP without any additions (lanes 1–2), or in the presence of PKG (lanes 3–4). The phosphorylated Sept3 was then dephosphorylated with alkaline phosphatase (AP, lanes 5–6). Proteins were subjected to immunoblot analysis. The membrane was probed with antibodies to phospho-Ser-91 Sept3. (C) As a control, the same samples from (A) were probed with sheep antibodies to wild-type Sept3.

recombinant protein was immunoprecipitated by anti-GFP polyclonal antibodies using Protein G–Sephadex. Aliquots of each protein were subjected to immunoblot (Figure 5A) or phosphorylated by PKG after immunoprecipitation (Figure 5B). Wild-type and $\text{S}^{92} \rightarrow \text{A}$ were expressed at similar levels, whereas $\text{S}^{91} \rightarrow \text{A}$ was expressed at a higher level in the cells (Figure 5A). Wild type Sept3 was phosphorylated by PKG, and Sept3- $\text{S}^{91} \rightarrow \text{A}$ was not phosphorylated despite the higher protein level (Figure 5B, upper panel). Interestingly, Sept3- $\text{S}^{92} \rightarrow \text{A}$ showed increased phosphorylation by PKG, which is consistent with the results obtained for the synthetic peptide displayed in Figure 3. The antibodies against phospho-Sept3 detected the same amount of phosphorylation of wild-type Sept3 and $\text{S}^{92} \rightarrow \text{A}$ (Figure 5B, lower panel). This is consistent with PKG phosphorylation of Ser-91 in both constructs, whereas PKG phosphorylates an additional site(s) in $\text{S}^{92} \rightarrow \text{A}$, which is not detected with this antibody. These results strongly support that Ser-91 is the major PKG phosphorylation site in Sept3.

Sept3 is a PKG substrate *in vitro* and its phosphorylation also increases in response to 8-pCPT-cGMP stimulation in nerve terminals [27]. The phosphorylation site in intact cells was investigated using isolated intact nerve terminals (rat brain synaptosomes) [38]. After stimulation of unlabelled synaptosomes with 8-pCPT-cGMP, samples were analysed by Western blotting with the phospho-specific antibodies, using Sept3 purified from rat brain as a control (Figure 5C). Most of the nerve-terminal Sept3 was associated with the peripheral membrane extract (lanes 3 and 5), whereas a small, but reproducible ($n = 4$), increase in phospho-Sept3 immunoreactivity was exclusively detected in the cytosolic fraction (lanes 2 and 4). These results show that Sept3 is phosphorylated on Ser-91 *in vivo* in response to extracellular stimuli that activate the cGMP signalling pathway, and suggests that phosphorylation of Sept3 produces its translocation from a peripheral membrane fraction to the cytosol.

DISCUSSION

Sept3 is one of two septins highly enriched in the brain. It is phosphorylated *in vitro* by PKG and is a phosphoprotein in nerve terminals *in vivo*. In the present study, we have demonstrated that Sept3 is phosphorylated by PKG on Ser-91 *in vitro* and that it is phosphorylated on the same site in nerve terminals. Endogenous phosphorylation in nerve terminals occurs constitutively, but is further increased in response to stimulation with a cell-permeant

Figure 5 PKG phosphorylates Sept3 on Ser-91 *in vitro* and *in vivo*

(A) Wild-type GFP-tagged Sept3, Sept3-S91A and Sept3-S92A were expressed in COS7 cells and then immunoprecipitated from the cell lysates with anti-GFP antibodies. Aliquots of total protein were subject to immunoblot analysis with sheep anti-Sept3 antibodies. (B) The recombinant proteins above were phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence (lanes 1, 3 and 5) or presence of PKG (lanes 2, 4 and 6). Half of the sample was run on gels and phosphoproteins were visualized by autoradiography (upper panel). The positions of GFP-Sept3 and the exogenous autophosphorylated PKG-I are shown with arrows. The other half of the sample was subjected to immunoblot analysis and probed with anti-phospho-Sept3 antibodies (lower panel). (C) Phosphorylation of Sept3 on Ser-91 in intact synaptosomes. Purified native Sept3 from rat brain was used as a control (lane 1). Rat brain synaptosomes were unstimulated (lanes 2 and 3) or incubated with 500 μM 8-pCPT-cGMP, (lanes 4 and 5) for 15 min, lysed and separated to cytosolic and peripheral membrane (M) fractions by centrifugation. The proteins were immunoblotted and probed with antibodies against Sept3 (upper panel). The nitrocellulose membrane was stripped with 0.2 M NaOH [30 min at room temperature (22 °C)] and re-probed with anti-phospho-Sept3 antibodies to detect Ser-91 phosphorylation (lower panel). Results are representative of four independent experiments.

cGMP analogue. Whereas Sept3 is almost exclusively associated with the nerve-terminal plasma membrane, phospho-Sept3 is exclusively cytosolic, suggesting that phosphorylation might induce translocation. Therefore Sept3 phosphorylation on Ser-91 may play a key role in the nerve terminal by regulation of the sub-cellular localization of the protein during stimulation.

We conclude that Ser-91 of Sept3 is the major, if not sole, phosphorylation site for PKG by using a broad strategy including phosphoamino acid analysis, amino acid sequencing, MS, synthetic peptide assays and point mutagenesis. The PKG-I phosphorylation site in Sept3 was determined by direct sequencing of proteolytic products combined with the chemical conversion of phosphoserine into S-propylcysteine. Ser-91 and Ser-92 were both apparently phosphorylated, but no other sites were detected. However, phosphorylation of Ser-92 was unexpected, as it does not reside in an optimal sequence context for PKG. We considered that it might be an *in vitro* artifact due to the prolonged phosphorylation time before site sequencing.

Alternatively, it could represent carry-over of the *S*-propylcysteine in the HPLC. To determine definitively whether Ser-92 was a phosphate acceptor, we phosphorylated Sept3 for a short time (to sub-stoichiometric levels) and determined the phosphorylation site by tandem MS. This unequivocally revealed a single phosphorylation site at Ser-91. Studies with synthetic peptides revealed that Ser-91 is the major site, but also revealed further complexities in exploring substrate specificity with peptides. A synthetic peptide containing both Ser-91 and Ser-92 was phosphorylated with a stoichiometry of 1 mol/mol, suggesting a single site. This was confirmed, since substitution of Ser-91 for Ala abolished all phosphorylation. However, phosphorylation of a peptide containing only Ser-91 (with a substitution of Ser-92 for Ala) was increased by 2-fold. This suggests that phosphorylation of Ser-87 occurs only after prior phosphorylation of Ser-91, and that Ser-87 phosphorylation might be retarded by the presence of Ser-92. No similar properties for PKG substrate specificity have been previously reported. Similar results were obtained in studies with site-directed mutagenesis of full-length Sept3. Whereas mutation of Ser⁹¹-Ala produced the expected block in phosphorylation of the recombinant protein, mutation of Ser⁹²-Ala increased the PKG phosphorylation. Immunoblots confirmed that the increase was on all site(s) distinct from Ser-91.

Sept3 phosphorylation revealed a high degree of selectivity for PKG relative to PKA [27]. The sequence context of the primary phosphate acceptor site in Sept3 is VSRKASSWNREEK, which agrees well with the motif necessary for efficient PKG phosphorylation determined in studies with peptide libraries on cellulose papers [39]. Those studies revealed the minimal motif RKX[ST] and highlighted a key role for additional basic amino acids on either side of the RK for optimal V_{max} . The kinase selectivity of Sept3 was further emphasized when the kinetics of phosphorylation of Sept3₈₆₋₉₈ was compared with those of PL₈₋₂₁. Whereas PKG and PKA phosphorylated PL₈₋₂₁ with very similar kinetics [30], Sept3₈₆₋₉₈ was a better substrate for PKG than for PKA. PKG and PKA are known to show overlapping substrate specificities, and the canonical PKA phosphorylation site (RRX[ST]) is also recognized by PKG [39]. Therefore the minimal motif determined for optimal PKG phosphorylation, RKXS/T, does not account for kinase selectivity, and little information is available regarding sequences that may contribute to this [29]. The presence of a Phe C-terminal on the phosphorylation site has been suggested as a negative determinant for PKA phosphorylation [40]. Sept3 also contains an aromatic amino acid (Trp) in a similar position, as does SF1 [29] (KKRKRSRWNQ), but whether this residue accounts for kinase selectivity remains to be determined. In contrast, other substrates for PKG that exhibit this selectivity, G substrate and VASP, do not have aromatic amino acids C-terminal to the phosphate acceptor site [41].

Sept3 is a phosphoprotein in intact nerve terminals, and its phosphorylation is increased in response to stimulation with cyclic nucleotides [27]. In the present study, we extended these findings to reveal the phosphorylation site and the potential roles for phosphorylation. Phosphorylation-site-specific antibodies revealed that elevated phosphorylation occurred on Ser-91 in response to a cGMP analogue, but does not rule out additional *in vivo* sites. We have also demonstrated that active PKG is enriched in the cytosol and peripheral membrane fractions of rat brain synaptosomes after detection by photoaffinity labelling, Western blot with PKG-I antibodies and by protein kinase activity assay (P. J. Robinson, X. Wang and J. Xue, unpublished work). Therefore Sept3 is quite probably a target for a cGMP-PKG signalling pathway *in vivo*.

Whereas Sept3 is primarily associated with peripheral membrane extracts of brain or nerve terminals, phosphorylation of

Sept3 in nerve terminals occurred exclusively in the cytosol. This suggests that phosphorylation might have induced a translocation from the peripheral membranes or cytoskeleton. Sept4 has been shown to be a membrane protein associated with phosphatidylinositol lipids, specifically with PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 [16]. The binding site for PtdIns lipids in Sept4 was shown to reside within a polybasic region in the N-terminal part of the protein, a region relatively conserved in sequence among most septins. In Sept3, this region is located between amino acids 52 and 58, close to the first GTP-binding motif at 68–76, but it is not known if Sept3 binds to PtdIns lipids. Further studies will determine whether PKG phosphorylation of Sept3 regulates its association with phospholipids, with specific protein partners, its GTPase activity or its filament assembly.

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