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Phosphorylation Analysis of G Protein-coupled Receptor by Mass Spectrometry: Identification of a Novel Phosphorylation Site in V2 Vasopressin Receptor

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Abstract

Phosphorylation plays vital roles in the regulation and function of the V2 vasopressin receptor (V2R), a G protein-coupled receptor (GPCR) that is responsible for maintaining water homeostasis in the kidney. Through a combination of immunoaffinity purification, immobilized metal affinity chromatography and nanoflow liquid chromatography tandem mass spectrometry, we identified a novel phosphorylation site (Ser²⁵⁵) in the third intracellular loop of human V2R. We showed that the third intracellular loop could be phosphorylated *in vitro* by protein kinase A, but not by Akt kinase, although sequence motif analysis predicated otherwise. The analytical procedures and methodologies described in this study should be generally applicable for identifying the endogenous phosphorylation sites in other GPCRs, overcoming the limitations of conventional approaches such as sequence motif analysis and site-directed mutagenesis.

Keywords

G protein-coupled receptor; V2 vasopressin receptor; Receptor phosphorylation; Immunoaffinity purification; Mass spectrometry; Immobilized metal affinity chromatography

INTRODUCTION

Phosphorylation plays vital roles in the regulation and function of G protein-coupled receptors (GPCR), a large family of seven trans-membrane receptors that sense molecules outside the cell and activate inside signal transduction pathways. For example, phosphorylation is a crucial mechanism in turning off receptor signaling as first described for rhodopsin ¹. The phosphorylation that leads to receptor uncoupling from G proteins is catalyzed by GPCR kinases (GRKs) that recognize the receptor as substrate after agonist binding ². This phosphorylation often modifies the cytosolic C-terminal tail and leads to arrestin binding and receptor internalization ². Phosphorylation of GPCRs by protein kinase A (PKA) or protein kinase C (PKC) has also been reported ^{3–5}, the activation of these kinases is the consequence of signaling triggered by the receptor after ligand binding. Sequence motif analysis was previously used to predict the putative phosphorylation sites in GPCRs. In several instances, the predicted phosphorylation sites were confirmed by *in vitro* experiments.

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V2 vasopressin receptor (V2R) is a GPCR which is responsible for maintaining water homeostasis in the kidney. V2R has been shown to be subjected to homologous but not to heterologous desensitization mediated by PKA, similar to the β_2 adrenergic receptor ⁶. V2R is known to couple to G proteins and stimulate adenylyl cyclase activity, but contrary to what happens with other receptors, activation of PKA by the increased levels of cAMP did not change the coupling ability of the receptor ⁶. From sequence motif analysis, the human V2R does not contain a PKA canonical acceptor site. Previous studies, by a combination of deletion, site-directed mutagenesis and phosphorylation carried out in intact cells, identified nine possible sites clustered at the C-terminal tail (Ser³⁵⁷, Thr³⁵⁹, Thr³⁶⁰, Ser³⁶², Ser³⁶³, Ser³⁶⁴, Thr³⁶⁹, Ser³⁷⁰ and Ser³⁷¹) that seem to be phosphorylated by GRKs ^{7, 8}. Although the approaches employed in these studies have been useful for studying GPCR phosphorylation, there are major limitations. For example, mutagenesis may alter the endogenous phosphorylation states.

The mass spectrometry-based approach, with its high sensitivity and specificity, has been increasingly applied to the protein phosphorylation analysis ^{9–12}. Through tandem MS, the amino acid sequence and phosphorylation sites can be determined simultaneously and unequivocally. Moreover, the improved enrichment of the phosphopeptides by using immobilized metal affinity chromatography (IMAC) allowed the detection of the less abundant phosphorylation sites ^{9–12}. In this study, we applied immunoaffinity purification, IMAC and nanoflow liquid chromatography/tandem mass spectrometry to identify the V2R residues that are phosphorylated under arginine vasopressin (AVP) stimulation, and identified a novel PKA phosphorylation site in the third intracellular loop of V2R.

EXPERIMENTAL METHODS

Materials

DMEM, penicillin/streptomycin, 0.5% trypsin/5 mM EDTA and FBS were from Invitrogen (Carlsbad, CA). COMPLETE™ protease inhibitor cocktail was from Roche (Indianapolis, IN). AVP and 3-isobutyl-1-methylxanthine were from Sigma (St. Louis, MO). [³H]AVP (specific activity, 44 Ci/mmol), [α -³²P] ATP (specific activity, 3000 Ci/mmol) and [γ -³²P] ATP (specific activity, 3000 Ci/mmol) were from PerkinElmer (Wellesley, MA). [³H] cAMP (specific activity, 44 Ci/mmol) was from Amersham (Arlington Heights, IL). All other reagents were obtained from Sigma.

Cell culture

HEK 293 cells stably expressing HA-tagged V2R were grown in DMEM-high glucose, supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 mg/ml).

Immunoaffinity purification and trypsin digestion of HA-tagged V2R

Fifteen 15-cm dishes of stably transfected cells expressing the HA-tagged V2R were treated with 10 nM AVP at 37 °C for 20 min. The plates were washed twice with PBS and lysed for 20 min on ice with 1.5 ml/dish RIPA buffer containing protease inhibitors and phosphatase inhibitors. The cells were scraped off from the dishes and homogenized in a Dounce homogenizer with 20 strokes of a tight-fitting pestle followed by two sonication treatments of 30 sec each. The cell lysate was centrifuged in a 50-ml centrifuge tube at 1200 × g for 10 min to remove cellular debris. After transferring the supernatant to a new 50-ml centrifuge tube, the supernatant was mixed with 150 μ l of anti-mouse IgG beads for 4 hrs at 4 °C and then centrifuged at 1200 × g for 10 min. The supernatant was transferred to a fresh tube, mixed with 150 μ l anti-HA agarose beads, and incubated overnight at 4 °C. The beads were spun down at 1200 × g for 10 min and transferred to a 1.5-ml siliconized microcentrifuge tube. The beads

were then washed three times with 1 ml RIPA buffer with phosphatase inhibitors, followed by three washes with 1 ml wash buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40) with phosphatase inhibitors. After loading the beads into a Bio-Rad Poly-Prep column (ID: 0.8 cm, length: 4 cm), the bound proteins were eluted with 600 μ l of 0.1 M glycine-HCl, pH 2.5 into a 1.5-ml siliconized microcentrifuge tube containing 24 μ l of 1 M Tris-HCl pH 8.0 for neutralization.

The eluate (~ 600 μ l) was reduced with 4 μ l of 100 mM DTT at 57 °C for 1 hr and subsequently alkylated with 10 μ l of 100 mM iodoacetamide at room temperature for 45 min in the dark. After neutralization of the iodoacetamide by 20 μ l of 100 mM DTT for 1 hr at room temperature, 5 μ l of trypsin (200 μ g/ml) was added. After overnight digestion at 37 °C, the reaction was terminated by the addition of 10 μ l of glacial acetic acid.

Immobilized metal affinity chromatography and nanoflow liquid chromatography/tandem mass spectrometry

Immobilized metal affinity chromatography (IMAC) was performed according to Ficarro *et al.*⁹, with minor modifications. A 200- μ m OD $\times$ 100- μ m ID fused-silica column (Polymicro Technologies, Phoenix, AZ) was packed with 8 cm of POROS 20 MC (PerSeptive Biosystems, Framingham, MA). The column was activated with 100 mM FeCl₃ and rinsed with 0.1% acetic acid to remove excess metal ions and to equilibrate the column. About 50 μ l of the V2R tryptic digest (out of ~ 600 μ l total) was then loaded onto the IMAC column. To remove nonspecific binding peptides, the column was washed with a solution of NaCl (100 mM) in acetonitrile, water, and glacial acetic acid (25:74:1, v/v). The column was then equilibrated with 0.1% acetic acid. For MS analysis, the IMAC column was connected to a fused-silica column of 200- μ m OD $\times$ 100- μ m ID packed with 8 cm POROS 10 R2 (Applied Biosystems, Foster City, CA). All column connections were made using 1 cm of 0.012-in ID $\times$ 0.060-in OD Teflon tubing (Zeus, Orangeburg, SC). Phosphopeptides were eluted onto the reverse-phase column with 10 μ l of 200 mM Na₂HPO₄. The reverse-phase column was then disconnected from the IMAC column and rinsed with several column volumes of 0.1% acetic acid to remove Na₂HPO₄.

The tryptic phosphopeptides enriched by IMAC were analyzed by nanoflow LC/MS/MS using a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex, Foster City, CA). The MS/MS spectra were acquired in information-dependent acquisition (IDA) mode. The HPLC gradient consists of holding at 0% Solvent B for 3 min, followed by increasing to 15% Solvent B in 3 min, then to 55% Solvent B in 24 min. Solvent A consists of water/acetonitrile (98:2, v/v) with 0.1% acetic acid. Solvent B consists of acetonitrile/water (90:10, v/v) with 0.1% acetic acid. A flow rate of ~ 200 nl/min was used to elute the peptides from the reverse-phase column into the ionization source of the mass spectrometer. Mass spectra were acquired in the Information-Dependent-Acquisition (IDA) mode. Collision-induced dissociation mass spectra were searched against the SWISS-PROT protein database by using the ProID algorithm (ABI/MDS-Sciex).

Expression and purification of GST-3rd intracellular loop of V2R and in vitro AKT and PKA kinase assays

A segment of human V2R encoding amino acid residues 232–271 was synthesized via PCR and sub-cloned into pGEX-4T. The GST fusion protein was purified to homogeneity by affinity chromatography using glutathione-agarose beads (Sigma, St. Louis, MO) as described by Smith and Johnson¹³. The purified fusion proteins (GST or GST-3rd intracellular loop of V2R) were dialyzed extensively against PBS.

About 1 μ g GST or GST-3rd intracellular loop of V2R proteins was incubated at 30 °C for 20 min in 25 μ l of kinase buffer (pH 7.5) containing 25 mM Tris-HCl, 10 mM MgCl₂, 2 mM

dithiothreitol, 5 mM β -glycerophosphate, 0.1 mM Na_3VO_4 , 50 μM ATP, 10 μCi of $[\gamma\text{-}^{32}\text{P}]$ ATP and 50 ng of either protein kinase A catalytic subunit β (Sigma, St. Louis, MO) or GST-Akt fusion protein from Cell Signaling Technology, Inc. (Danvers, MA). The kinase activity of PKA was tested using Kemptide as a substrate, and the kinase activity of Akt was tested using GSK3 (14–27) peptide as a substrate. The reactions were then stopped by adding 6.5 μl of $5 \times$ SDS gel loading buffer. Proteins were subjected to the analysis by SDS-PAGE, Coomassie blue staining and autoradiography.

RESULTS AND DISCUSSION

To identify the human V2R residues that are phosphorylated under arginine vasopressin (AVP) stimulation, we treated the stably transfected HEK293 cells with 10 nM AVP for 20 minutes. Purification of human V2R was accomplished by immunoprecipitation of HA-hV2R using a monoclonal anti-HA agarose conjugated antibody. Following reduction and alkylation as described in Experimental Section, the purified receptors were digested with trypsin overnight. The resulting tryptic phosphopeptides were enriched by immobilized metal affinity chromatography (IMAC)^{9, 14, 15}, followed by the analysis using nanoflow reverse-phase HPLC coupled with a high-resolution tandem mass spectrometer (Q-STAR XL). Tandem MS spectra were searched against the SWISS-PROT database using the ProID algorithm, yielding the identification of a tryptic phosphopeptide ($^{253}\text{TGpSPGEGAHVSAAVAK}^{268}$) derived from the human V2R. Fig. 1 shows the MS/MS spectrum of the triply charged ion ($[\text{M} + 3\text{H}]^{3+}$) of this phosphopeptide. The assignment of phosphorylation site at Ser²⁵⁵ is further verified by both N- and C-terminal product ions (b_2 , b_3 , y_{13} , y_{14} and y_{15}) containing Ser²⁵⁵. All sequence-informative product ions are listed in Table 1. This phosphopeptide could only be detected when cells were treated with AVP, suggesting Ser²⁵⁵ phosphorylation is ligand-dependent event. We didn't detect any other V2R phosphopeptides, including the putative phosphopeptides from the C-terminal tail. It is possible that the phosphopeptides derived from the C-terminal tail of V2R are hyper-phosphorylated, and are too hydrophilic to be retained on the reverse-phase matrices⁹. This is a limitation that still needs to be addressed.

Sequence analysis revealed that Ser²⁵⁵ is conserved in the V2R orthologs of mouse, rat, cattle, dog, pig and horse. To predict which kinase could phosphorylate Ser²⁵⁵, we examined the full-length receptor protein using Motif Scan software from MIT (http://scansite.mit.edu/motifscan_seq.phtml). Under medium-stringency the software predicted Akt as a potential kinase for Ser²⁵⁵, whereas under low-stringency the software added PKA as an additional kinase for Ser²⁵⁵. The kinase activities of Akt and PKA towards Ser²⁵⁵ were measured using an *in vitro* kinase assay with the purified GST-3rd intracellular loop of V2R as a substrate. As shown in Fig. 2, the GST-3rd intracellular loop of V2R was phosphorylated by PKA (lane 5) but not by Akt (lane 2). Although the Gs/adenylyl cyclase signaling pathway makes PKA a likely kinase for V2R, this is the first time that a PKA phosphorylation site has been identified in V2R. β_2 adrenergic receptor ($\beta_2\text{AR}$) has been shown to be phosphorylated by PKA at a canonical PKA site (RRSS) in the third intracellular loop¹⁶. However, the same canonical PKA site (RRSS) does not exist in V2R. According to Kennelly and Krebs¹⁷, Ser²⁵⁵ is a non-canonical PKA phosphorylation site (RRXXS).

All previously proposed phosphorylation sites in V2R are located within the C-terminal tail, and have been shown to inhibit receptor recycling upon ligand removal^{8, 18}. Mutagenesis of single serines or threonines in the C-terminal tail reduced phosphorylation to different degrees and had variable effects on V2R recycling^{8, 19}. Ser²⁵⁵ represents the first phosphorylation site identified in the 3rd intracellular loop of V2R (Figure 3). Functional studies of Ser²⁵⁵ phosphorylation may include the evaluation of its effects on receptor folding, ligand-binding, internalization and recycling^{20–23}. Given the recent crystal structure of β_2 adrenergic receptor

lacks the native 3rd intracellular loop (modified for crystallization purpose)²⁴, the structure and function of the 3rd intracellular loop of GPCRs may deserve more research attentions.

In conclusion, we carried out endogenous phosphorylation analysis of GPCR through immunoaffinity purification, immobilized metal affinity chromatography and nanoflow LC/MS/MS, and identified a novel, AVP-dependent phosphorylation site (Ser²⁵⁵) in the third intracellular loop of V2R. We demonstrated that Ser²⁵⁵ could be phosphorylated by PKA *in vitro*. The analytical strategies demonstrated in this study should be generally applicable to the identification of novel phosphorylation sites in other GPCRs, overcoming the limitations of conventional approaches such as sequence motif analysis and site-directed mutagenesis.

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REFERENCES

1. Hurley JB, Spencer M, Niemi GA. Vision Res 1998;38:1341–1352. [PubMed: 9667002]
2. Pitcher JA, Freedman NJ, Lefkowitz RJ. Annu. Rev. Biochem 1998;67:653–692. [PubMed: 9759500]
3. Innamorati G, Sadeghi H, Birnbaumer M. J. Biol. Chem 1998;273:7155–7161. [PubMed: 9507030]
4. Olivares-Reyes JA, Jayadev S, Hunyady L, Catt KJ, Smith RD. Mol. Pharmacol 2000;58:1156–1161. [PubMed: 11040065]
5. Oppermann M, Freedman NJ, Alexander RW, Lefkowitz RJ. J. Biol. Chem 1996;271:13266–13272. [PubMed: 8662816]
6. Birnbaumer M, Antaramian A, Themmen AP, Gilbert S. J. Biol. Chem 1992;267:11783–11788. [PubMed: 1376312]
7. Innamorati G, Sadeghi H, Eberle AN, Birnbaumer M. J. Biol. Chem 1997;272:2486–2492. [PubMed: 8999963]
8. Innamorati G, Sadeghi HM, Tran NT, Birnbaumer M. Proc. Natl. Acad. Sci. U. S. A 1998;95:2222–2226. [PubMed: 9482866]
9. Ficarro SB, McClelland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM. Nat. Biotechnol 2002;20:301–305. [PubMed: 11875433]
10. Garcia BA, Shabanowitz J, Hunt DF. Methods 2005;35:256–264. [PubMed: 15722222]
11. Cantin GT, Shock TR, Park SK, Madhani HD, Yates JR 3rd. Anal. Chem 2007;79:4666–4673. [PubMed: 17523591]
12. Cantin GT, Yi W, Lu B, Park SK, Xu T, Lee JD, Yates JR 3rd. J Proteome Res 2008;7:1346–1351. [PubMed: 18220336]
13. Smith DB, Johnson KS. Gene 1988;67:31–40. [PubMed: 3047011]
14. Andersson L, Porath J. Anal. Biochem 1986;154:250–254. [PubMed: 3085541]
15. Muszynska G, Andersson L, Porath J. Biochemistry (Mosc) 1986;25:6850–6853.
16. Hausdorff WP, Bouvier M, O'Dowd BF, Irons GP, Caron MG, Lefkowitz RJ. J. Biol. Chem 1989;264:12657–12665. [PubMed: 2545714]
17. Kennelly PJ, Krebs EG. J. Biol. Chem 1991;266:15555–15558. [PubMed: 1651913]
18. Le Gouill C, Innamorati G, Birnbaumer M. FEBS Letters 2002;532:363–366. [PubMed: 12482593]
19. Innamorati G, Le Gouill C, Balamotis M, Birnbaumer M. J. Biol. Chem 2001;276:13096–13103. [PubMed: 11150299]
20. Birnbaumer M, Gilbert S, Rosenthal W. Mol. Endocrinol 1994;8:886–894. [PubMed: 7984150]
21. Erlenbach I, Wess J. J. Biol. Chem 1998;273:26549–26558. [PubMed: 9756892]

22. Gouill CL, Darden T, Madziva MT, Birnbaumer M. FEBS Letters 2005;579:4985–4990. [PubMed: 16115624]
23. Tan CM, Brady AE, Nickols HH, Wang Q, Limbird LE. Annu. Rev. Pharmacol. Toxicol 2004;44:559–609. [PubMed: 14744258]
24. Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI, Kobilka BK. Nature 2007;450:383–387. [PubMed: 17952055]

Abbreviations

V2R, V2 vasopressin receptor; AVP, arginine vasopressin; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; β_2 AR, β_2 adrenergic receptor; PKA, protein kinase A; GST, glutathione S-transferase; MS, mass spectrometry; IMAC, immobilized metal affinity chromatography; LC, liquid chromatography.

Fig. 1. MS/MS identification of a tryptic phosphopeptide of V2R

The phosphopeptide is derived from the trypsin digestion of the immunoaffinity-purified V2R, and identified by searching its MS/MS spectra ($[M+3H]^{3+}$ at m/z 506.9 is shown in the inset) against the SWISS-PROT database using the ProID software. The amino acid sequence of the phosphopeptide is $^{253}\text{TGpSPGEGAHVSAAVAK}^{268}$ (pS denotes a phosphorylated serine). The underlined are the observed b/y sequence ions (or their corresponding H_3PO_4 -loss ions) that are predicted from the amino acid sequence (Top).

Fig. 2. Phosphorylation of the GST-3rd intracellular loop of V2R

Equal amounts (~1 µg) of GST (*lanes 1 and 4*) or GST-3rd intracellular loop of V2R (*lanes 2 and 5*) were incubated with either Akt (*lanes 1 and 2*) or PKAcβ (*lanes 4 and 5*) in the presence of [γ -³²P] ATP. *Lanes 3 and 6* show the autophosphorylation of Akt and PKA, respectively.

Fig. 3. Schematic diagram of V2R

The Ser²⁵⁵-containing phosphopeptide is located in the third intracellular loop.

Table 1

MS/MS product ions of the $[M+3H]^{3+}$ ion of a V2R tryptic phosphopeptide, $^{253}\text{TGpSPGEGAHVSAAVAK}^{268}$. These product ions are identified by database searching using the ProID algorithm, and verified by manual examination.

	Residue	Immonium ion	a	b	y	y-NH ₃
1	T	74.1	74.0	102.6		
2	G		131.1	159.1	1417.6	
3	pS	140.0	298.1	326.1	1360.6	
4	P	70.1	395.1		1193.6	
5	G				1096.6	
6	E	102.1				
7	G					
8	A		709.3			
9	H	110.1			782.5	
10	V	72.1			645.4	
11	S				546.3	
12	A				459.3	
13	A				388.3	371.2
14	V	72.1			317.2	
15	A				218.1	201.1
16	K	101.1			147.1	130.1