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Mutational analysis of sites in sepiapterin reductase phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II

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Abstract

Sepiapterin reductase (SPR) catalyzes the last step in the pathway of tetrahydrobiopterin biosynthesis in tissues. SPR is phosphorylated by Ca^{2+}-dependent protein kinases, which indicates that Ca^{2+}-activated protein kinases may play a role in the regulation of SPR in vivo. Phosphorylation sites of rat sepiapterin reductase (rSPR) by Ca^{2+}/calmodulin-dependent protein kinase II were determined in the present study. Using specific monoclonal anti-phospho-Ser and -Thr antibodies, we found that only Ser residues of rSPR were phosphorylated. We constructed several point mutants of SPR by systematically replacing the three Ser residues by Ala ones. These mutants showed that all three Ser residues, i.e. S46, S196, and S214, of rSPR were phosphorylated. We also recognized that only Ser-213 of human SPR was phosphorylated. Each of these serine residues in SPR was found in the consensus sequence (Arg-X-X-Ser/Thr) of the phosphorylation site. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Sepiapterin reductase; Ca^{2+}/calmodulin-dependent protein kinase II; Phosphorylation site

1. Introduction

Sepiapterin reductase (SPR) catalyzes the last step of the biosynthetic pathway of tetrahydrobiopterin (BH4) [1]. BH4 is an essential cofactor for tyrosine hydroxylase and tryptophan hydroxylase, which are the rate-limiting steps in the biosynthesis of monoamine neurotransmitters in the central nervous system, and for phenylalanine hydroxylase, which catalyzes the formation of tyrosine in the liver [2]. Abnormality in the metabolism of BH4 results in neurological diseases such as atypical phenylketonuria (hyperphenylalaninemia) [3]. BH4 is biosynthesized from GTP by three enzymes including SPR, but only in SPR no inherited abnormality had been found. Most recently, however, an autosomal recessive SPR deficiency in monoamine neurotransmitter deficiencies was reported [4]. BH4 also acts as an activator for all three nitric oxide synthase (NOS) isoforms [5,6]. Furthermore, BH4 plays a regulatory role in human skin melanogenesis by forming a stable complex with the α-melanocyte stimulating hormone (α-MSH) [7,8].

Ca^{2+}/calmodulin-dependent protein kinase II (CaM KII) is a ubiquitous enzyme mediating responses to changes in the intracellular concentration

Abbreviations: SPR, sepiapterin reductase; rSPR, rat SPR; hSPR, human SPR; BH4, 5,6,7,8-tetrahydrobiopterin; CaM KII, Ca^{2+}/calmodulin-dependent protein kinase II; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PVDF, polyvinylidene fluoride

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of calcium ions [9,10]. CaM KII is the most abundant protein kinase in the brain and is particularly abundant at synapses, where it plays a crucial role in the regulation of synaptic transmission [11,12]. As previously reported, phenylalanine hydroxylase, which catalyzes the conversion of phenylalanine to tyrosine in the presence of BH4, is phosphorylated by CaM KII [13]. As well as phenylalanine hydroxylase, tyrosine hydroxylase, a key enzyme in catecholamine biosynthesis, is phosphorylated by various protein kinases including CaM KII [14]. Furthermore, tryptophan hydroxylase, the rate-limiting enzyme in the biosynthesis of serotonin, is also phosphorylated by CaM KII [15]. The enzyme activity or the interaction of stimulatory proteins with the above hydroxylases is controlled by CaM KII-dependent phosphorylation [16–18]. Therefore, it is not surprising that the biosynthesis of neurotransmitters is regulated by calcium ions through phosphorylation by multifunctional Ca^{2+}-activated protein kinases such as CaM KII.

Recently, we reported that SPR, the key enzyme of BH4 production, is phosphorylated by CaM KII [19] and that it becomes more sensitive to Ca^{2+}-activated protease (calpain) than the non-phosphorylated form of SPR [20]. These findings suggest that SPR may be in control of the catalysis by BH4-requiring enzymes by regulating BH4 biosynthesis via CaM KII-dependent phosphorylation of itself. In this study, we constructed various point mutants of SPR by site-directed mutagenesis, determined the sites on the SPR subunit phosphorylated by CaM KII, and then measured the kinetic parameters of the point mutant SPRs phosphorylated/non-phosphorylated by CaM KII with respect to sepiapterin and NADPH.

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP (4000 Ci/mmol) was obtained from ICN (USA). Protein kinase C (PKC), cAMP-dependent protein kinase (PKA), CaM KII, cGMP-dependent protein kinase (PKG), MAP kinase, tyrosine kinase, casein kinase II, calmodulin, endoprotease Lys-C, ATP, and monoclonal anti-phospho-Ser/Thr/Tyr antibody were obtained from Sigma (USA). The Sephasil protein column used was purchased from Pharmacia Biotech (Sweden).

2.2. Cloning and expression of rat (r) SPR and human (h) SPR

Clones encoding rSPR and hSPR were amplified by the polymerase chain reaction (PCR). Amplified rSPR and hSPR cDNAs were ligated into pTrxFus and pET16b expression vectors, respectively, and expressed in *Escherichia coli*. Expressed proteins were purified by ammonium sulfate fractionation and affinity chromatography [21].

2.3. Phosphorylation experiment

Phosphorylation of 50 μg of wild-type or mutated SPRs by protein kinases was conducted at 25°C for 60 min in a final volume of 100 μl. The reaction with CaM KII was performed in a reaction mixture containing 50 mM Tris–HCl buffer (pH 7.5), 0.5 μg CaM KII, 0.5 μM calmodulin, 100 μM ATP, 2 mM magnesium acetate, and 0.15 mM $CaCl_2$. Phosphorylations by PKC, PKA, and PKG were performed as described by Katoh et al. [19]. Phosphorylation by MAP kinase was performed in 50 mM HEPES (pH 7.5), 1 mM dithiothreitol, 100 μM ATP, and 5 mM magnesium chloride. Phosphorylation by tyrosine kinase was performed in 50 mM Tris–HCl (pH 7.4), containing 100 μM ATP and 3 mM manganese chloride, and that by casein kinase II, in 50 mM Tris–HCl (pH 7.5) with 100 μM ATP.

2.4. Immunoblotting with anti-phospho-amino acid antibody

SDS–PAGE of the subunit SPR on a 12.5% gel was performed according to Laemmli [22], and proteins on the gel were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was rinsed with 50 mM Tris–HCl (pH 7.5) and 150 mM NaCl (A buffer) and blocked overnight with A buffer containing 3% skim milk. The membrane was incubated for 2 h with a diluted solution of the primary antibody (biotin-labeled monoclonal anti-phospho-Ser/Thr/Tyr antibody) made with A buffer containing 3% bovine serum albumin and then washed

with A buffer containing 1% Triton X-100 two times (10 min each). The membrane was then incubated with a solution of streptavidin–biotin-labeled peroxidase complex (Wako, Japan) for 20 min and washed with A buffer three times (10 min each). For visualization of the immunoreactive proteins, the membrane was incubated with Luminol luminescence solution (Wako). The excited membrane in the enhanced chemiluminescence reaction mixture was exposed to X-ray film. Quantification of the immunoreactivity corresponding to the phospho-SPR bands was done by densitometry, using NIH image software.

2.5. Detection of ^{32}P in phosphorylated amino acids

Native rSPR (0.5 mg), purified from rat erythrocytes [23], was incubated for 10 min at 25°C in a reaction mixture containing 50 mM Tris–HCl buffer (pH 7.5), 0.5 μg CaM KII, 0.5 μM calmodulin, 100 μM [γ-^{32}P]ATP (3000 cpm/pmol), 2 mM magnesium acetate, and 0.15 mM $CaCl_2$. After SDS–PAGE of the reaction mixture as described by Laemmli [22], the radioactive band was cut out from the gel and hydrolyzed in 6 N HCl at 110°C for 3 h. Amino acids were separated by thin-layer chromatography (TLC). The excited plate was exposed to X-ray film and analyzed by densitometry.

2.6. Fractionation by protease

Purified recombinant wild-type rSPR (0.3 mg of protein) was phosphorylated by CaM KII and digested with endoprotease Lys-C at 37°C for 12 h. Fragments were separated by reverse-phase HPLC using a Sephasil protein column (4.6×250 mm) and elution with a linear gradient of 0–72% acetonitrile in 0.06% trifluoroacetic acid at a flow rate of 1 ml/min under detection at 215 nm. Amino acid sequences of the purified fragments were determined with a PPSQ-21 gas-phase protein sequencer (Shimadzu, Japan) with the chemicals and program supplied by the manufacturer.

2.7. Construction of point mutants of rSPR

Point mutants were constructed using a Quik-Change site-directed mutagenesis kit (Stratagene, CA, USA). The mutagenic sense primers were 5′-GACGCGATGCTGCGGCAACTGAAGGAGGA-ACTCTG-3′ for changing S46 to A, 5′-GGGTGC-TGGCCTATGCCCCAGGGCCCCTGGACACC-3′ for changing S196 to A, and 5′-GCAGCAGTTGG CCCGAGAAACCGCCATGGACCCAG-3′ for changing S214 to A. Antisense primers were prepared as the same sequences on the opposite strands of the plasmid. The double point mutants (S46-A196-A214, A46-S196-A214, A46-A196-S214) and triple mutant (A46-A196-A214) were constructed by performing PCR two and three times, respectively. The point mutant of hSPR (S213 change to A) was constructed using primer 5′-CCGGGAGACCGCC-GTGGACC-3′. The DNA sequences of all mutants were checked by the dideoxynucleotide chain-termination method with a dye terminator sequencing kit (Applied Biosystems, USA). Expressed mutated rSPR proteins in *E. coli* were purified by ammonium sulfate fractionation and affinity chromatography [21] and checked by Western blotting with rSPR antibody.

2.8. SPR assay and kinetic studies

SPR activity was photometrically measured by the reduction of sepiapterin [24]. Steady-state kinetic experiments with SPR were performed as described previously [23]. The standard assay mixture contained 100 mM sodium phosphate buffer, pH 6.4, containing 100 nmol sepiapterin and 200 nmol NADPH. The reaction was initiated by the addition of enzyme, and activity was monitored by the change in absorbance caused by the reduction of sepiapterin at 420 nm (Hitachi Spectrophotometer U-3210). Assays were performed at 25°C.

2.9. Protein determination

Protein concentration was determined by measuring the absorbance at 280 nm, with bovine serum albumin as a standard (Hitachi Spectrophotometer U-3210).

3. Results and discussion

Purified recombinant wild-type rSPR was incu-

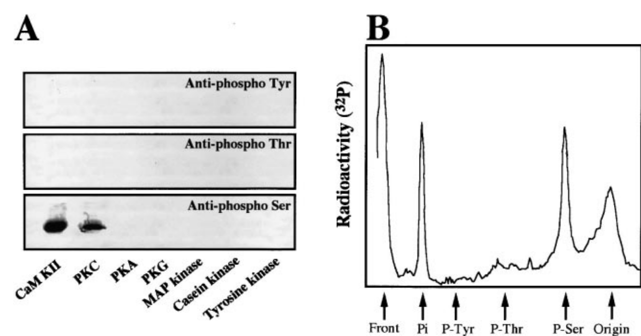

Fig. 1. Amino acid residue of rSPR phosphorylated by CaM KII. (A) Wild-type rSPR (50 μg) was incubated in the phosphorylating systems of several protein kinases (final volume of 100 μl). Phosphorylated amino acids were detected using monoclonal anti-phospho-Ser, -Thr, and -Tyr antibodies. (B) Native rSPR (500 μg) was added to a reaction mixture containing [γ-^{32}P]ATP. Purified radioactive SPR separated by SDS–PAGE was then hydrolyzed in HCl, and the amino acids were separated by TLC. The excited plate was exposed to X-ray film, after which the film was analyzed by densitometry.

bated in the phosphorylating systems of CaM KII, PKC, PKA, PKG, MAP kinase, casein kinase II, or tyrosine kinase. After SDS–PAGE, phosphorylated amino acids were detected using monoclonal anti-phospho-Ser, -Thr, and -Tyr antibodies (Fig. 1A). The recombinant rSPR was specifically phosphorylated by CaM KII and PKC only (Fig. 1A), as was previously reported by Katoh et al. [19] for the native SPR purified from rat erythrocytes. Although CaM KII is a Ser/Thr protein kinase, Ser was the only type of residue of recombinant rSPR phosphorylated by CaM KII (Fig. 1A). When the native SPR purified from rat erythrocytes was incubated in the phosphorylating systems of CaM KII in the presence of [γ-^{32}P]ATP and then hydrolyzed in HCl, radioactivity was certainly detected in phospho-Ser by TLC (Fig. 1B). Therefore, in subsequent experiments the phosphorylation sites of recombinants and mutants of rSPR were detected by immunoblotting with the anti-phospho-Ser antibody.

To determine the critical sites of phosphorylation in rSPR by CaM KII, we cleaved maximally phosphorylated rSPR with endoprotease Lys-C, and separated the resulting peptide fragments by HPLC. The elution profile is shown in Fig. 2A. Each peptide was collected and detected by dot blot analysis with monoclonal anti-phospho-Ser antibody. The phosphorylated peptides, SPRP1 and SPRP2, were obtained from the column after 36 min (∼40% acetonitrile) and 45 min (∼50% acetonitrile), respectively. These peptides were applied to the gas-phase protein sequencer. The N-terminal sequences of the peptides, Val-Pro-Met-Glu-Gly-Gly- (SPRP1) and Ala-Ala-Arg-Asp-Met-Leu-Tyr-Gln- (SPRP2), were obtained and conformed to the amino acid sequence of rSPR (Fig. 2B, double underlines). The consensus sequences of the phosphorylation motifs (X-Arg-X-X-Ser*/Thr* or X-Arg-X-X-Ser*/Thr*Val, asterisks indicating the phosphorylated residue) specifically recognized by CaM KII have been reported [25,26]. rSPR [27,28] also has three identical motifs (X-Arg-X-X-Ser/Thr; Fig. 2B, underlines), and these fit the amino acid sequence of the phosphorylated peptides (SPRP1 and SPRP2) perfectly, thus suggesting that these three Ser residues (S46, S196, and S214) are phosphorylated by CaM KII.

Table 1
Kinetic parameters of phosphorylated and non-phosphorylated SPRs

	Sepiapterin			NADPH		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
Non-phosphorylated rSPR (wild-type)	12.6	9.7	0.8	2.8	11.1	4.0
Phosphorylated rSPR (wild-type)	11.5	8.5	0.7	2.7	10.7	4.0
Non-phosphorylated rSPR (A46A196A214)	12.2	8.9	0.7	2.5	10.5	4.2
Non-phosphorylated hSPR (wild-type)	14.3	1.1	0.08	10.0	1.1	0.1
Phosphorylated hSPR (wild-type)	13.7	1.3	0.09	12.3	1.5	0.1
Non-phosphorylated hSPR (S213→A)	13.5	1.3	0.09	11.5	1.3	0.1

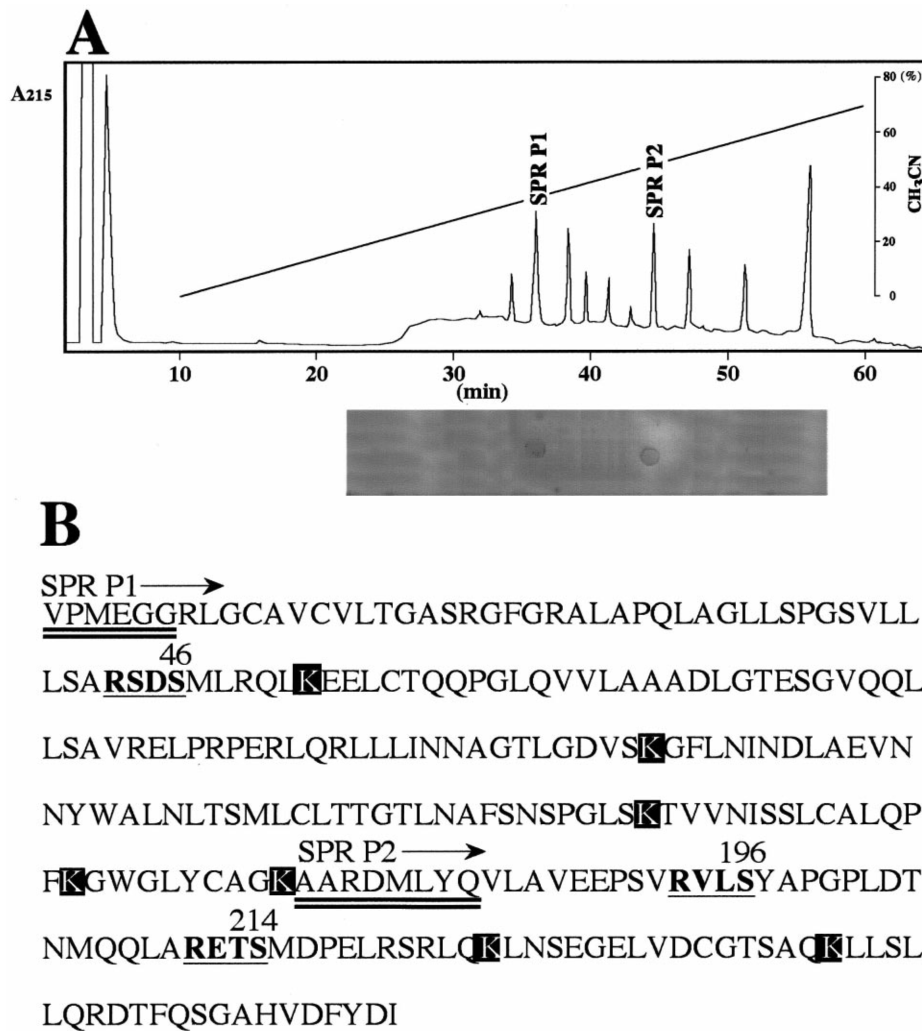

Fig. 2. Peptide mapping and localization of phosphorylation site motifs of rSPR. (A) Wild-type rSPR was phosphorylated by CaM KII. Phosphorylated rSPR (0.3 mg of protein) was digested with endoprotease Lys-C, and the peptide fragments were separated by reverse-phase HPLC. Peptides were eluted by a linear gradient of 0–72% acetonitrile, and each peptide was collected and detected by dot blotting with monoclonal anti-phospho-Ser antibody. (B) Primary structure of rSPR. Double underlines indicate N-terminal amino acid sequences of SPRP1 and SPRP2, underlines indicate CaM KII phosphorylation motif (Arg-X-X-Ser/Thr), and closed boxes indicate lysine residues.

To prove that these Ser residues were phosphorylated by CaM KII, we prepared mutant rSPRs (S46-A196-A214, A46-S196-A214, A46-A196-S214, and A46-A196-A214), in which two or three Ser residues were changed to Ala, by site-directed mutagenesis, expressed the mutant rSPRs in *E. coli*, and purified them by ammonium sulfate fractionation and affinity chromatography. Purified mutant rSPRs were incubated in the phosphorylating system of CaM KII. After SDS–PAGE, the proteins were electroblotted onto a PVDF membrane, and phosphorylated protein was detected by monoclonal anti-phospho-Ser antibody (Fig. 3A). The mutants in which one Ser residue remained (S46-A196-A214, A46-S196-A214, and A46-A196-S214) were phosphorylated by CaM KII, but the mutant in which all three Ser had been changed to Ala (A46-A196-A214) was not phosphorylated at all. This result shows that each of the three Ser residues was phosphorylated by CaM KII. Also, we examined the phosphorylation site of hSPR. The primary structure of hSPR as deduced from its cDNA sequence [29] contains only one CaM KII

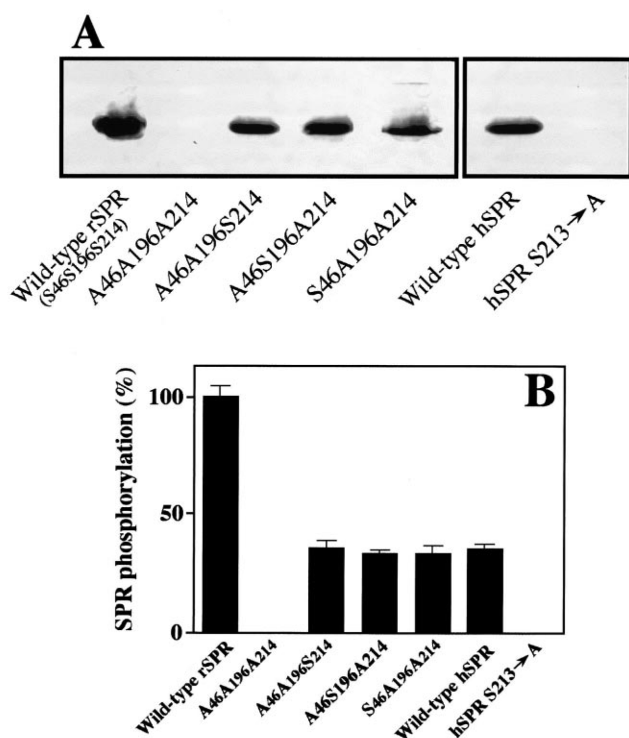

Fig. 3. Phosphorylation of wild-type and mutant rSPRs by CaM KII. Wild-type and mutant rSPRs were phosphorylated by CaM KII. After SDS–PAGE, proteins were electroblotted onto a PVDF membrane. (A) Immunoblot with monoclonal anti-phospho-Ser antibody. (B) Quantification of SPR phosphorylation. Data are means ± S.D. of three experiments.

phosphorylation motif (Arg-X-X-Ser/Thr), which involves S213. So we constructed a point mutant hSPR in which S213 was changed to Ala by site-directed mutagenesis. This mutant of hSPR was not phosphorylated by CaM KII (Fig. 3A), indicating that S213 of hSPR was phosphorylated by CaM KII. Phosphorylation levels of SPR mutants reacted with CaM KII are shown in Fig. 3B. Phosphorylation levels of double point mutants (S46-A196-A214, A46-S196-A214, and A46-A196-S214) were nearly one-third of the level for the wild-type rSPR. The level of phosphorylation of the wild-type hSPR phosphorylation was also about 30% of that of the wild-type rSPR. It was earlier reported that the maximal incorporation of phosphate into rSPR and hSPR subunits by CaM KII was 3 and 1 ^{32}P mole/mole enzyme subunit, respectively [19,30,31]. From these reports and the results of this study, we can conclude that the phosphorylation sites recognized by CaM KII are S46, S196, and S214 of rSPR, and S213 of hSPR.

The effect of phosphorylation of these Ser residues on the enzyme activity was also examined. Mutant or wild-type SPR was incubated with CaM KII under the conditions in which SPR was maximally phosphorylated [19], and then the kinetic parameters of SPR were measured with respect to sepiapterin and NADPH (Table 1). The non-phosphorylated mutants of rSPR (A46-A196-A214) and hSPR (S213 changed to Ala) showed values of K_m and k_{cat} for sepiapterin and NADPH similar to those of the non-phosphorylated and phosphorylated wild-types of rSPR and hSPR, respectively. These results for rSPR are quite similar to those reported earlier by Katoh et al. [19]. As for other BH4-generating enzymes, GTP cyclohydrolase I was phosphorylated by casein kinase II and PKC, and phosphorylated GTP cyclohydrolase I showed increased activity compared with the non-phosphorylated enzyme [32]. Also, 6-pyruvoyltetrahydropterin synthase was phosphorylated by PKG, and a mutant of this enzyme (S19A) showed only about 30% of the activity of the wild-type when both recombinants were expressed in COS-1 cells [33]. BH4-requiring enzymes such as phenylalanine hydroxylase and tryptophan hydroxylase are regulated in their activities by phosphorylation by CaM KII [16,18]. Tyrosine hydroxylase, however, does not undergo a change in its activity when phosphorylated by CaM KII [17], as was seen in the phosphorylation of SPR in this study (Table 1). On the other hand, it is quite interesting that tyrosine hydroxylase, which is once phosphorylated at Ser 19 by CaM KII, can bind 14-3-3 protein and show high activity [17]. Also, it was observed that SPR phosphorylated by CaM KII becomes markedly sensitive to calpain, a Ca^{2+}-activated protease [20,30]. This finding suggests that phosphorylation of SPR by Ca^{2+}-dependent protein kinase may affect the susceptibility of SPR to Ca^{2+}-activated proteases or other functions rather than the activity of this enzyme.

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