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Phosphorylation of *smg* p21B/*rap*1B p21 by cyclic GMP-dependent protein kinase

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smg p21B/*rap*1B p21, a member of *ras* p21-like small GTP-binding protein superfamily, has been shown to be phosphorylated by cyclic AMP-dependent protein kinase (protein kinase A). We show here that this protein was also phosphorylated by cyclic GMP-dependent protein kinase (protein kinase G) in a cell-free system. The same serine residue (Ser^{179}) in the C-terminal region was phosphorylated by both protein kinases G and A. The K_m and V_{max} values of *smg* p21B for protein kinase G were 5×10^{-7} M and 4×10^{-9} mol/min/mg, and those values for protein kinase A were 1×10^{-7} M and 3×10^{-8} mol/min/mg.

ras p21; *smg* p21/*rap*1 p21; GDP/GTP exchange protein; Protein kinase

1. INTRODUCTION

cGMP is known to be of crucial importance in signal transduction, particularly in retinal rod, smooth muscle, and cerebellar Purkinje cells. In rod, cGMP opens the Na^+ channel by directly binding to this channel [1]. In smooth muscle, Ca^{2+} causes muscle contraction, whereas cGMP or cAMP causes muscle relaxation (for a review, see [2]). In Purkinje cells, cGMP may be related to long-term depression [3,4]. The mode of action of cAMP has been well established, and its action is mediated by protein kinase A (for a review, see [5]). The mode of action of cGMP in smooth muscle and Purkinje cells has not been defined, but by analogy with cAMP, it is possible that the cGMP action is mediated by protein kinase G.

In smooth muscle, the vasoconstrictors induce the activation of the phosphoinositide-specific phospholipase C which leads to vasoconstriction through inositol-1,4,5-trisphosphate/Ca^{2+}-calmodulin-dependent myosin light chain kinase system (for a review, see [6]). This receptor-linked phospholipase C activation is antagonized by both cGMP and cAMP (for a review, see [7]). Moreover, cAMP inhibits myosin light chain kinase (for a review, see [8]) and lowers the cytosolic Ca^{2+} level by increasing uptake of this ion by the intracellular calcium store [9]. However, the direct substrate proteins for protein kinases G and A remain to be identified.

The *smg*/*rap*1 p21 family, which is one of the major small G protein families in smooth muscle, is directly phosphorylated by protein kinase A [10–14]. The *smg*/*rap*1 p21 family consists of two members, *smg* p21A/*rap*1A p21/K*rev*-1 p21 and *smg* p21B/*rap*1B p21, and belongs to the *ras* p21-related small G protein superfamily (for reviews, see [15–17]). Protein kinase A phosphorylates *smg* p21B at Ser^{179} [13] and *smg* p21A at Ser^{180} [14], which are located between the polybasic region and the geranyl-geranylated cysteine residue in the C-terminal region. This C-terminal region is essential for *smg* p21 to bind to membranes and to interact with the stimulatory GDP/GTP exchange protein named *smg* GDS [18–20]. The protein kinase A-catalyzed phosphorylation of *smg* p21 initiates the *smg* GDS-induced activation [13,21]. It has been shown that *rap*1B p21 is also phosphorylated by cerebellar granular calmodulin-dependent protein kinase, but the site of this phosphorylation remains to be identified [22].

In the present study, we examined whether *smg* p21B is phosphorylated by protein kinase G. This paper reports that protein kinase G as well as protein kinase A phosphorylates *smg* p21B at the same site in the C-terminal region of the protein.

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Abbreviations: protein kinase A, cyclic AMP-dependent protein kinase; protein kinase G, cyclic GMP-dependent protein kinase; GDS, GDP dissociation stimulator; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

Protein kinase G was purified to homogeneity from bovine lung as

described [23]. The catalytic subunit of protein kinase A was purified to near homogeneity from rabbit skeletal muscle as described [24] with slight modifications. *smg* p21B was purified to near homogeneity from human platelet membranes as described [25]. cGMP, cAMP, and ATP were obtained from Yamasa Syoyu Corp. (Chiba, Japan). [γ-^{32}P]ATP (370 MBq/ml) was obtained from Amersham. L-1-Tosylamide-2-phenyl-ethyl-chloromethyl ketone-treated trypsin and soybean trypsin inhibitor were obtained from Sigma. Other materials and chemicals were obtained from commercial sources.

2.2. *Phosphorylation of* smg *p21B by protein kinase G*

smg p21B was incubated with protein kinase G for the indicated periods of time at 30°C in a reaction mixture (50 μl) containing 20 mM Tris-HCl at pH 7.5, 100 μM [γ^{32}-P]ATP (2,000–5,000 cpm/pmol), 10 mM Mg-acetate, and 10 μM cGMP. The reaction was stopped by the addition of an SDS-stopping solution (25 μl) containing 0.2 M Tris-HCl at pH 6.7, 9% SDS, 6% 2-mercaptoethanol, and 15% glycerol. The sample was boiled and subjected to SDS-PAGE using 14% gels (6.7 cm long) layered on 20% gels (3.3 cm long) [26]. The gel was dried and exposed to an X-ray film. The radioactive bands were excised from the gel, and the radioactivity was counted.

2.3. *Determination of the phosphorylated site of* smg *p21B*

smg p21B (44 μg of protein) was phosphorylated by protein kinase G (115 ng of protein) for 60 min as described above except that [γ-^{32}P]ATP (10,000–15,000 cpm/pmol) was used. The phosphorylated sample was dialyzed overnight against a large volume of 20 mM Tris-HCl at pH 7.5 containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 0.5% sodium cholate to remove free [γ-^{32}P]ATP, and then digested with trypsin (0.88 μg of protein) for 30 min at 30°C in a reaction mixture containing 10 mM Tris-HCl at pH 7.5, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 0.25% sodium cholate as described [13]. The digestion was stopped by the addition of soybean trypsin inhibitor (2.6 μg of protein). The digested sample was then subjected to Bakerbond WP-octyl column chromatography as described [13]. The radioactive peaks were collected, evaporated to dryness using Speed-Vac Concentrator (Savant), and analyzed by a peptide sequencer (Applied Biosystems model 470A). In another set of experiments, the sample of the ^{32}P-phosphorylated peptide was divided into 5 aliquots and subjected to four cycles of the manual Edman degradation [13]. One of the 5 aliquots was set aside for product analysis after each cycle of the degradation cycle and subjected to high voltage paper electrophoresis at 1,000 V for 30 min in HCOOH/H_2O (1:99) at pH 2.1. The autoradiograph of the paper was made, and the radioactivity of [^{32}P]phosphoserine was counted by Fuji bio-image analyzer (BAS 2000).

2.4. *Determination of protein concentrations*

Protein concentrations were determined by densitometric tracing of protein bands on an SDS-polyacrylamide gel with bovine serum albumin as a standard protein as described [27].

3. RESULTS AND DISCUSSION

Protein kinase G as well as protein kinase A phosphorylated *smg* p21B as shown in Fig. 1. The protein kinase G-catalyzed phosphorylation of *smg* p21B was time-dependent and about 0.6 mol of phosphate was maximally incorporated into 1 mol of *smg* p21B. The reason for this low stoichiometry for the phosphorylation is unknown. The amino acid phosphorylated by protein kinase G was only serine, and neither threonine nor tyrosine was phosphorylated, as described for the protein kinase A-catalyzed phosphorylation.

When *smg* p21B was digested to a limited extent by trypsin, two fragments were produced: one was the N-terminal fragment with a M_r value of about 20,000, and the other was the C-terminal fragment consisting of Lys[177]-Lys-Ser-Ser-Cys[181] (geranyl-geranylated and methylated) as described [13]. The N-terminal fragment showed GDP/GTP-binding and GTPase activities but lacked a membrane-binding activity, whereas the C-terminal fragment showed membrane-binding activity [20]. Protein kinase A phosphorylated the serine residue

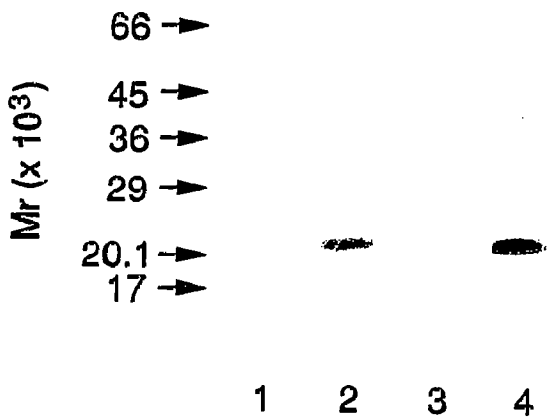

Fig. 1. Phosphorylation of *smg* p21B by protein kinase G. *smg* p21B (77 ng of protein) was incubated with either protein kinase G (115 ng of protein) or the catalytic subunit of protein kinase A (400 ng of protein) for 30 min. (Lane 1) With protein kinase G in the absence of cGMP and cAMP; (lane 2) with protein kinase G in the presence of 10 μM cGMP; (lane 3) with protein kinase G in the presence of 10 μM cAMP; (lane 4) with the catalytic subunit of protein kinase A. The results shown are representative of three independent experiments.

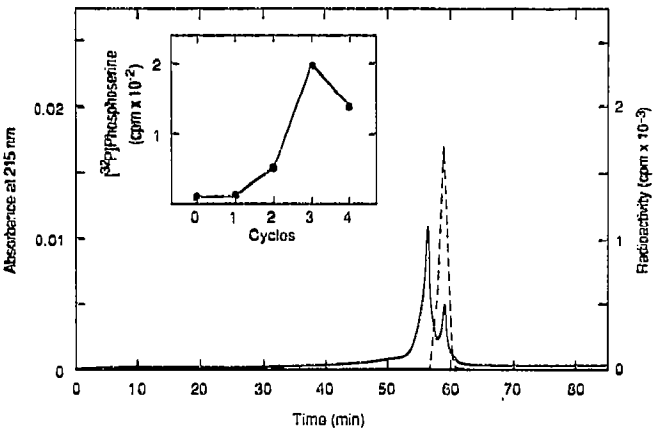

Fig. 2. Determination of the site of the protein kinase G-catalyzed phosphorylation of *smg* p21B. *smg* p21B was fully phosphorylated by protein kinase G with [γ-^{32}P]ATP and then digested with trypsin. The digestion was stopped by soybean trypsin inhibitor. The digested sample was analyzed by Bakerbond WP-octyl column chromatography. The absorbance was recorded at 215 nm for both the sample and the control buffer. The absorbance of the control buffer was subtracted from that of the sample and is illustrated in the figure. (———) absorbance; (---) radioactivity. The result of the Edman degradation of the ^{32}P-phosphorylated peptide is shown in the Inset. The results shown are representative of three independent experiments.

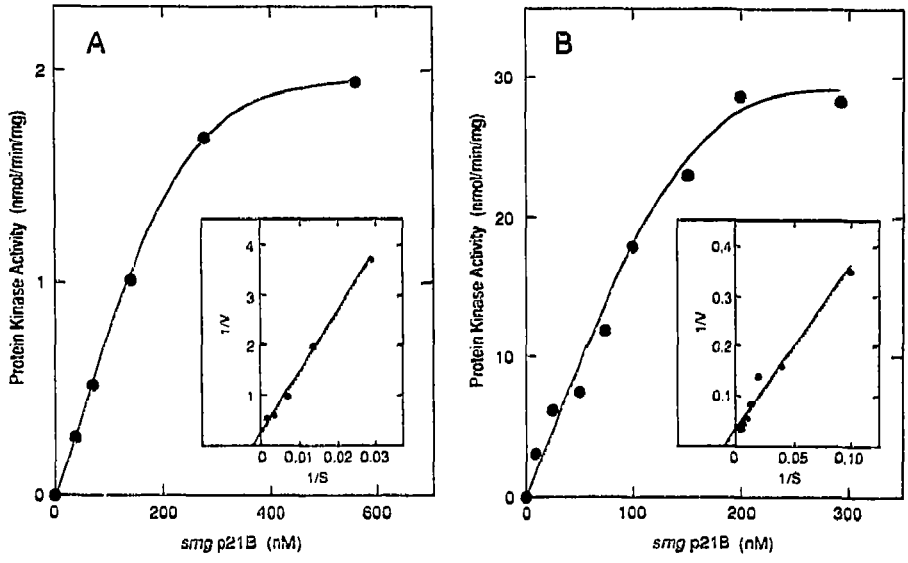

Fig. 3. Kinetic analysis of the phosphorylation of *smg* p21B by protein kinases G and A. Various concentrations of *smg* p21B were phosphorylated by protein kinase G (115 ng of protein) for 4 min or by the catalytic subunit of protein kinase A (400 ng of protein) for 2 min. All of the reaction mixtures contained 0.2% sodium cholate and 58 mM NaCl which were included in the preparation of *smg* p21B. (A) With protein kinase G. (B) With protein kinase A. The Inset shows the double-reciprocal plots. The results shown are representative of three independent experiments.

in this isolated C-terminal fragment [13]. When *smg* p21B was fully phosphorylated by protein kinase G and then digested by trypsin to a limited extent under the same conditions as used previously [13], all of the radioactivity was recovered in the C-terminal fragment as shown in Fig. 2. The Edman degradation analysis revealed that Ser^{179} was the site of the protein kinase G-catalyzed phosphorylation. This site was the same as that phosphorylated by protein kinase A [13]. The K_m and V_{max} values of *smg* p21B for protein kinase G were 5.0×10^{-7} M and 4.2×10^{-9} mol/min/mg, and those for protein kinase A were 1.1×10^{-7} M and 3.2×10^{-8} mol/min/mg, as shown in Fig. 3.

We have shown here that *smg* p21B is phosphorylated by protein kinase G. We previously reported that the protein kinase A-catalyzed phosphorylation of *smg* p21B makes it sensitive to the action of *smg* GDS and initiates the conversion from the GDP-bound inactive form to the GTP-bound active form [13,21]. We have not examined here the effect of the protein kinase G-catalyzed phosphorylation of *smg* p21B on the sensitivity to the *smg* GDS action. However, it is likely that the protein kinase G-catalyzed phosphorylation of *smg* p21B makes it sensitive to the action of *smg* GDS since protein kinase G phosphorylates the same site as that phosphorylated by protein kinase A. We previously proposed a possible mode of activation of *smg* p21 by protein kinase A as follows [13]. In resting cells, *smg* p21 binds to membranes through its C-terminal region. When the serine residue in this region is phosphorylated by protein kinase A, this phosphorylation makes *smg* p21 sensitive to the action of *smg* GDS to make a complex. This *smg* p21–*smg* GDS complex then dissociates from the membrane and translocates to the cytoplasm where the GDP-bound inactive form of *smg* p21 is converted to the GTP-bound active form. The same mode of activation of *smg* p21 by protein kinase G is probable.

It is possible that *smg* p21 is phosphorylated by protein kinase G in response to extracellular signals that elevate cGMP in certain tissues, and that at least some of the actions of cGMP are mediated by *smg* p21. Further studies are essential for understanding the modes of activation and action and function of *smg* p21 in signal transduction.

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