PHOSPHORYLATION OF SYNTHETIC PEPTIDES BY (^{32}P)ATP AND CYCLIC GMP-STIMULATED PROTEIN KINASE

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SUMMARY. Cyclic GMP-stimulated protein kinase from pig lung has been shown to phosphorylate synthetic peptides. The rate of phosphorylation was about one order of magnitude higher than that for mixed histones at a comparable concentration, i.e. 0.1 mM. The peptides represented sites, phosphorylatable by cyclic AMP-stimulated protein kinase, in pyruvate kinase type L from rat liver, calf thymus histone H2B and the α-subunit of rabbit muscle phosphorylase b kinase. The shortest pyruvate kinase peptide that could be phosphorylated at a significant rate by cyclic GMP-stimulated protein kinase was Arg-Arg-Ala--Ser-Val-Ala, which is one amino acid residue longer than the minimal substrate of cyclic AMP-stimulated protein kinase. The apparent K_m was 0.3 mM which is about 10 times higher than that with cyclic AMP-stimulated protein kinase. The K_m was only slightly decreased upon successive extension of the peptide in the N-terminal direction to Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala. Modification of the sequence showed the importance of two adjacent arginyl residues, and substitution of arginine for the C-terminal alanine abolished the measurable activity. Thus, it has been demonstrated that there are both differences and similarities in substrate specificity of the two protein kinases.

INTRODUCTION

Cyclic GMP-stimulated protein kinase (EC 2.7.1.37), discovered by Greengard and Kuo (1), has been shown to differ from cyclic AMP-stimulated protein kinase (EC 2.7.1.37) not only in cyclic nucleotide specificity, but also in molecular size, stability and substrate specificity (1-11). However, little is known about the physiological substrates for cyclic GMP-stimulated protein kinase. The phosphorylation of both muscle proteins and an intestinal brush border membrane protein has been shown to be stimulated by cyclic GMP indicating that these proteins are substrates of cyclic GMP-stimulated protein kinase (12,13). Mixed histones are phosphorylated by both of the cyclic nucleotide-stimulated protein kinases mentioned. It has also been shown that calf thymus histone H2B is phosphorylated at serine 32 and serine 36 irrespective of the protein kinases used (14). This indicates that there are some similarities in the substrate specificity of the two enzymes.

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In recent experiments, synthetic peptides have been shown to serve as substrates for cyclic AMP-stimulated protein kinase (15-17). Thus Arg-Arg-Ala--Ser-Val, corresponding to the phosphorylated site in rat liver pyruvate kinase type L (EC 2.7.1.40) (18), is the minimal peptide that can be phosphorylated at a significant rate by cyclic AMP-stimulated protein kinase (17). It was shown that the two arginyl residues are essential for phosphorylation (17). Therefore, synthetic peptides having similar amino acid sequences corresponding to the phosphorylatable sites of histone H2B, rat liver pyruvate kinase (18) and the α-subunit of rabbit muscle phosphorylase kinase b kinase (19), were tested as substrates for cyclic GMP-stimulated protein kinase from pig lung. Recently, it has also been shown that Leu-Arg-Arg-Ala--Ser-Leu-Gly is phosphorylated by cyclic GMP-dependent protein kinase from rat cerebellum (20).

MATERIALS AND METHODS. Cyclic GMP-stimulated protein kinase was purified from pig lung according to Nakazawa and Sano (5). The peak fractions from the DEAE-cellulose step were used. The enzyme activity was increased 3 to 8 times in the presence of 50 nM cyclic GMP, the specific activity of the enzyme being 2200 to 2400 units per mg in the presence of cyclic GMP. The enzyme activity was increased less than 30% by 50 nM cyclic AMP. The activity was measured as described in ref. (5). One unit of protein kinase catalyzed the incorporation of 1 pmol of phosphate per min into mixed histones.

(^{32}P)ATP was prepared according to the method described by Engström (21) and purified as described by Mårdh (22). It was mixed with unlabelled ATP to give a specific radioactivity of 25-40 cpm/pmol.

Peptides were synthetized and purified as described previously (17).

Phosphorylation of peptides. The incubations were performed at pH 7.0 and 30°C for 2 min in 40 µl of 2.5 mM potassium phosphate buffer containing magnesium acetate (50 mM), theophylline (2.5 mM), sodium fluoride (10 mM), glycerol (2.5%) (v/v), EDTA (0.5 mM), ammonium sulfate (15 mM), 2-mercaptoethanol (12.5 mM) and (^{32}P)ATP, peptide and cyclic nucleotide at the concentrations indicated. The reaction was started by the addition of 3 to 6 units of cyclic GMP-stimulated protein kinase and was interrupted by adding 20 µl of concentrated acetic acid. The samples were then mixed with 20 µl of a solution containing 20 mM ATP and 50 mM EDTA. The ^{32}P-labelled phosphopeptide was separated from $^{32}P_i$ and (^{32}P)ATP by chromatography of 50 µl of the incubation mixture on an AG 1x8 BioRad ion exchanger column equilibrated and eluted with 30% (v/v) acetic acid as described in (16).

RESULTS AND DISCUSSION

Phosphorylation of peptides. The phosphorylation of synthetic peptides is shown in Table 1. Under the conditions given, 1 unit of cyclic GMP-stimulated protein kinase catalyzed the incorporation of about 20 pmol of (^{32}P)phosphate per min into 100 µM Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala in the presence of 200 nM cyclic GMP and 100 µM (^{32}P)ATP. This rate was about one order of magnitude higher than that for mixed histones under similar conditions. The essential difference in substrate specificity between the two protein kinases

Table 1. Relative rates of phosphorylation of peptides with (^{32}P)ATP by cyclic GMP-stimulated protein kinase from pig lung.

In experiments to measure the relative phosphorylation rate, the concentration of (^{32}P)ATP was 100 μM and that of peptides 100 μM. In experiments to determine K_m values the concentration of (^{32}P)ATP was 500 μM, with varying peptide concentrations. The concentration of cyclic GMP was 200 nM and the incubations were performed at 30°C for 2 min at pH 7.0 as described under MATERIALS AND METHODS.

Amino acid sequence of peptides derived from:	Relative rates of phosphorylation:	Apparent K_m value: (mM)
Pyruvate kinase type L from rat liver:		
Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala	100	0.2
Val-Leu-Arg-Arg-Ala-Ser-Val-Ala	78	0.2
Leu-Arg-Arg-Ala-Ser-Val-Ala	58	0.6
Arg-Arg-Ala-Ser-Val-Ala	55	0.3
Arg-Ala-Ser-Val-Ala	0	
Ala-Ser-Val-Ala	0	
Leu-Arg-Arg-Ala-Ser-Val	0	
Arg-Arg-Ala-Ser-Val	0	
Modified peptides:		
Leu-Arg-Arg-Ala-Ser-Val-Arg	0	
Arg-Leu-Arg-Ala-Ser-Val-Ala	0	
Phosphorylase kinase from rabbit muscle, α-chain:		
Phe-Arg-Arg-Leu-Ser-Ile-Ser	63	0.2
Phe-Arg-Arg-Leu-Ser-Ile	13	0.3
Histone H2B from calf thymus:		
Arg-Lys-Arg-Ser-Arg-Lys-Glu-Ser-Tyr-Ser	35	0.1
Arg-Lys-Arg-Ser-Arg-Lys	0	
Arg-Lys-Glu-Ser-Tyr-Ser	0	

was demonstrated by the fact that Arg-Arg-Ala-Ser-Val, the minimal peptide phosphorylated by cyclic AMP-stimulated protein kinase, was not phosphorylated at a significant rate by cyclic GMP-stimulated protein kinase; while Arg-Arg--Ala-Ser-Val-Ala was the shortest peptide phosphorylated by cyclic GMP-stimulated protein kinase. This does not simply reflect the addition of one amino acid residue at the C-terminal end, since Leu-Arg-Arg-Ala-Ser-Val-Arg was shown not to be a substrate. The apparent requirement of at least two amino acid residues on the C-terminal side of the phosphorylatable site did not appear to apply for peptides representing the phosphorylatable site of the α-subunit of phosphorylase kinase, since Phe-Arg-Arg-Leu-Ser-Ile was phosphorylated at a significant rate. However, addition of a seryl residue to the

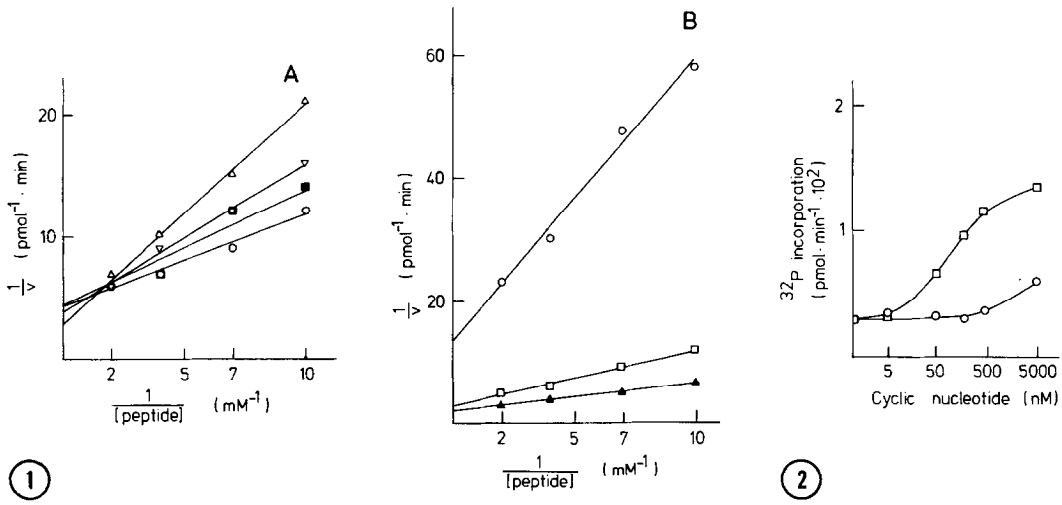

Figure 1. Lineweaver-Burk plots of data from phosphorylation experiments with synthetic peptides. Incubations were performed in the presence of 200 nM cGMP and 500 μM (^{32}P)ATP for 2 min. Slopes and intercepts of the lines were calculated by the least squares method, while the points represent experimental data. A: Δ—Δ, Leu-Arg-Arg-Ala-Ser-Val-Ala; ▽—▽, Arg-Arg-Ala-Ser-Val-Ala; ■—■, Val-Leu-Arg-Arg-Ala-Ser-Val-Ala; O—O, Gly-Val-Leu-Arg-Arg-Ala-Ser--Val-Ala. B: O—O, Phe-Arg-Arg-Leu-Ser-Ile; □—□, Phe-Arg-Arg-Leu-Ser-Ile--Ser; ▲—▲, Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala.

Figure 2. The activity of cyclic GMP-stimulated protein kinase at various concentrations of cyclic GMP and cyclic AMP. The standard assay conditions were employed with 500 μM (^{32}P)ATP and 100 μM peptide. The peptide Gly-Val--Leu-Arg-Arg-Ala-Ser-Val-Ala was used as substrate in the presence of cyclic GMP (□—□) and cyclic AMP (O—O).

C-terminal end of this peptide increased the rate of phosphorylation considerably. The requirements for phosphorylation could not be extrapolated to peptides representing the phosphorylatable seryl residues 32 and 36 in histone H2B. Phosphorylation was not detected with hexapeptides covering one or the other seryl residue and containing two amino acid residues on the C-terminal side of the phosphorylatable seryl residue. However, a peptide covering both sequences was found to be a substrate. Thus the minimum peptide substrate from histone H2B remains to be determined. The importance of having two adjacent arginyl residues is demonstrated by the fact that the peptide Arg-Leu-Arg-Ala-Ser-Val-Ala was not detectably phosphorylated. A similar result has been reported for cyclic AMP-stimulated protein kinase (17,23).

The results shown in Fig. 1 A suggested that the structure on the N-terminal side of the arginyl residues of the pyruvate kinase peptides had a slight influence in phosphorylation. However, the peptides investigated in

this experiment showed apparent V_{max} and K_m of the same order of magnitude.

As seen in Fig. 1 B, the two peptides representing the phosphorylatable site of the α-chain subunit of phosphorylase kinase displayed identical apparent K_m, but a fivefold difference in apparent V_{max}. This pattern is different from that seen with cyclic AMP-stimulated protein kinase and pyruvate kinase type L peptides, where a change from one to two amino acid residues on the C-terminal side of the phosphorylatable serine residue decreases the apparent K_m about four times but increases apparent V_{max} only about one third (17).

Optimal conditions for peptide phosphorylation. The incorporation of phosphate into the peptide Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala was found to increase linearly with increasing enzyme concentration. As shown in Figure 2 the activity of cyclic GMP-stimulated protein kinase from pig lung was increased severalfold by increasing the concentration of cyclic GMP from 5 to 500 nM. Cyclic AMP added at the same concentration had no effect on enzyme activity. Elevating the cyclic nucleotide concentration from 500 nM to 5 μM gave only a slight additional rise in stimulation by cyclic GMP but considerable stimulation by cyclic AMP. The latter observation could be explained by an interaction of cyclic AMP with cyclic GMP-binding sites in the enzyme at high concentrations of cyclic AMP.

Conclusions. It has been shown in the present study that peptides can serve as substrates for cyclic GMP-stimulated protein kinase. The peptides that were phosphorylated represented known substrates for cyclic AMP-stimulated protein kinase as well. However, the results demonstrate both similarities and differences in substrate specificity for the two types of protein kinases.

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