Volume 220, number 1, 47–51 FEB 04915 August 1987

Recombinant type I regulatory subunit of the cAMP-dependent protein kinase is biologically active

Stephan Oehen, JoAnne Parsons, David A. Jans and Brian A. Hemmings

Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel, Switzerland

Received 27 April 1987

The cDNA for the porcine type I regulatory subunit (R_I) of the cAMP-dependent protein kinase (cAMP-PK) was cloned into two different bacterial expression vectors: pKK223 and pUC18. Recombinant R_I was produced by bacteria transformed with either construct, and purified by affinity chromatography. Both the native R_I from the pKK223 construct and the R_I with an amino terminal extension of eight amino acids from the pUC18 construct were found to be completely native with regard to inhibition of the catalytic subunit activity and cAMP binding.

Bacterial expression vector; *lac* promoter; Plasmid pUC18; Plasmid pKK223; Protein kinase

1. INTRODUCTION

The cAMP-dependent protein kinase is an interesting enzyme, both in structure and function. The kinase is composed of two regulatory (R) subunits (either type I or type II) and two catalytic (C) subunits [1]. In the heterodimeric conformation, R_2C_2, the C subunit is completely inactive. Following the binding of cAMP by the regulatory subunits the C subunit is released and hence becomes catalytically active [2]. The R subunit thus carries out two unique functions in that it binds the C subunit with high affinity, thereby suppressing the catalytic activity of this subunit; and it binds cAMP with high affinity which results in a conformational change and release of the C subunit [3,4].

The functions of the R subunit are localised in at least two structural domains, i.e. cAMP binding and C subunit binding regions [1,5,6]. In addition the type I regulatory subunit (R_I) appears to exist as a dimer held together by disulphide bonds between Cys-50 in the amino-terminus of the protein [7]. In order to understand the functions of these various domains, and to determine the mechanism whereby cAMP binding in the carboxy-terminus of the protein can be transmitted to the amino-terminus, and hence reduce the affinity of R for C, we set up a bacterial expression system to produce recombinant R_I. In this paper we describe two expression systems which result in the biosynthesis of biologically active R_I subunit.

Correspondence address: B.A. Hemmings, Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel, Switzerland

Abbreviations: cAMP-PK, cAMP-dependent protein kinase; IPTG, isopropylthiogalactoside

2. MATERIALS AND METHODS

2.1. *Materials*

The source of most materials used in this study has been described [8,9]. cAMP-agarose type III was obtained from Pharmacia and Affigel 10 from BioRad. Isopropylthiogalactoside (IPTG) was from BRL.

2.2. *Plasmids and bacterial strains*

Plasmid pR_I15 which contains the full length cDNA of porcine R_I in pBR322 [9] was used for

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

the construction of the expression vectors and the JM103 strain of *E. coli* was used for transformation.

2.3. *DNA sequencing*

DNA sequencing was performed using the Sanger dideoxy chain termination method [10].

2.4. *Purification of proteins and preparation of antisera*

The R_I and C subunits were purified from rabbit and bovine skeletal muscle respectively, by published procedures [11]. Antisera to the bovine skeletal muscle R_I was prepared as described [11]. Affinity purified antisera to R_I were prepared by passage over columns of R_I covalently attached to agarose (Affigel 10). Bound antisera were eluted using 6 M urea and dialyzed immediately into 10 mM Mes-NaOH, pH 6.9, 1 mM EDTA and 150 mM NaCl.

2.5. *Western blot analysis*

Crude cell-free extracts, or partially purified protein fractions were separated on 10% polyacrylamide gels containing 0.1% SDS. Following electrophoresis proteins were transferred to nitrocellulose using the methods of Towbin et al. [12]. Nitrocellulose filters were blocked using 3% (w/v) BSA and 3% (w/v) ovalbumin in TBS. Recombinant R_I was detected using affinity purified antisera (prepared as described above) followed by incubation with ^{125}I-labelled protein A. Following washing the blots were subjected to autoradiography at -70°C with two intensifying screens.

2.6. *Assay of the cAMP-dependent protein kinase*

C subunit activity was determined using the peptide assay (Kemptide) as described [11,13]. To assay for R subunit activity, purified rabbit skeletal muscle C subunit (10 μl) was preincubated with protein fractions (10 μl) containing the recombinant R_I, and then added to the kinase assay mix. Typically the kinase reaction was started by the addition of [γ-^{32}P]ATP and incubated at 30°C for 10 min prior to termination by spotting the reaction mix onto P-81 paper followed by washing with 0.5% (v/v) phosphoric acid. The C subunit was purified from rabbit skeletal muscle as described in [11].

2.7. *Isolation of recombinant R_I*

For the isolation of recombinant R_I produced by pKK233 and pUC18 constructs, cultures of JM103 were grown in LB broth containing 1 mM IPTG for 20 h at 37°C. Cell-free extracts were prepared by treatment of cell pellets with lysozyme (10 mg·ml^{-1}) in the presence of 0.2% Triton X-100, 10 mM Mes-NaOH, pH 6.9, followed by sonication (3 × 60 s). Cell debris was removed by centrifugation at 20000 × *g* for 30 min at 4°C and the resulting supernatant was applied directly to a cAMP-agarose affinity column (1 × 2 cm) previously equilibrated with 10 mM Mes-NaOH, pH 6.9, 1 mM EDTA, 150 mM NaCl and 0.2% Triton X-100. The column was then washed extensively with 2 M NaCl in 10 mM Mes-NaOH, pH 6.9, 1 mM EDTA. The bound R subunits were eluted by incubation of the column at room temperature for 2 h in the presence of 5 mM cAMP in 10 mM Mes-NaOH, pH 6.9, 1 mM EDTA. Following elution the R_I subunits were dialyzed extensively against 10 mM Mes-NaOH, pH 6.9, 1 mM EDTA, 150 mM NaCl. Preparation of cAMP free recombinant R_I subunit was carried out as follows. The extensively dialyzed material from the cAMP-agarose column was made up to 6 M urea by the addition of solid urea and allow to sit on ice for 30 min. This material was then dialyzed extensively against 10 mM Mes-NaOH, pH 6.9, 1 mM EDTA, 150 mM NaCl. In order to obtain homogeneous preparations of recombinant R_I the protein was subjected to ion-exchange chromatography on a Mono Q column (Pharmacia).

2.8. *Protein estimation*

Protein was estimated by the method of Bradford [14] using BSA as a standard.

3. RESULTS AND DISCUSSION

3.1. *Construction of R_I expression vectors*

The cDNA for the porcine R_I, pR_I15, which contains the complete coding region for the protein [9] was used for all the constructions described below. The 1426 bp *Eco*R_I fragment of pR_I15, which extends 70 bp 5′ of the initiator methionine and 200 bp past the TGA terminator codon was isolated, and then partially digested with *Nco*I (fig.1a). The 1350 bp *Nco*I-*Eco*R_I fragment was

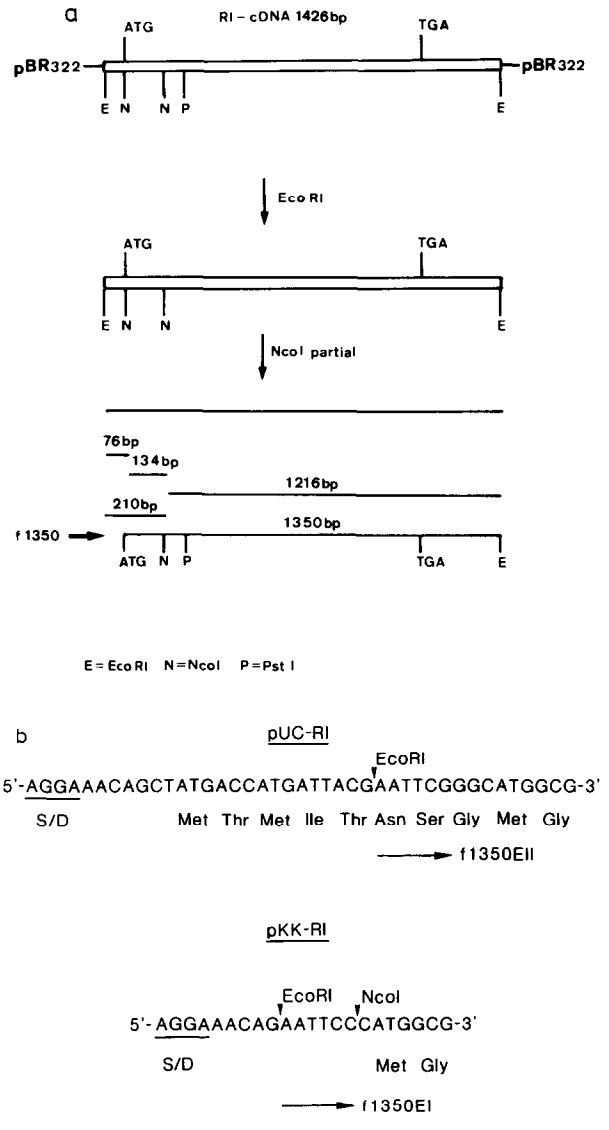

Fig.1. Construction of bacterial expression vectors for porcine R_I. (a) Isolation of the R_I coding sequences. The 1426 bp cDNA for the porcine R_I was digested with *Eco*R_I followed by partial digestion with *Nco*I. The 1350 bp fragment (f1350) which starts at the initiator methionine was isolated, treated with Klenow and used for the addition of linkers. Finally the 1350 bp fragment was ligated into the pKK223-2 and pUC18 vectors. (b) Sequence of the expression vectors pKK-R_I and pUC-R_I showing the position of the Shine and Delgano sequence relative to the initiator methionine. Restriction endonuclease sites are indicated, as is the Shine and Dalgarno sequence, which is underlined and denoted by S/D.

subsequently blunt ended and *Eco*R_I linkers, GGAATTCC and CCCGAATTCGGG, were added to produce f1350EI and f1350EII, respectively. The fragments 1350EI and 1350EII were subsequently ligated into the *lac* based vectors pKK233 [15–17] and pUC18 [18], respectively. The DNA sequence at the 5′-region with respect to the vector sequences is shown in fig.1b. In the pKK-R_I construction the Shine and Dalgarno sequence is 12 bp upstream from the initiator methionine and hence the recombinant protein produced has no extension of the amino-terminus, and is therefore 'native'. The pUCR_I construct produces a protein which contains an extension of eight amino acids at the amino-terminus (fig.1b).

Both constructs were used to transform bacterial strain JM103 and several clones were obtained with the R_I cDNA in the correct orientation.

3.2. *Expression of porcine R_I in bacteria*

Bacteria transformed with pKK-R_I and pUC-R_I were cultured and induced for 6 h or 20 h with 1 mM IPTG. Cell-free extracts were prepared and analyzed by Western blotting using an affinity purified antisera to bovine R_I [11]. The results (fig.2) showed that both constructs produced immunoreactive protein, and that the expression of recombinant R_I was tightly controlled in the case of the pKK-R_I construct. The pUC-R_I construct, in contrast, was somewhat leaky, resulting in the production of detectable cross-reacting material even in the absence of IPTG. However, with the pUC-R_I construct much larger amounts of immunoreactive protein were detected following induction by IPTG.

3.3. *Purification of recombinant R_I from pKK-R_I and pUC-R_I*

The recombinant R_I was purified from JM103 cultures which had been induced by 1 mM IPTG for 16 h. Typically the cell pellet was disrupted by lysozyme/sonication treatment, centrifuged and directly applied to a cAMP-agarose affinity column. Following extensive washing with 2 M NaCl the R_I protein was eluted with 5 mM cAMP. This procedure usually produced a protein which was approx. 30–50% pure as judged by SDS-gel electrophoresis. It was subsequently purified to homogeneity using ion-exchange chromatography on a Mono Q column. Approx. 50–100 μg of

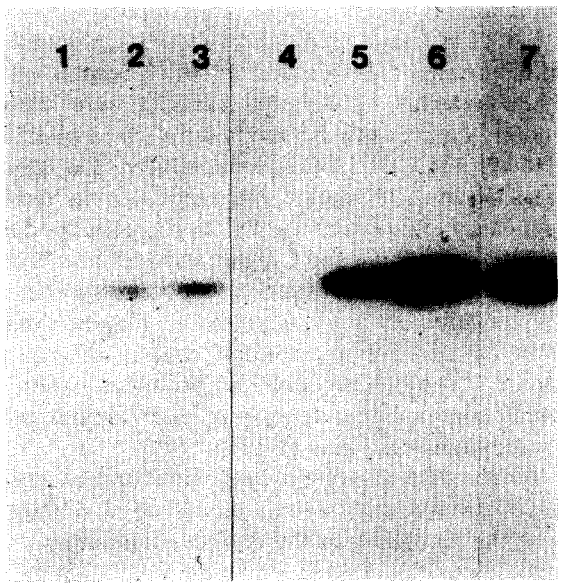

Fig.2. Western blot analysis of recombinant R_I synthesis by pKK-R_I and pUC-R_I. Cultures of JM103 carrying pKK-R_I or pUC-R_I were grown to an A_{600} of 0.2, and then cultures allowed to grow for a further 6 h in the presence or absence of 1 mM IPTG. Cell-free extracts were prepared and samples (50 μg protein) analyzed by Western blotting using an affinity purified antibody to R_I. Lanes: 1, JM103/pKK223 (+IPTG); 2, JM103/pKK-R_I (−IPTG); 3, JM103/pKK-R_I (+IPTG); 4, JM103/pUC18 (+IPTG); 5, JM103/pUC-R_I (−IPTG); 6, JM103/pUC-R_I (+IPTG); 7, purified bovine R_I (0.06 μg protein).

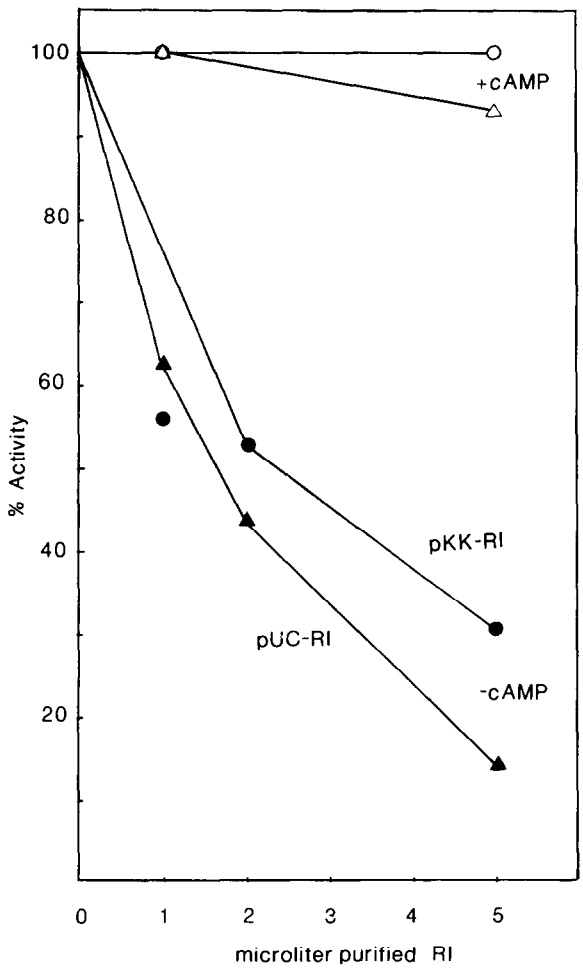

Fig.3. Inhibition of C subunit by recombinant R_I preparations from the pKK-R_I and pUC-R_I constructs. Assays were carried out as described in section 2. R_I from the pKK-R_I construct in the absence of 10 μM cAMP (●); R_I from the pKK-R_I construct in the presence of cAMP (○); R_I from the pUC-R_I construct in the absence of cAMP (▲); R_I from the pUC-R_I construct in the presence of cAMP (△). The protein concentration of recombinant R_I stock was 10 μg/ml.

recombinant R_I was obtained per liter from each of the two constructs. To obtain cAMP-free recombinant R_I the protein was treated with 6 M urea for 30 min and then dialyzed extensively. This procedure resulted in the production of a biologically active protein (see below).

3.4. *Recombinant R_I inhibits C subunit activity*

Preparations of recombinant R_I from both constructs were tested for their ability to inhibit the catalytic activity of the purified C subunit from rabbit skeletal muscle. Both R_I preparations inhibited C activity and in both cases the inhibition could be reversed by the addition of 10 μM cAMP to the assay mix (fig.3), indicating normal association-dissociation of the recombinant R_I and the C subunit.

This was further investigated by examining the K_a for activation of the holoenzyme formed from recombinant R_I from the pUC-R_I construct and rabbit skeletal muscle C subunit (fig.4). Half-maximal activation of the holoenzyme was obtained at 1×10^{-7} M cAMP, similar to that observed for the native porcine enzyme [19]. Recombinant R_I from the pKK-R_I construct showed similar results (not shown).

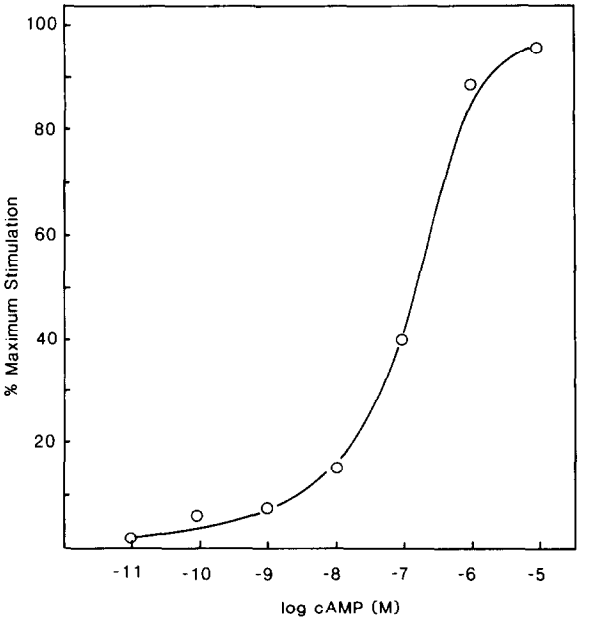

Fig.4. Activation of holoenzyme formed from recombinant R_I (pUC-R_I) and rabbit skeletal muscle C subunit by cAMP. Assays were carried out as detailed in section 2.

4. CONCLUSIONS

(i) The data presented in this paper demonstrate that recombinant R_I subunit is biologically active. The fact that the protein produced by the pUC18-R_I construct is fully active is interesting in that, in contrast to that from the pKK-R_I construct, the R_I in this instance contains 8 amino acids at the NH_2 terminus. This implies that N-terminals are probably not important for biological activity.

(ii) These R_I expression systems will make it possible to probe structure-function relationships using site-directed mutagenesis.

During the course of this work, Saraswat et al. [20] reported the expression of the bovine R_I subunit in *E. coli*. These workers also used a pUC construct producing a protein with an additional 10 amino acids at the amino-terminus.

ACKNOWLEDGEMENTS

The authors would like to thank Michael Altus, Brian Rudkin and David Pearson for critically reading the manuscript.

REFERENCES

[1] Carlson, G.M., Bechtel, P.J. and Graves, D.J. (1979) Adv. Enzymol. 50, 41–115.
[2] Flockhart, D.A. and Corbin, J.D. (1979) CRC Rev. 12, 133–186.
[3] Stephen, B., White, H.D., Siegel, J.B. and Krebs, E.G. (1981) Proc. Natl. Acad. Sci. USA 78, 1591–1595.
[4] Kerlavage, A.R. and Taylor, S.S. (1982) J. Biol. Chem. 257, 1749–1754.
[5] Corbin, J.D., Sugden, P.H., West, L., Flockhart, D.A., Lincoln, T.M. and McCarthy, D. (1978) J. Biol. Chem. 253, 3997–4003.
[6] Zetterqvist, O. and Ragnarsson, U. (1982) FEBS Lett. 139, 287–290.
[7] Titani, K., Sasgawa, T., Ericsson, L.H., Kumar, S., Smith, S.B., Krebs, E.G. and Walsh, K.A. (1984) Biochemistry 23, 4193–4199.
[8] Botterell, S., Jans, D.A. and Hemmings, B.A. (1987) Eur. J. Biochem., in press.
[9] Nowak, I.E., Seipel, K., Schwarz, M., Jans, D.A. and Hemmings, B.A. (1987) Eur. J. Biochem., in press.
[10] Sanger, F. (1981) Science 214, 1205–1210.
[11] Hemmings, B.A. (1986) FEBS Lett. 196, 126–130.
[12] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[13] Hemmings, B.A. (1985) Curr. Top. Cell. Regul. 27, 117–132.
[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[15] Amman, E., Brosius, J. and Ptashne, M. (1983) Gene 25, 167–178.
[16] De Boer, H.A., Comstock, L.J. and Vasser, M. (1983) Proc. Natl. Acad. Sci. USA 80, 21–25.
[17] Brosius, J. (1984) Gene 27, 161–172.
[18] Vieira, J. and Messing, J. (1982) Gene 19, 259–268.
[19] Krebs, E. and Beavo, J.A. (1973) Annu. Rev. Biochem. 48, 923–959.
[20] Saraswat, L.D., Filutowicz, M. and Taylor, S.S. (1986) J. Biol. Chem. 261, 11091–11096.