Expression of the Type I Regulatory Subunit of cAMP-dependent Protein Kinase in *Escherichia coli*

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An expression vector has been constructed for the type I regulatory subunit of cAMP-dependent protein kinase. A cDNA clone for the bovine R'-subunit has been inserted into pUC7. When *Escherichia coli* JM105 was transformed with this plasmid, R-subunit was expressed in amounts that approached 4 mg/liter. The expressed protein was visualized in total cell extracts by photolabeling with 8-azidoadenosine 3':5'-monophosphate followed from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose. Expression of R-subunit was independent of isopropyl-β-d-thiogalactopyranoside. R-subunit accumulated in large amounts only in the stationary phase of growth, and the addition of isopropyl-β-d-thiogalactopyranoside during the log phase of growth actually blocked the accumulation of R-subunit. Maximum expression (20 mg/liter) was achieved when *E. coli* 222 was transformed with the R'-containing plasmid. *E. coli* 222 is a strain that contains two mutations; it is cyA- and also has a mutation in the catabolite gene activator protein (crp*) that enables the protein to bind to DNA in the absence of cAMP.

The expressed R'-subunit was a soluble, dimeric protein, and no significant proteolysis was apparent in the cell extract. The purified R'-subunit (a) bound 2 mol of cAMP/mol of R monomer, (b) reassociated with C-subunit to form holoenzyme, and (c) migrated as a dimer on sodium dodecyl sulfate-polyacrylamide gels in the absence of reducing agents. The expressed protein was also susceptible to limited proteolysis, yielding a monomeric cAMP-binding fragment having a molecular weight of 35,000. In all of these properties, the expressed protein was indistinguishable from R' purified from bovine tissue even though the R-subunit expressed in *E. coli* represents a fusion protein that contains 10 additional amino acids at the amino terminus that are provided by the lac Z' gene of the vector. This NH₂-terminal sequence was confirmed by amino acid sequencing.

cAMP-dependent protein kinase, thought to be the primary mediator of cAMP action in eukaryotic cells, is composed of both regulatory (R) and catalytic (C) subunits (1). The inactive holoenzyme is an aggregate of both subunit types, RₐCₐ, and activation by cAMP promotes dissociation into R₂- (cAMP), and two monomeric C-subunits which are catalytically active. Different forms of the kinase have been identified and by convention are referred to as types I and II based on their elution from DEAE-cellulose (2). These forms can be distinguished on the basis of size and autophosphorylation and also are antigenically distinct (3, 4). In addition, there are soluble and membrane-associated forms of the kinase (5–7). Most evidence suggests that the catalytic subunits are very similar (8) and that those features which distinguish the various holoenzyme forms can be attributed exclusively to the R-subunits (9, 10). Bovine R' has been cloned recently and appears to be the product of a single gene (11). The type II regulatory subunits, on the other hand, represent a larger family of proteins. At least two unique gene products have been identified (12), and a qualitative comparison of various tissues suggests that this diversity may be widespread (13, 14). The reason for this diversity is not yet apparent.

Despite differences, there are general features that are conserved in all R-subunits. These include cAMP binding, formation of a dimeric protein following dissociation by cAMP, and a recognition site for catalytic subunit. Furthermore, a well-defined domain structure appears to be retained in each R-subunit. In general, the protein can be divided into thirds based on the amino acid sequence. The NH₂-terminal third of the molecule contains a hinge region that is highly susceptible to proteolytic cleavage (15–17), contains an essential recognition site for the C-subunit (18), and is the major site of interaction between the two protomers of the R dimer (19). Finally, it is the NH₂-terminal segment, at least in the R'-subunit, that contains the primary antigenic determinant, both in the dissociated R-subunit and in the holoenzyme (12, 17). The COOH-terminal two-thirds of the molecule contain two in tandem gene duplicated segments (20, 21) which represent two cAMP-binding sites. Our knowledge of the structure of the cAMP-binding sites has been greatly facilitated by their homology with the catabolite gene activator protein (CAP') in *Escherichia coli* (22). Homologies in amino acid sequences initially established the relatedness of the cAMP-binding domains in these two proteins (23). Comparison of photolabeling of R with the crystal structure of CAP has confirmed that cAMP binds in an analogous manner in both proteins and that the protein folding in each cAMP-binding domain of R is similar to that which is seen in CAP (24).

In order to understand this molecule at the molecular level, it will be necessary to solve the crystal structure of the protein. In conjunction with this, the use of site-directed mutagenesis can provide an important tool for asking questions about functional sites and about the specific conformational changes associated with cAMP-dependent protein kinase. As a first

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‡‡ The abbreviations used are: CAP, catabolite gene activator protein; SDS, sodium dodecyl sulfate; IPTG, isopropyl-β-D-thiogalactopyranoside; 8-N₃cAMP, 8-azidoadenosine 3':5'-monophosphate.

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step in this direction, we report here the expression of the bovine R1 gene in E. coli under the control of the lac promoter. The R1-subunit is expressed at high levels, is soluble, and appears to be functionally equivalent to the protein purified from bovine tissue.

EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from the following companies: histone HIA, phenylmethylsulfonyl fluoride, isopropyl-β-D-thiogalactopyranoside (IPTG), and bovine serum albumin, Sigma; Nα-p-tosyl-L-lysine chloromethyl ketone, and 15 mg/liter phenylmethylgalactopyranoside (IPTG), and bovine serum albumin, Sigma; Nu-p-ethane spacer), Pharmacia; nitrocellulose (0.45 pm) and electrophoresed on 12.5% polyacrylamide gels (1.5 mm) containing high in E. coli, we devised the following method for the accurate characterization by restriction analysis.

Proteins—C-subunit was purified from porcine heart (28) and R from porcine skeletal muscle (29).

Construction of the Expression Vector—Isolation of cDNA from clone 62C12 and construction of the expression vector are summarized in Fig. 1. DNA manipulations were carried out according to Maniatis et al. (29). Following transformation, cells were plated on LB plates containing ampicillin (60 μg/ml). Replicates of these plates were prepared on nitrocellulose filters and screened with a restriction fragment that was labeled with 32P at its 5'-end using T4 polynucleotide kinase and [γ-32P]ATP. Positive colonies were further characterized by restriction analysis.

Detection of Regulatory Subunit—Since basal levels of cAMP are high in E. coli, we devised the following method for the accurate detection of R in crude extracts independent of exogenous cAMP. This method could be used for rapid screening. Protein samples were electrophoresed on 12.5% polyacrylamide gels (1.5 mm) containing sodium dodecyl sulfate according to the method of Laemmli (30). Proteins were then transferred from the gels to nitrocellulose filters using an electroblotting apparatus (Hoefer Scientific Instruments). The filters were incubated for 4-6 h at 500 mA in 20 mM Tris, 154 mM glycine, 20% methanol (pH 8.3). The nitrocellulose was incubated for 1 h at room temperature with 0.05% Tween 20 in 150 mM sodium chloride and 10 mM Tris (pH 7.5) and then washed at room temperature with the same buffer except that Tween 20 was omitted. The filter was incubated with 8-N3[32P]cAMP (20 nm) in the same buffer for 30 min in the dark at room temperature. After washing with ice-cold buffer, the filter was irradiated with a UV S-11 lamp (254 nm) for 5 min, washed again, blotted dry, and subjected to autoradiography. The R-subunit also was detected by Millipore filtration using a Beckman HPLC system using a cyano column (IBM) according to the procedure of Hunkapiller and Hood (32).

Formation of Holoenzyme—Holoenzyme was prepared as described previously (24), and kinase activity was assayed spectrophotometrically (33).

RESULTS

Construction of the Expression Vector—In order to construct an expression vector for the cloned R1 gene, a full-length cDNA insert was isolated from a derivative of a pBR322 clone, 62C12, which contains the complete coding sequence of bovine R1 (11). The procedure for isolation of the restriction fragment containing R1 is summarized in Fig. 1. The R restriction fragments indicated were ligated into pUC7, and following transformation, colonies were screened to determine which ones contained the full-length inserts. Twenty colonies were selected, and the DNA from each was prepared and digested with BglI in order to establish the orientation of the insert. The DNA from three clones gave a restriction pattern which indicated that the R coding segment was inserted in the correct orientation with regard to the lac Z′ gene (Fig. 1). These clones were further characterized for expression of the protein.

Expression of Regulatory Subunit—The initial transformation was done with E. coli JM105. Expression of the protein could be monitored in several ways. Because this protein has a high affinity for cAMP, we chose to use photoaffinity labeling with 8-N3[32P]cAMP as a method for detecting functional protein. Since the intracellular concentration of cAMP in E. coli is high, it was not feasible to photolabel directly in total cell extracts. Instead, photolabeling was carried out following SDS-polyacrylamide gels electrophoresis of total extracts as described previously. As indicated in Fig. 2, one clone expressed a cAMP-binding protein in significant amounts. The clones which had the R1 insert in the opposite orientation did not produce detectable amounts of protein, and no photolabeled protein was visualized in cells transformed with the parent pUC7 plasmid. Surprisingly, two other clones which contained the BglI restriction mapping, also contained plasmid with the insert in the correct orientation did not express detectable amounts of protein.

The photolabeled protein had a molecular weight that was slightly larger than R1 purified from bovine tissue. This is consistent with the pUC construct where the AUG start codon is supplied by the lac Z′ gene in the plasmid, yielding a fusion protein which contains 10 additional amino acids at the NH2 terminus. The expressed protein was not degraded significantly in crude extracts.

Although pUC7 contains the lac promoter, addition of IPTG during the log phase of growth blocked the production of R-subunit (Fig. 3). In the absence of IPTG, R-subunit accumulated in large amounts only in the stationary phase of growth. Very low levels of R-subunit were detected during the first 10 h of growth (data not shown). Quantification of photolabeled protein was washed with buffer 1 containing 2 mM NaCl until the absorbance at 280 nm reached 0 and then washed again with buffer 1. The R-subunit was eluted with 2 volumes of cAMP (50 mM) in buffer 1 at 30 °C for 1 h. The elution was repeated at least once.

The eluate from the cAMP-agarose resin was monitored for the presence of R-subunit and dialyzed overnight. After removing the supernatant solution, the resin was washed with buffer 1 containing 2 mM NaCl until the absorbance at 280 nm reached 0 and then washed again with buffer 1. The R-subunit was eluted with 2 volumes of cAMP (50 mM) in buffer 1 at 30 °C for 1 h. The elution was repeated at least once.

The eluate from the cAMP-agarose resin frequently contained some proteolytic degradation products of R in addition to full-length R. The degradation products were removed by gel filtration on a 2 × 70-cm Sephadex G-150 column equilibrated with 25 mM potassium phosphate, 5 mM 2-mercaptoethanol, 5 mM EDTA, and 150 mM NaCl (pH 6.5). Fractions (2 ml) were collected at a flow rate of 7 ml/h. The R-subunit eluted close to the void volume and was well-resolved from the 25-kDa proteolytic fragment.

Protein Sequencing—Sequencing was carried out on an Applied Biosystems Gas-Phase Sequencer. Two nanomoles of R-subunit were sequenced, and the phenylthiohydantoin derivatives were analyzed on a Beckman HPLC system using a cyano column (IBM) according to the procedure of Hunkapiller and Hood (32).
protein using purified \( \text{R'} \) as a standard indicated that up to 4 mg of R-subunit were being produced per liter of culture.

Characterization and Purification of the Expressed Protein—Having demonstrated that R-subunit was being expressed, we next compared the behavior of the expressed fusion protein with that of the native protein purified from bovine tissue. Selective photolabeling with \( \gamma^{32}\text{P}\)ATP and used to identify those clones which contained the full-length insert. Sites for restriction enzymes EcoRI (E) and BglII (B) are indicated. The three restriction fragments resulting from digestion with BglII are indicated by dashed arrows and were used to identify the orientation of the insert. The orientation of the lac Z' gene, ampicillin-resistance gene (Amp), tetracycline-resistance gene (tet), and origin of replication (ori) are indicated by arrows.

In order to more fully characterize the protein, the expressed \( \text{R'} \)-subunit was purified to homogeneity according to the procedure described under “Experimental Procedures.” Following disruption in a French pressure cell, the R-subunit was found primarily in the supernatant fraction (Fig. 5). Determination of cAMP binding after dialysis of the redissovled ammonium sulfate pellet indicated a yield of 2–4 mg \( \text{R'} \)-subunit/liter of original culture, which was consistent with the initial estimate based on photolabeling. The R-subunit was purified further by affinity chromatography. Following elution from the affinity resin at 30 °C, both the native protein and a significant amount of proteolytic fragment were observed (Fig. 6). The R-subunit was purified to homogeneity by gel filtration on Sephadex G-150 with the intact R-subunit eluting in a position that corresponded to a dimeric protein.

The intact R-subunit had a molecular weight of 48,000 on SDS-polyacrylamide gels. The amino-terminal sequence of this protein was Thr-Met-Ile-Thr-Asn-Ser-Pro-Pro-Asp-Ser-Val-Cys. Another feature that is observed with purified \( \text{R'} \) is that the protomers of the dimer are cross-linked by interchain disulfide bonds. When the expressed protein was subjected to electrophoresis in the absence of 2-mercaptoethanol, it also migrated as a dimer (Fig. 4).

In order to more fully characterize the protein, the expressed \( \text{R'} \)-subunit was purified to homogeneity according to the mobility of bovine \( \text{R'} \). Lanes 1–4, clones that contain the \( \text{R'} \) gene in the same orientation as the lac Z' gene (lanes 1 and 3 represent the same clone); lane 5, a clone which contains the \( \text{R'} \) insert in an orientation that was opposite to the lac Z' gene; lane 6, a clone that was transformed with pUC7 alone.
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Fig. 4. Demonstration of interchain disulfide bonding. *E. coli* (JM105) transformed with the R'-containing plasmid was harvested at 16 and 23 h. The pelleted cells were dissolved in gel loading buffer with or without 2-mercaptoethanol. Samples were electrophoresed, electrotransferred to nitrocellulose, and photolabeled. Molecular weights of cross-linked dimeric protein (92,000), monomer (47,000), and proteolytic fragment (35,000) for bovine R' are indicated by arrowheads.

Fig. 5. Purification of R-subunit from *E. coli* transformed with the R'-containing plasmid. Samples taken at different stages of purification were dissolved in gel loading buffer and subjected to electrophoresis on an SDS-12.5% polyacrylamide gel. Both supernatant (SUP) and pellet (PEL) fractions from each centrifugation and after ammonium sulfate precipitation (PTN) were included. Proteins were transferred to nitrocellulose and photolabeled. Bovine R' is included as a standard (STD) on the far right.

Fig. 6. Purification of R-subunit from *E. coli* and formation of holoenzyme. *E. coli* JM105, transformed with the pUC plasmid containing R', was harvested after 24 h. Left: first and second lanes, total cell extract; third lane, protein eluted from the affinity resin. (The smaller band in this lane (M, 35,000) corresponds to a proteolytic fragment of the R-subunit and was removed subsequently by gel filtration). Right, purified R-subunit was incubated with bovine heart C-subunit. Following removal of excess C-subunit, formation of holoenzyme was assessed for dependence of activity on cAMP and by gel electrophoresis. Free C-subunit (C) and R-subunit (R) are indicated in the first and third lanes, respectively. The reconstituted holoenzyme is in the center lane. Proteins were visualized with Coomassie Blue.

Fig. 7. Expression of R-subunit in *E. coli* 222. *E. coli* 222 was transformed with pUC7 and with pUC7 containing the R' insert (pLST-1). Cells were pelleted from overnight cultures, dissolved in gel loading buffer, and subjected to electrophoresis. Protein was photolabeled as described in "Experimental Procedures." *E. coli* JM105 transformed with the same plasmid was included as a control (left). R' standard is indicated on the right. Only the portion of the gel showing radioactivity is shown.

**DISCUSSION**

The cDNA corresponding to the complete coding region of the bovine type I regulatory subunit of cAMP-dependent
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protein kinase was inserted into the HincII site of pUC7. When the repressor overproducing strain of E. coli, JM105, was transformed with a plasmid that contained the R' insert in the same orientation as the lac Z' gene, a clone was isolated which expressed up to 4 mg of R-subunit/liter of culture. Two other transformants were isolated from the same ligation mixture which, on the basis of DNA analysis, were identical to the above clone. Nevertheless, these two clones did not produce large amounts of R-subunit. Further experimentation is now underway to ascertain what percentage of transformants produces R-subunit and to determine whether the lack of expression in these clones is due to a host mutation. The plasmid that has consistently yielded transformants that express R-subunit was stored as a 20% glycerol stock from cells harvested in the mid-log phase of growth, and this plasmid is subsequently referred to as pLST-1.

The expression of R-subunit did not follow the typical pattern of IPTG induction that might be anticipated for a gene linked to the lac promoter. The molecular basis for this expression is not apparent. The R-subunit obviously binds cAMP with a high affinity and thus will titrate any cAMP that is produced. However, this should not lead to activation of the lac promoter. Although there are some reports that the R-subunit may interact with DNA (36), there is no evidence as yet that suggests that any R-subunit binds specifically and with a high affinity to DNA. The homologies of the R-subunit with CAP make this an intriguing speculation (37); however, based on the structural data available, there are no clear homologies between any R-subunit and the DNA-binding domain of CAP. Nagamine and Reich (37) cite a specific sequence in the R-subunit that may be analogous to a DNA recognition sequence in CAP; however, this sequence lies in the middle of the cAMP-binding domains of R. When these domains are compared in more detail by building the R-subunit sequences into the crystal structure of CAP, it is highly unlikely that this region would appear as a structure analogous to the DNA-binding sequence in CAP.2 If the R-subunit does interact with DNA directly, the sites of interaction almost certainly must be confined to the amino-terminal region of the protein.

Although further experimentation will be required in order to understand the mechanism being utilized for the expression of R-subunit, the protein can nevertheless be expressed reproducibly in large amounts. Maximum induction was observed when media was inoculated with cultures grown to OD₅₆₀ = 0.1–0.5 and then allowed to grow into late stationary phase in the absence of IPTG. Very little R was visualized in the log phase of growth, and large amounts accumulated only in the stationary phase. Addition of IPTG during the log phase of growth blocked the expression of R-subunit. Maximum expression of R-subunit was seen in E. coli strain 222. This is a strain which contains a double mutation. It lacks adenylate cyclase (cyr-) and also contains a variant of CAP (crp') that binds DNA in a cAMP-independent manner (27). This strain expressed nearly 10-fold more R-subunit than did E. coli JM105, which is a strain that constitutively overexpresses the lac repressor.

Visualizing the R-subunit by photolabeling with 8-N₃[c³²P] cAMP following electrotransfer to nitrocellulose provided a convenient method for detection which obviated the need to remove basal cAMP from crude extracts. Apparently, the protein refolds sufficiently following electrotransfer to nitrocellulose to restore its high affinity binding for cAMP. The high selectivity is apparent since no other proteins are labeled to any significant extent in the total cellular extract. This procedure thus appears to be a very specific method for detecting R-subunits following denaturation and separation on the basis of size.

The cAMP-binding protein that was expressed had a molecular weight that was slightly larger than native R' based on gel mobilities and was consistent with the design of the construct which should yield a fusion protein. The sequence of the construct was confirmed by amino acid sequencing. Proteolysis did not appear to be a major problem in the initial extraction even though this protein is particularly susceptible to limited proteolysis. The proteolytic fragment that was observed later in the purification was identical, based on gel mobilities, to the endogenously generated fragment of the native protein, and this also is consistent with the predicted construct. This susceptibility to limited proteolysis also confirmed that the expressed protein is folding in a manner that is analogous to the native protein.

Although the expressed protein contained 10 additional residues at the amino-terminal end, it behaved like the protein purified from mammalian tissues. The expressed R-subunit was a soluble dimer, and the specificity of interaction between the protomers was confirmed by the appearance of interchain disulfide bonds. This is similar to R' from porcine skeletal muscle (19), and although it has not been ascertained that this disulfide bonding is physiological, it nevertheless does indicate that the cysteine residues from adjacent chains are in close proximity in the dimer both in the mammalian protein and in the expressed R-subunit.

Another property that is associated with the amino-terminal region of the protein is the interaction with the C-subunit, and this function also appears to be intact. When purified R-subunit was incubated with C-subunit, holoenzyme formed that was fully dependent on added cAMP for activity. Activation of the holoenzyme by cAMP indicated that the cAMP-binding sites were functional, and this was confirmed by photoaffinity labeling with 8-N₃cAMP and by successful purification with a cAMP affinity resin. Quantitation of the cAMP-binding sites established that both classes of binding sites were functional in that the expressed protein and that the expressed protein binds maximally 2 mol of cAMP/R-monomer.

By the above criteria, the expressed regulatory subunit appeared to be functioning in a manner totally analogous to its mammalian counterpart. This construct thus provides an efficient mechanism for further probing this molecule by directed mutagenesis. In addition to the functional sites described here, the molecule is known to have other functions such as specific interaction with microtubule-associated protein II (38), calcineurin (39), and p75 in brain (40). Other functions include a role as a phosphatase inhibitor (41) and a potential role as a topoisomerase I (36). These reports provide new and relatively unexplored insights into the role that this molecule may play in cAMP-mediated regulation in addition to its well-established role as an inhibitor of the catalytic subunit.

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