Isolation and characterization of cDNA clones encoding polypeptides related to a *Dictyostelium discoideum* cyclic AMP binding protein

GERARD BAIN,† CAROLINE E. GRANT‡ and ADRIAN TSANG*

Department of Biology, McGill University, Montreal, Quebec, Canada H3A 1B1

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By screening a cDNA library with a cDNA encoding the *Dictyostelium discoideum* cAMP-binding protein CABP1, under conditions of reduced stringency, we have isolated clones which code for two closely related molecules. Hybrid selection experiments indicated that these cDNAs encoded polypeptides with molecular masses of 34 (p34) and 31 (p31) kDa, both of which were recognized by anti-CABP1 monoclonal antibodies. Sequence analysis revealed that the clones were identical except for the presence of a 102 nucleotide segment inserted in-frame in the p34 cDNAs, just downstream of the translation initiation codon. DNA blot analysis suggested that p34 and p31 were encoded by the same gene. This hypothesis was strongly supported by the observation that both polypeptides were generated when a single cDNA was expressed under the control of the actin 15 promoter in *D. discoideum* cells. RNA blot analysis indicated that the cDNAs were complementary to three developmentally regulated transcripts of sizes 1.15 kb, 1.25 kb and 1.4 kb. Comparison of the derived amino acid sequences of p34 and p31 with those of the two subunits of CABP1 indicated that these polypeptides were very closely related, and that the corresponding genes probably arose by duplication followed by sequence divergence. Finally, the carboxy termini of these four polypeptides demonstrated 50% similarity to two polypeptides encoded by a bacterial plasmid which confers resistance to tellurium anions.

Introduction

The cellular slime mould *Dictyostelium discoideum* grows vegetatively as single-celled amoebae. Starvation triggers a process of development and differentiation, which ultimately leads to the formation of a multicellular fruiting body composed of two distinct cell types, spores and stalk cells (see Loomis, 1982, for a review). The molecule cAMP plays a pivotal role in the developmental cycle of *D. discoideum*. It acts as a chemoattractant to direct the aggregation of individual amoebae after development has begun, regulates the differentiation of both spores and stalk cells, and controls the expression of many developmentally regulated genes (reviewed in Gerisch, 1987). While most of these effects are mediated by the interaction between extracellular cAMP and a cell-surface cAMP receptor (reviewed in Firtel et al., 1989), it also appears that intracellular cAMP plays an important role in some of these processes (Kimmel, 1987; Kay, 1989; Riley et al., 1989).

In an attempt to identify possible pathways which could mediate the effects of intracellular cAMP on the development of *D. discoideum*, we have isolated and characterized a novel cAMP binding protein, CABP1 (Tsang & Tasaka, 1986). This protein consists of two polypeptide subunits, CABP1A and CABP1B. Western blot analysis using anti-CABP1 monoclonal antibodies indicates that there are several CABP1-related polypeptides present in developing cells (Kay et al., 1987; Tsang et al. 1988).

Two of the polypeptides which crossreact with the anti-CABP1 antibodies are of particular interest because of their developmental regulation and intracellular localization. These molecules, which have apparent molecular masses of 34 kDa (p34) and 31 kDa (p31), are not detected in vegetatively growing cells, but appear early in development (Kay et al., 1987). They are localized primarily in the nucleus (Kay et al., 1987). In this paper, we describe the molecular cloning and...
characterization of cDNAs encoding p34 and p31. We show that these two polypeptides are very closely related to the two subunits of CABP1 and provide evidence that they are the products of a single gene.

Methods

Isolation of cDNA clones. A cDNA library was constructed according to standard procedures (Maniatis et al., 1982) from a mixture of polyA+ RNAs prepared from NC4 cells that had developed for 12 and 20 h as described by Kay et al. (1987). The library was screened under conditions of low stringency with a nick-translated cDNA clone encoding a portion of CABP1 (Grant & Tsang, 1990) as described by Maniatis et al. (1982). To isolate full-length cDNAs, a Sgt11 cDNA library, kindly provided by Dr Peter Devreotes (Klein et al., 1988), was screened under high stringency conditions using a nick-translated cDNA isolated from the cDNA library described above.

DNA sequencing. The nucleotide sequences of the cDNAs were determined by the chain termination method (Sanger et al., 1977) using [35S]dATP (Amersham) as the radiolabel.

DNA blot hybridization. Genomic DNA was isolated from strain AX2 by centrifugation through CsCl density gradients (Firtel & Bonner, 1972), and Southern blotting was performed as described by Maniatis et al. (1982). The filters were prehybridized and hybridized at either 37 °C (moderate stringency) or 42 °C (high stringency). They were washed briefly in 2× SSC, 0.1% SDS at room temperature and then twice for 30 min in 2× SSC, 0.1% SDS at 65 °C (moderate stringency) or 0.1× SSC, 0.1% SDS at 68 °C (high stringency).

RNA blot hybridization. Five micrograms of polyA+ RNA isolated from NC4 cells at various times of development was fractionated on 1.5% (w/v) agarose formaldehyde gels and transferred to Genescreen membrane (New England Nuclear) (Maniatis et al., 1982). The membrane was probed using the high-stringency conditions employed for DNA blot analysis as described above.

Construction of actin 15-cDNA fusion. Oligonucleotide-directed mutagenesis (Kunkel, 1985) was used to introduce a HindIII site at the translation initiation codon of the clone pDdM34. The presence of the desired mutation was confirmed by DNA sequence analysis (Sanger et al., 1977). Digested of the resulting plasmid with HindIII released a fragment containing all the coding region of the cDNA plus 3' untranslated sequences. This fragment was then inserted into the expression vector pB10Act15KHK (obtained from Dr J. Williams, Imperial Cancer Research Fund, Clare Hall Laboratories, U.K.).

Transformation of D. discoideum. Plasmid DNA was introduced into strain AX2 by the calcium phosphate coprecipitation technique (Early & Williams, 1987). Approximately 150 independent transformants were pooled for analysis.

Hybrid selection, cell-free translation, and immunoprecipitation. These techniques were performed as described previously (Grant & Tsang, 1990).

Metabolic labelling. Bacterially-grown cells were harvested, washed three times with KKP (20 mm-potassium, phosphate, pH 6.2), and then resuspended to a final density of 5×10^7 cells ml⁻¹ in KKP. One millilitre of this suspension was spread over the surface of a 47 mm diameter polycarbonate membrane filter (Nuclepore) supported on two layers of filter paper (Whatman no. 1) saturated with KKP. After 3 h incubation at 22 °C, the filters were transferred to a clean, dry Petri dish and 20 μCi (740 KBq) [35S]methionine was added directly to them. After a 3 h labelling period, the cells were washed off the filters, lysed and analysed by immunoprecipitation as described previously (Tsang & Tasaka, 1986).

Results

Isolation of cDNAs encoding CABP1-related polypeptides

To isolate clones encoding related polypeptides, we screened a cDNA library with a 0.67 kb cDNA containing a portion of the CABP1 coding sequence (Grant & Tsang, 1990) under conditions of reduced stringency. Besides CABP1 cDNAs, this approach led to the isolation of a single related cDNA species, termed pC5D9. In order to identify this clone, messenger RNAs complementary to pC5D9 were isolated by hybrid selection, translated in vitro, and analysed by immuno-
precipitation followed by SDS-PAGE. As Fig. 1 shows, under stringent conditions pC5D9 selects RNA species which encode polypeptides with molecular masses of 34 and 31 kDa, both of which are recognized by the anti-CABP1 antibody. This observation suggests that pC5D9 contains sequences complementary to the transcripts coding for p34 and p31, and that these two transcripts share substantial similarity at the nucleotide level.

The insert in pC5D9 is only about 400 nucleotides long. To isolate full-length cDNAs for p34 and p31, a λgt11 cDNA library, kindly provided by Dr Peter Devreotes (Klein et al., 1988), was screened under highly stringent conditions using pC5D9 as a probe. Twelve positive clones were obtained after screening approximately 200000 plaques.

Sequence analysis of cDNAs

To facilitate further analysis, the longest cDNAs were subcloned into Bluescript plasmids. Fig. 2 describes the resulting clones and their restriction map as well as the strategy employed to determine their nucleotide sequences.

The two longest cDNAs, pDdM34 and pDdM31, have been sequenced completely on both strands. Each clone contains only a single long open reading frame flanked by 5' and 3' untranslated sequences. The nucleotide and deduced amino acid sequences of the cDNAs are illustrated in Fig. 3.

Remarkably, pDdM34 and pDdM31 are completely identical except for the presence of a 102 nucleotide segment located at the 5' end of pDdM34, just downstream of the translation initiation codon. This element does not disrupt the open reading frame, thereby leading to the production of a protein containing an extra 34 amino acids compared to the polypeptide encoded by pDdM31.

DNA blot analysis

Southern analysis was performed using the 3' BamHI–EcoRI fragment common to both pDdM34 and pDdM31 as a probe (probe 1 in Fig. 2). As Fig. 4(a) shows, only a single band hybridized to the probe under conditions of high stringency for all enzymes tested. Even if the stringency of hybridization was reduced, the only additional bands recognized by this probe contain the CABP1 gene (Fig. 4b). Therefore, the transcripts complementary to pDdM34 and pDdM31 appear to be derived from a single gene.

RNA blot analysis

To examine the pattern of expression of the transcripts complementary to pDdM34 and pDdM31, polyA+ RNA isolated from cells at various times of development was analysed by RNA blotting. Highly stringent conditions were employed to prevent hybridization to CABP1 RNAs. As is shown in Fig. 5, three transcripts with lengths of approximately 1.15 kb, 1.25 kb and 1.4 kb were detected during growth and at all times of development, although their relative amounts were variable.
Fig. 3. Nucleotide and deduced amino acid sequences of cDNAs. The sequence of the two longest cDNAs, pDdM34 and pDdM31, was determined completely on both strands. The remaining clones have not been sequenced entirely on both strands. The 5' and 3' borders of all cDNAs are indicated. The 3' end of pC5D9 was not determined. The border that is marked represents the 3' end of our sequencing data for this clone. The sequence which is underlined represents the 102 nucleotide element which is absent from pDdM31. Nucleotide numbers are on the left while amino acid numbers are on the right.
Comparison of polypeptides encoded by pDdM34 and pDdM31 to CABPl and related molecules

As described earlier, the polypeptides encoded by pDdM34 and pDdM31 are identical except for the presence of a 34 amino acid segment inserted near the amino terminus of the larger molecule. Similarly, we have found that the two subunits of CABPl are also identical, except for the presence of an additional 37 amino acids at the amino terminus of CABPlA. We have recently demonstrated that these subunits are the products of a single gene (Grant & Tsang, 1990), and that they are generated by an alternative splicing mechanism (Grant et al., 1990). An alignment of the deduced amino acid sequences of the polypeptides encoded by pDdM34 and pDdM31 with those of CABPlA and CABPlB is shown in Fig. 6.

The similarity between these four molecules is remarkable. The amino-terminal sequences are virtually identical, with only two amino acid differences in the first 45 residues. Significantly, the sequence which is specific to the polypeptide encoded by pDdM34 almost exactly matches the region of CABPl which is found only in CABPlA. Furthermore, the nucleotide sequence encoding this element is flanked precisely by the splice consensus signals GT/AG, suggesting that the transcript which is complementary to pDdM31 is generated by a splicing process very similar to the one which produces CABPlB (Grant et al., 1990).

Immediately following this highly conserved amino terminus, the polypeptides diverge considerably. This region in CABPl, which is 94 amino acids in length, is very distinctive in its amino acid content. It contains a high proportion of proline and glutamine residues, as well as five copies of the nine amino acid repeat QPAQQYQAP (Grant & Tsang, 1990). In the molecules encoded by pDdM34 and pDdM31, however, this region contains only 46 residues. Even though it is considerably shorter than the same element in CABPl, its overall character remains very similar since it is also very rich in...
Fig. 6. Alignment of the amino acid sequences of CABPl and related polypeptides. The amino acid sequences of the polypeptides encoded by pDdM34 and pDdM31 were aligned with CABPl and two related molecules, designated ORF4 and ORF5, encoded by a bacterial plasmid which confers resistance to tellurium anions, by pairwise comparison using the ALIGN program (Pearson & Lipman, 1988). The underlined sequences are absent from CABPl and the polypeptide encoded by pDdM31. Identical amino acids are represented by dots, while dashes indicate gaps.

No other proteins whose sequences are present in the GenBank, EMBL or NBRF databases have been found to possess significant similarity to these molecules.

Expression of actin 15-pDdM34 fusion gene in Dictyostelium cells

Southern blot analysis (Fig. 4) suggests that the polypeptides encoded by pDdM34 and pDdM31 are the products of a single gene. To obtain further evidence for this hypothesis, the pDdM34 cDNA clone was expressed in D. discoideum cells under the control of the actin 15 promoter using the expression vector pB1OAct15BKH. The expression construct codes for a translational fusion in which the amino-terminal 14 amino acids are contributed by vector sequences while the remainder is derived from the open reading frame present in pDdM34.
Fig. 7. Expression of pDdM34 under the control of the actin 15 promoter. (a) The details of the act 15-pDdM34 fusion are shown. The nucleotide sequences provided by the expression vector pBlOAct15BKH are shown in small letters, while those present in pDdM34 are in capitals. The numbers above the sequence represent amino acid position in the fusion, while those below the sequence indicate the codon position in the open reading frame of pDdM34. After introducing the expression construct into D. discoideum cells, the transformants were analysed by metabolic labelling with $^{35}$Smethionine followed by immunoprecipitation (b). The two subunits of CABPl, 1A and 1B, are labelled, along with p34 and p31. Lane 1, immunoprecipitated products obtained from control AX2 cells; lane 2, immunoprecipitated products obtained from cells transformed with the expression vector lacking the pDdM34 insert; lane 3, immunoprecipitated products obtained from cells transformed with the actin 15-pDdM34 fusion construct.

(Fig. 7a). Stable transformants were isolated and analysed by metabolic labelling with $^{35}$Smethionine followed by immunoprecipitation with a monoclonal antibody which recognizes CABPl as well as a number of antigenically related polypeptides (Tsang & Tasaka, 1986). As Fig. 7(b) clearly shows, transformants carrying this construct express large amounts of two polypeptides which migrate slightly more slowly than the endogenous CABP1-related p34 and p31 molecules. This result indicates that the information required to generate both these polypeptides appears to be present in pDdM34. Furthermore, it strongly supports the hypothesis that these two polypeptides are generated from one gene.

Attempts to use the overexpressing strain for a biochemical analysis of the polypeptides encoded by pDdM34 have not been successful. A more detailed examination of this strain has revealed that the overexpression of these polypeptides is not very stable. In addition, immunofluorescence experiments have demonstrated that the overexpressed molecules appear to be forming aggregates in the cytoplasm and therefore may not be functional (data not shown).

Discussion

Based on the data presented here, it is very likely that pDdM34 and pDdM31 respectively encode the CABP1-related polypeptides p34 and p31, which cross-react with anti-CABP1 antibodies and also copurify with CABP1 (Tsang & Tasaka, 1986). First, hybrid selection analysis of the cDNA clone pC5D9 (Fig. 1) indicates that this sequence is complementary to mRNAs which encode these two molecules. Second, when a cDNA library was screened using this clone as a probe, two different classes of cDNAs were isolated, represented by pDdM34 and pDdM31. Third, the sizes of these two clones agree well with the sizes of two of the transcripts which are detected by Northern blotting (Fig. 5). Finally, these two polypeptides appear to be the products of a single gene (Figs 4 and 7) and are very similar to the two subunits of CABP1, which are also produced from a single gene by alternative splicing (Grant et al., 1990). Taken together, these data strongly suggest that pDdM34 and pDdM31 are complementary to the mRNAs which encode p34 and p31.

RNA blot analysis demonstrated that three developmentally regulated transcripts with sizes of 1.15 kb, 1.25 kb and 1.4 kb are complementary to pDdM34 and pDdM31 (Fig. 5). The 100 bp difference in size between the two smaller transcripts, in conjunction with their overall length, suggests that the cDNA clones are derived from them. The precise origin of the 1.4 kb mRNA species is unknown at present. We do not believe that this transcript is generated from another closely related gene. Southern analysis demonstrated that only a single gene encodes p34 and p31 (Fig. 4a). Under low stringency conditions, p34/31 probes crossreacted with only one additional gene, that which encodes CABP1 (Fig. 4b). CABP1 probes, however, do not hybridize to the 1.4 kb transcript under conditions of high stringency (Grant et al., 1990). Therefore, this mRNA probably corresponds to a third transcript which is generated by the p34/31 gene. The determination of its precise origin and function will require additional analysis.

The three transcripts detected by probes derived from pDdM34 and pDdM31 are present during both growth and development, although their levels are quite variable (Fig. 5). In contrast, p34 and p31 cannot be detected in growing cells by immunoblotting. They appear soon after starvation and then remain at relatively constant levels throughout development (Kay et al., 1987). We do not know the reason for the discrepancy between the expression of the mRNAs and that of the polypeptides. The significance of the fluctuation in transcript levels during development is also not known.

Based on the high similarity between their polypeptide products (Fig. 6), it is probable that the CABP1 and...
p34/31 genes arose by duplication. Subsequent sequence divergence has led to a major change in only one domain, with the remainder of the two genes maintaining close similarity. The virtual identity of the amino termini of p34/31 and CABP1 in contrast to the relatively weaker similarity displayed by the remainder of the proteins suggests that this region of the polypeptides is crucial for function. Furthermore, this area contains the segments which are removed from the CABP1 and p34/31 primary transcripts to generate CABP1B and p31. The strong similarity displayed by the remainder of the two genes and CABP1 also raises the question of whether these proteins possess the cAMP binding activity which are removed from the CABP1 and p34/31 primary transcripts. These differences suggest that these molecules do not perform exactly the same functions in the cell.

The functional relationship between p34 and p31 is not clear. Since CABP1 operates as a heterodimer between CABP1A and CABP1B (Tsang & Tasaka 1986), it is possible that p34 and p31 associate to form one functional protein. Alternatively, all four polypeptides may be required to form an active complex. More study is required to clarify whether or not this is the case.

The discovery of very high similarity between CABP1, p34/31 and two bacterial polypeptides encoded by a plasmid which confers resistance to tellurium anions (Jobling & Ritchie, 1988) (Fig. 7) was completely unexpected. This close similarity suggests that these molecules have a common origin. It also implies that they probably have similar functions. Unfortunately, the mechanism which imparts tellurium resistance in bacteria is not known, so we can gain no clues as to the function of p34/31 and CABP1. The results described in this paper directly demonstrate that the D. discoideum cAMP binding protein CABP1 belongs to at least a small family of related polypeptides. Additional distinct cDNA clones which encode other molecules which are recognized by anti-CABP1 monoclonal antibodies are currently being analysed in our laboratory. These studies should allow us to determine the precise size of the CABP1 family and may also provide some clues as to the functions of these polypeptides.

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References


