Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 431-439

CLONING AND SEQUENCING OF A FULL LENGTH cDNA CORRESPONDING TO HUMAN CELLULAR RETINOL-BINDING PROTEIN

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Received June 5, 1985

We have isolated and sequenced a cDNA clone corresponding to the human cellular retinol-binding protein (CRBP). The deduced amino acid sequence, which encompasses 134 amino acid residues, shows significant homology with several low molecular weight proteins which bind hydrophobic ligands. No homology to the plasma retinol-binding protein was observed. Southern and Northern blot analyses suggest that the CRBP gene is present in a single copy in the haploid genome and that it is transcribed in a single mRNA species.

Vitamin A is indispensable for the well being of all mammals. Deficiency of the vitamin induces impaired dark adaptation, which is a corollary to the role of the vitamin in the visual process. Other consequences of vitamin A deficiency are hyperkeratosis of several epithelia, sterility, infections etc. (1). Inasmuch as these effects may be secondary to the deficiency, consensus has not been reached as regards the general molecular mode of operation of the vitamin. However, it seems established that retinoids (vitamin A and related compounds) play an important role in cell differentiation. Thus, it has been shown that retinoids regulate keratin biosynthesis in terminally differentiating

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0006-291X/85 $1.50

epidermal cells (2). Also murine embryonal carcinoma cells and human promyelocytic leukemia cells (HL60) are stimulated to differentiate into endodermal cells and granulocytes, respectively, after exposure to such compounds (3, 4). Furthermore, retinoids have been implicated in the suppression of malignant and transformed cells induced by chemical carcinogens, radiation and transforming growth factors (5-7).

Under physiological conditions large amounts of vitamin A are stored in the liver. The hydrophobic vitamin is mobilized by associating with a specific carrier protein, the retinol-binding protein (RBP), which in plasma occurs bound to transthyretin (reviewed in ref. 8). On the surface of vitamin A-requiring cells there exists a receptor recognizing RBP (9, 10). In view of the insolubility of vitamin A in aqueous media it is not surprising that intracellularly it is bound to specific proteins. Two such proteins have been identified and due to their ligand-binding specificities they are called the cellular retinol-binding protein (CRBP) and the cellular retinoic acid-binding protein (CRABP), respectively (reviewed in ref. 11). These proteins display similar physico-chemical properties and exhibit approximately 40% amino acid sequence homology (11, 12).

Vitamin A in plasma occurs almost exclusively in the alcohol form, retinol. Therefore, it is conceivable that CRBP serves as an acceptor protein for retinol transferred across the plasma membrane. In order to gain knowledge about the flux of vitamin A in a target cell and to ultimately define its molecular mode of action it appears mandatory to explore the

functions of CRBP and CRABP. As an initial effort in this direction we report here the cloning and sequence determination of a cDNA corresponding to human CRBP.

MATERIALS AND METHODS

Screening the cDNA library

A human liver cDNA library in the expression vector pEX was kindly provided by Dr. K. Stanley (13). The library was screened by the colony blot procedure using antibodies against rat CRBP at a dilution of 1:500 (3), and HRP-conjugated goat anti-rabbit IgG (Institut Pasteur) at a dilution of 1:1000 as the second antibody.

Nucleotide Sequence Analysis

Sequence analysis was performed using the method of Maxam and Gilbert (14).

Enzymes and Chemicals

Restriction endonucleases and DNA polymerase I were obtained from Biolabs and Boehringer Mannheim, respectively. The ^{32}P labelled compounds were purchased from Amersham Buchler, Braunschweig.

Northern and Southern Blot Analysis

RNA extraction and Northern blot analysis were carried out as previously described (15). High molecular weight DNA from peripheral blood was digested with EcoRI, HindIII and TaqI, size fractionated on a 0.8% agarose gel and transferred to gene screen membrane. The filter was hybridized to the ^{32}P labelled cDNA fragment (specific activity 2×10^8 cpm/μg DNA) as in (16).

RESULTS

Cloning of cDNA Corresponding to Cellular Retinol-Binding Protein

A human liver cDNA library in the expression vector pEX (13) was screened using antibodies raised against rat CRBP (17). Out of 70,000 clones several were positive using the colony blot technique (13). The clone showing the strongest signal after purification, called CRBP-clone 1, was further characterized. It had an insert of about 840 base pairs, the restriction map of which is shown in Figure 1 together with

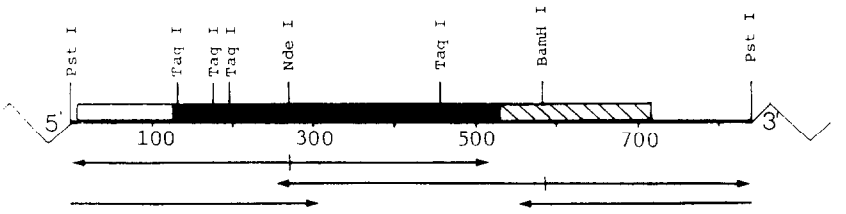

Fig. 1. Restriction map of the cDNA clone coding for the Cellular Retinol-Binding Protein.
The coding part of the insert is indicated by the solid bar while the 5' and 3' flanking regions are represented by open and slashed boxes, respectively. The arrows show the direction of the sequence as determined by the chemical degradation procedure after labelling the ends.

an outline of the strategy used for sequencing. Analysis of the sequence (Fig. 2) reveals an initiation codon in position 126 followed by an open reading frame up to nucleotide 530, encoding 134 amino acids. The coding portion is bounded by 125 and 183 bp of 5' and 3' untranslated regions, respectively. A potential poly(A) addition site AATAAA preceeds the poly(A) tail of 19 nucleotides in the 3' flanking sequence.

Following the first ATG in the nucleotide sequence an uninteruppted open reading frame of 402 bp exists. The amino acid sequence deduced from this nucleotide stretch is virtually identical to the protein sequence previously determined for rat CRBP (18). This firmly establishes that the isolated cDNA clone corresponds to human CRBP. Out of the 134 amino acids that seem to constitute the mature human protein 129 residues are identical to the rat homologue. The five differences observed occur in the NH_2-terminal, positions 5 and 12, and COOH terminal regions, positions 121, 125 and 134, of the protein (Fig. 2). The high degree of homology between the human and rat CRBPs is further emphasized by the observation that three of the amino acid replacements may be accounted for by single point mutations.

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GGGGGGGGGCGGAGGGCGCTCATTTCCGGGCCGCCCACCACCCGCGTAGCACCGGCAGCC   60

GCTGTCCCGGCAGTCTCCAGCCGTCCCGCCCGCTTGTGGCCAAACTGGCTCCAGTCACTC  120

      -1  +1             (Asn)                 10    (Ser)
      Met Pro Val Asp Phe Thr Gly Tyr Trp Lys Met Leu Val Asn
CCGAA ATG CCA GTC GAC TTC ACT GGG TAC TGG AAG ATG TTG GTC AAC 167

                          20
Glu Asn Phe Glu Glu Tyr Leu Arg Ala Leu Asp Val Asn Val Ala
GAG AAT TTC GAG GAG TAC CTG CGC GCC CTC GAC GTC AAT GTG GCC   212

     30                                   40
Leu Arg Lys Ile Ala Asn Leu Leu Lys Pro Asp Lys Glu Ile Val
TTG CGC AAA ATC GCC AAC TTG CTG AAG CCA GAC AAA GAG ATC GTG   257

                          50
Gln Asp Gly Asp His Met Ile Ile Arg Thr Leu Ser Thr Phe Arg
CAG GAC GGT GAC CAT ATG ATC ATC CGC ACG CTG AGC ACT TTT AGG   302

     60                                   70
Asn Tyr Ile Met Asp Phe Gln Val Gly Lys Glu Phe Glu Glu Asp
AAC TAC ATC ATG GAC TTC CAA GTT GGG AAG GAG TTT GAG GAG GAT   347

                          80
Leu Thr Gly Ile Asp Asp Arg Lys Cys Met Thr Thr Val Ser Trp
CTG ACA GGC ATA GAT GAC CGC AAG TGC ATG ACA ACA GTG AGC TGG   392

     90                                   100
Asp Gly Asp Lys Leu Gln Cys Val Gln Lys Gly Glu Lys Glu Gly
GAC GGA GAC AAG CTC CAG TGT GTG CAG AAG GGT GAG AAG GAG GGG   437

                         110
Arg Gly Trp Thr Gln Trp Ile Glu Gly Asp Glu Leu His Leu Glu
CGT GGC TGG ACC CAG TGG ATC GAG GGT GAT GAG CTG CAC CTA GAG   482

    120(Ala)           (Thr)                130
Met Arg Val Glu Gly Val Val Cys Lys Gln Val Phe Lys Lys Val
ATG AGA GTG GAA GGT GTG GTC TGC AAG CAA GTA TTC AAG AAG GTG   527

134
(His)
Gln ***
CAG TGA GGCCCAAGCAGACAACCTTGTCCCAACCAATCAGCAGGATGTGTGAGCCAG   584

GATCCCTCTTTGCACAGCATGAGGCAAAAATGTCCAGCCACCCCTAGGCATCTGTTAGC   643

AGAGTCTGTCTCTTGGCTTTGTCACTTTTCCTTTTCTTAAAACAAAGCCATGCCAATAA   702

AGTGACCTGTGTTC poly(A)                                         716
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Fig. 2 DNA sequence of the CRBP cDNA clone.
The lower line shows the nucleotide sequence of the clone; the upper line the deduced amino acid sequence. On the right side is reported the nucleotide numbering, while the position of the amino acids is indicated above the corresponding codons. The amino acids within parentheses are the substitutions observed in the rat CRBP sequence.

Northern and Southern Blot Analyses

To determine the size of the CRBP-specific mRNA, Northern blot analysis was performed on total RNA isolated from human and rat liver, respectively. As shown in Fig. 3 a single species of mRNA of approximately 650 to 750 nucleotides in length was identified in both cases. This information clearly suggest that the characterized cDNA clone represents a full length or near full length copy of the mature CRBP mRNA.

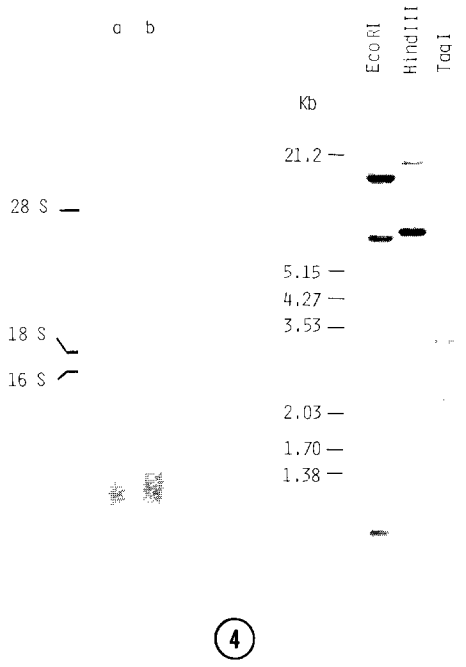

Fig. 3. Northern blot analysis.
Ten µg of total RNA extracted from human liver (lane a) and 10 µg of total RNA from rat liver were size fractionated on a 1.2% formaldehyde-agarose gel and transferred onto a nitrocellulose paper. The filter was then hybridized to a ^{32}P labelled 550 bp cDNA fragment coding for CRBP. The migration of ribosomal RNAs used as molecular weight markers is indicated.

Fig. 4 Southern blot analysis
Ten µg/slot of chromosomal DNA from peripheral blood were digested with EcoRI, HindI and TaqI restriction enzymes, electrophoresed on a 0.8% agarose gel and transferred to gene screen membrane. The filter was then hybridized to the nick-translated cRBP DNA as described.

Southern blot analysis on human chromosomal DNA extracted from peripheral blood was also carried out. Since CRBP appears to be a member of a large protein faimly (see below) stringent hybridication conditions were used to minimize cross-hybridizations to CRBP-related sequences. The results are summarized in Fig. 4. The hybridization patterns are consistent with each haploid genome containing one CRBP gene. In fact, the three hybridizing fragments generated by TaqI are the minimum that can be expected from the distribution of the four sites present in the cDNA sequence.

DISCUSSION

The role of CRBP in the metabolism of vitamin A is not yet known. The protein occurs in widely differing amounts in several tissues (17, 19, 20) suggesting that the gene is controled by complex mechanisms. This notion is reinforced by the observations that the levels of CRBP increase during perinatal development and during progression of certain types of experimental and spontaneous tumours (21-24). However, the finding that liver and kidney cells express considerably greater amounts of CRBP than other cell types examined is consistent with this protein having an intracellular transport function (see ref. 17). This notion receives additional support from the amino acid sequence deduced from the cDNA clone of human CRBP reported here. Thus, CRBP display considerable homology to the amino acid sequences of several low molecular weight proteins, i.e. myelin protein P2 (25), two fatty acid-binding proteins (26, 27) and protein 422 cloned from the adipocyte cell line 3T3-LI (28), which all may be engaged in the intracellular transport of hydrophobic ligands.

CRBP also displays considerable homology to CRABP particularly in the NH_2-terminal region (12). It is conceivable that this structural homology signifies that the two proteins exhibit similar molecular modes of operation. In contrast, no homology is observed between CRBP and plasma RBP even though they bind exactly the same ligand. The three-dimensional structure of RBP has been elucidated and it reveals a ß-barrel structure with retinol situated in a central cavity of the protein (28). Neither the NH_2-terminal nor the COOH terminal region of RBP is involved in the actual binding of the vitamin. Despite lack of similarity in

primary structure between CRABP and RBP it would not be surprising should it turn out that the two proteins display similarities in their three-dimensional structures. It appears likely that due to its hydrophobicity retinol is deeply burried also within the CRBP molecule. In this context it is interesting to observe that the amino acid substitutions that distinguish human from rat CRBP occur in the NH_2- and COOH-terminal regions leaving residues 13 to 120 identical in the two proteins. Thus, it may tentatively be suggested that CRBP like RBP does not use the terminal regions of the polypeptide chain in binding the ligand.

Should the function of CRBP be that of intracellularly transporting retinol, the question about the destination of this transport remains. One possibility that has been considered is the transport of retinol to the nucleus, which would imply that CRBP may serve a function similar to that of steroid hormone receptors. It has been shown that CRBP containing retinol is able to bind to isolated liver cell nuclei with high affinity and in a saturable fashion (29, 30). Whether such events are accompanied by the selective transcription of one or more genes remains to be elucidated.

Be that as it may the availability of a cDNA clone for CRBP will prove useful in the further dissection of the intracellular metabolism of vitamin A.

ACKNOWLEDGEMENTS

We wish to thank Carol Featherstone for critical reading of the manuscript and Christina Plöen and Heide Seifert for secretarial help. This work was supported by grants from The Swedish Medical Research Council.

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