Cloning, Structure, and Expression of a Rat Binding Protein for Polychlorinated Biphenyls

HOMOLOGY TO THE HORMONALLY REGULATED PROGESTERONE-BINDING PROTEIN UTEROGLOBIN*

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Certain metabolites of polychlorinated biphenyls (PCBs) are retained in the Clara cells and in the airway lumen of rodent lung due to their interaction with a secretory 13-kDa protein. Here, we report the isolation of a cDNA encoding the rat lung PCB-binding protein. The identity of the PCB-binding protein is supported by expression of the cDNA in Cos-1 cells where the homogenates from transfected cells show specific binding of 4,4'-bis([14C]methylsulfonyl)-2,2',5,5'-tetrachlorobiphenyl, a high affinity ligand for the PCB-binding protein. Also a monospecific antiserum to the PCB-binding protein recognizes a 13-kDa protein in the homogenates of transfected cells but not in the corresponding fraction of mock-transfected cells. Northern blot analysis of total RNA from different rat tissues demonstrates that the cDNA detects a ~600-base pair mRNA which appears to be solely expressed in lung. Interestingly, DNA sequence analysis and prediction of the amino acid sequence reveals that the PCB-binding protein shares 53% positional amino acid identity with uteroglobin, a progesterone-binding protein found in rabbit uterus and lung. Furthermore, amino acids shown by x-ray crystallography to delineate the central cavity of uteroglobin, which fits progesterone, are highly conserved in the two proteins.

Respiratory disorders have been described for human subjects accidentally or occupationally exposed to polychlorinated biphenyls (PCBs)⁴ (Shigematsu et al., 1978; Warshaw et al., 1979) and more than 60 different methylsulfonyl-PCBs have been purified from rat lung and consists of two apparently identical subunits held together by disulfide bridges (Lund et al., 1988a) and a physicochemically similar protein has been characterized in human bronchoalveolar lavage fluid (Lund et al., 1986). In view of the respiratory disorders observed in PCB-exposed human subjects and the pathway for the accumulation of methylsulfonyl-PCBs in lung described above it is tempting to speculate in a causative relationship. To test such a hypothesis would require insight into the physiological function of the PCB-binding protein. In this paper we describe the cloning and expression of a cDNA encoding the rat lung PCB-binding protein. The DNA sequence analysis reveals a structural relationship between the PCB-binding protein and uteroglobin, a hormonally regulated steroid-binding protein in rabbit (Bailly et al., 1983). The kinship of these proteins suggest novel approaches to the elucidation of the physiological role of the PCB-binding protein and of its role in PCB-induced lung toxicity.

EXPERIMENTAL PROCEDURES AND RESULTS²

DISCUSSION

In this paper we describe the cloning, structure, and expression of a cDNA corresponding to a rat lung PCB-binding protein. When expressed in Cos-1 cells, the cDNA directs the synthesis of a protein that is recognized by a monospecific antiserum to the rat PCB-binding protein. Also, the expressed protein has ligand binding properties that are indistinguishable from those of the purified rat PCB-binding protein (Lund et al., 1988b).

² Portions of this paper (including "Experimental Procedures," "Results," and Figs. 1–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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1 The abbreviations used are: PCB, polychlorinated biphenyl; ([14C]MeSO₂), TCB, 4,4'-bis([14C]methylsulfonyl)-2,2',5,5'-tetrachlorobiphenyl; HPLC, high performance liquid chromatography; PHES, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfite-polyacrylamide gel electrophoresis.

2 Portions of this paper (including "Experimental Procedures," "Results," and Figs. 1–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
FIG. 5. Alignment of the deduced amino acid sequence of the rat PCB-binding protein with that of uteroglobin. The amino acid sequence of the rat PCB-binding protein (rUGL) was aligned with the amino acid sequence of uteroglobin (Bailly et al., 1983) using the program FASTA (Pearson and Lipman, 1988). Amino acid identities are connected by straight lines. Amino acids in the PCB-binding protein are numbered above the sequence and amino acids in uteroglobin are numbered below the sequence; residues in the putative secretory leader sequences are assigned negative numbers, whereas amino acids in the mature proteins are assigned positive numbers. Underlined are amino acids in uteroglobin that are localized to the central ligand-binding cavity (bold) and the cysteins that participate in disulfide bridge formation (italic).

et al., 1985, 1988b), whereas the electrophoretic mobility of the expressed protein is slightly different from that of the purified protein, possibly due to differences in post-translational modification. RNA blotting experiments indicate that the mRNA for the PCB-binding protein is solely expressed in the lung and genomic DNA blotting experiments suggest that the protein may be encoded by a single copy gene. The deduced protein sequence of the PCB-binding protein reveals a structural similarity (53% positional amino acid identity in a 90-amino acid overlap) to a steroid-binding protein, uteroglobin (Menne et al., 1982, Bailly et al., 1983). Uteroglobin has been previously thought to be exclusively expressed in rabbits and other members of the order Lagomorph (Savouret and Milgrom, 1983) although some studies suggest an immunological cross-reactivity with a protein in humans (Dhanireddy et al., 1988). The observed similarity between uteroglobin and the PCB-binding protein may prove to be of particular importance with regard to our future structural and functional analysis of the PCB-binding protein.

The three-dimensional structure of uteroglobin is known in detail by means of x-ray crystallography (Mornon et al., 1980; Morize et al., 1987; Bailly and Deletré, 1989). Uteroglobin is a dimer of two identical monomers (A and B) of 70 amino acids held together by two disulfide bridges between residues 3 and 69 (38[A]-69[B] and 69[A]-38[B]). It is a globular protein that can be included in a sphere with a radius of 17 Å. Most of the hydrophobic residues are totally or partially buried inside the protein and most of the hydrophobic groups are exposed on the surface. An oblong hydrophobic pocket is present centrally in the protein and is delimited by hydrophobic residues Ile-2, Phe-6, Val-9, Ile-10, Leu-13, Leu-14, Leu-25, Gly-38, Met-41, Leu-45, Ile-56, Met-57, Leu-59, and Ile-63 and by 2 polar residues, Tyr-21 and Thr-60. These amino acids may be important for the specificity of ligand binding. In particular the hydroxyl groups of Tyr-21 and Thr-60 can interact with oxygens in progesterone, a putative physiological ligand for the protein. It is of great interest that most of these particular residues are present in the PCB-binding protein and that they are identically spaced as in uteroglobin (Fig. 5). It is therefore our hypothesis that the three-dimensional structure of the PCB-binding protein is very similar to that of uteroglobin, keeping the conserved hydrophobic amino acids as well as the equivalents of Tyr-21 and Thr-60 in a central ligand binding cavity. Amino acids in the PCB-binding protein that through this structural analogy to uteroglobin would be assigned to the surface of the protein are not identical to the same extent. This may explain why an antiserum to the rat PCB-binding protein does not recognize a protein in lung fractions from other species and why antibodies to uteroglobin do not react with the PCB-binding protein (Lund et al., 1988b). Our model thus envisages two homologous proteins (i.e. of common evolutionary origin) in which divergent evolution has left a modest overall amino acid identity (53%) but where the structural basis for ligand binding has been conserved. To us the data further imply that ligand binding is linked to the function of the proteins.

The extension of this model would therefore suggest similar functions of the PCB-binding protein and uteroglobin. To what extent can this help in the elucidation of the physiological role of the PCB-binding protein and of the toxicological implications of its PCB interactions? Preliminary results regarding the amino-terminal sequence of the PCB-binding protein prompted us to test whether methylsulfonyl-PCBs bind purified uteroglobin and if they fit its central hydrophobic cavity as studied by molecular graphics and interactive energy minimizations, and in both instances they do (Gillner et al., 1988). The limited availability of purified uteroglobin prevented a detailed analysis of the binding affinities for different PCB metabolites. Clearly both proteins bind progesterone with similar affinities (Beato and Baier, 1975; Lund et al., 1985). The physiological significance of the binding of progesterone to uteroglobin is not well understood, but it has been suggested that since rabbit uteroglobin is present in uterine fluids early in pregnancy it may function to protect the developing conceptus from toxic levels of progesterone (reviewed in Savouret and Milgrom, 1983). Other proposed mechanisms of action of uteroglobin include protease inhibition and immunological masking of antigens (Savouret and Milgrom, 1983). Perhaps most intriguing is the recent suggestion that uteroglobin can inhibit phospholipase A₂ (Miele et al., 1988) since exposure of humans to PCBS is associated with inflammatory symptoms from the airways and an accumulation of methylsulfonyl-PCBs in the lungs of exposed subjects is well documented (Shigenatsu et al., 1978; Haraguchi et al., 1984). Bronchoalveolar lavage fluid from healthy volunteers has also been shown to contain a binding protein for 4,4′-bist([1]H)methylsulfonyl)-2,2′,5,5′-tetrachlorobiphenyl which is physicochemically similar to the rat PCB-binding protein (Lund et al., 1986). The availability of a full-length cDNA for the PCB binding protein should allow for the expression of sufficient quantities of the protein to test if it is also an inhibitor of phospholipase A₂ and to address the role of ligand binding in this putative function. Finally, purification and cloning of corresponding cDNA for the human PCB-binding protein will show to what extent the shared ligand binding properties reflect a structural relation to the rat PCB-binding protein and uteroglobin.

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Supplemental Material:

"Closing, structure and expression of a rat binding protein for polychlorinated biphenyls: a hypothesis for the hormonally regulated progestin-binding protein suppressor gene." by

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**EXPERIMENTAL PROCEDURES**

All enzymatic manipulations and reagents were based on standard procedures (Sambrook et al., 1989).

**Protein expression.** The rat long PCB-binding protein was purified as previously described (Lund et al., 1988).

The final fraction was subjected to preparative SDS-PAGE and the protein was visualized by rehydration of the gel in 1 M KCl. The 13-kDa protein was then excised (Stetter and Belfrage, 1983), dialyzed against H2O and the protein was sequenced with an Applied Biosystems model 477A sequencer equipped with an on-line HPLC for PTH amino acid analysis.

**cDNA cloning.** Two non-overlapping "gene-specific" 5' oligonucleotides were synthesized based on the expression sequence of the rat PCB-binding protein and a rat cDNA library (100-1000 nucleotides). A rat long cDNA library in a mammalian expression vector produced by Mr. K. Lorentzen and Dr. M. Morten (Department of Medicine, School of Medicine, Sweden) was prepared in E. Coli Y1000. DupliGene screening (Colonyscreen screen, DuPont) were filled from each clone and individually screened with either of the two "gene-specific" that had been labeled with [35S]-ATP and [3H]polyadenylic kinase. Filters were pre-processed overnight in 2X SSC, 0.1% SDS, 1 and EDTA at 56°C and were then prehybridized for 6 h in 2X SSC, 1% SDS. After hybridization, the filters were washed at 56°C. The next day the filters were washed at 56°C, 2 X 2 min, 2 X 30 min in 2X SSC, 0.2 SDS, and 5 X SSC, 0.2 M Na-acetate, and washed in 20% formic acid. Two clones remained positive after hybridization. Both clones were subjected to Southern hybridization with EcoRI-digested DNA by standard techniques. The probe used was generated using PstI and BamHI restriction endonucleases. The EcoRI-digested DNA was hybridized to EcoRI-digested DNA by hybridization to the rat cloned using the radiolabeled "gene-specific" (Wallace and Miyada, 1987). The DNA sequence was then analyzed using PstI and EcoRI restriction enzymes to determine the location of the DNA sequence as indicated by autoradiography. The radiolabeled DNA was then analyzed using PstI and EcoRI restriction enzymes to determine the location of the DNA sequence as indicated by autoradiography. The radiolabeled DNA was then analyzed using PstI and EcoRI restriction enzymes to determine the location of the DNA sequence as indicated by autoradiography.

**Expression in Cos-1 cells.** The full-length cDNA was ligated into the EcoRI site of plasmid pN71 (Andonner et al., 1989) and transformed into E. Coli DH10B. The desired recombinant containing the cDNA inserted in the correct orientation was selected by restriction mapping and sequencing. The resulting plasmid (pN71PCB1C) was purified by standard methods (Sambrook et al., 1989). Two colonies were then transformed into the DE3 strain of E. Coli using the DE3 strain of E. Coli (Hannick and Danna, 1986) followed by selection treatment for 5 hours (Hannick and Danna, 1986). A cell homologue from Cos-1 cells was prepared at the indicated time points following transformation by scraping the cells with PBS, washing twice with PBS, and then homogenizing the cells with glass/membrane homogenizer. The protein content of the homogenate was determined by the benzene assay method and then analyzed by SDS-PAGE method and Western blotting as described previously (Lund et al., 1988; Lund, 1988) using [125I]polyethylene glycol (PEG) bound to protein A-Sorbent (provided by Dr. J. Danna and Dr. M. Morten). The homogenate was then analyzed by SDS-PAGE and Western blotting as described previously.

**RESULTS**

The rat long PCB-binding protein was purified to homogeneity and the nucleotide sequence was sequenced. Based on the amino acid sequence and a rat cDNA library, two non-overlapping "gene-specific" that were used to screen a rat long cDNA library in a mammalian expression vector produced a hydrogenating. The rat long cDNA sequence as determined by sequencing was sequenced from a positive clone. The cloned amino acid sequence contains the amino-terminal sequence determined by sequencing (underlined in Fig. 1) preceded by a 19 amino acid hydrophobic leader sequence. The leader sequence presumably represents a proline signal sequence and it is consistent with the proposed signal sequence (Wendel and Miyada, 1986). The deduced open reading frame of the cDNA encodes a protein of 96 amino acid residues with a predicted M; of 10,641 and the predicted protein is equivalent to the rat long cDNA. The predicted protein contains the nucleotide sequence of the rat PCB-binding protein which has a calculated M, of 12,000 determined by SDS-PAGE.
Structure of a PCB-binding Protein

PAGE (Land et al., 1984b). SDS-PAGE analysis of the dicarbocyclic PCB-binding proteins indicates a Mr of 13,000 (Land et al., 1984b) whereas the predicted Mr of the monomer as deduced from the cDNA is 16,932. SDS-PAGE of the dicarbocyclic PCB-binding proteins would therefore appear to underestimate the actual Mr.

To confirm that the cDNA encodes the PCB-binding protein, the cDNA in Cos-1 cells was transfected using a vector, pCMV5, into which the expression is driven by the promoter-enhanced region of the human cytomegalovirus (Adamsen et al., 1984). Cos-1 cells transfected with the plasmid pCMV5/CBP1 or containing the full-length cDNA were harvested at 24 and 48 hours post-transfection and homogenates were prepared. As shown in Fig. 2, the appearance of protein in the homogenates from Cos-1 cells transfected with pCMV5/CBP1 is similar to that observed for the natural homologous protein. In contrast, transfection of Cos-1 cells with pCMV5/CBP6 led to the appearance of a protein of similar size and mobility in the homogenate (Fig. 3A). When the expression curve was transformed according to Wolf (1987) to a ratio of 417 folding of homologous protein and a K_M of 0.4 M were obtained (Fig. 3B).

The K_M indicates that the level of expression is quite high, with the PCB-binding protein constituting approximately 0.5% of the homologous protein from transfected Cos-1 cells.

We next determined the tissue distribution of the PCB-binding protein mRNA. Total RNA was prepared from various tissues and then size fractionated by agarose gel electrophoresis. The size-fractionated RNA was hybridized directly in the gel with 32P-labeled cDNA probes generated by the random hexamer method. Of the tissues tested, only the lung expressed a 0.6-kb mRNA detected by the PCB-binding protein cDNA (Fig. 4). No evidence for multiple mRNA species was obtained. Further hybridization experiments using restriction enzymes of the genomic DNA showed a simple pattern of hybridization bands, suggesting a single gene or at least a low copy number gene for the PCB-binding protein in the rat genome (data not shown).

Finally, the deduced amino acid sequence of the rat PCB-binding protein was used to search protein sequence databases in data banks. Of great interest was the finding that the rat PCB-binding protein shows a 21% positional amino acid identity with novel euxanthins (Bally et al., 1983). This is shown by the alignment of the two proteins illustrating the extensive similarities (Fig. 4A). This suggests that the amino acids in euxanthins that have been shown by x-ray crystallography to accommodate the central alkyl hydrophobic cavity thought to bind to PCBs (Morrow et al., 1986; Morrow et al., 1987), are similar to the protein. As can be seen in Fig. 5, three positive liquid binding amino acids show a high degree of identity, both with regard to the actual amino acids as well as to the spacing between them. Furthermore, most amino acid differences between the two proteins were conserved.