Structure and Function of a Clara Cell Secretory Protein: A Determinant of Methylsulfonyl-PCB Bioaccumulation

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1. Introduction

Respiratory disorders have been described for human subjects accidentally exposed to polychlorinated biphenyls (PCBs) and large numbers of different methylsulfonyl-PCBs have been identified in lungs from such individuals (1, 2). These sulfur-containing PCB metabolites have been shown in animal studies to arise during enterohepatic circulation of PCB-glutathione conjugates. Depending on the positions of the chlorine atoms and the methylsulfonyl moieties, certain PCB methyl sulfones accumulate in the lungs and kidneys of rats and mice (3). Using such a metabolite which had been radioactively labeled, a selective *in vivo* localization of the injected compound in the apical cytoplasm of the non-ciliated, bronchiolar Clara cells of rodent lung could be demonstrated (4, 5). Subsequent work identified and purified a binding protein for 4,4'-bis(methylsulfonyl)-2,2',5,5'-tetrachlorobiphenyl from both rat lung and human broncholaveolar lavage fluid which reversibly bind the PCB metabolite with high affinity and specificity (6, 7). Monospecific antibodies were used to clearly show that the protein is localized to the secretory granules of the Clara cells and in the epithelial lining fluid, a localization which perfectly mirrors the *in vivo* disposition of the radioactively labeled 4,4'-bis(methylsulfonyl)-2,2',5,5'-tetrachlorobiphenyl (8). The only putative endogenous ligand found for the protein was the steroid hormone progesterone suggesting that perhaps the respiratory symptoms observed in humans could be due to methylsulfonyl-PCBs mimicking and/or interfering with an endocrine pathway in lung.

To obtain further information regarding the possible function of the rat and human PCB binding proteins, the corresponding cDNAs were isolated and the primary structures determined (6, 9). From this and subsequent analysis, it has become clear that the PCB binding proteins constitute homologues of uteroglobin, a progesterone-binding protein initially discovered in uterine secretions of pregnant rabbits, and that they are identical to rat and human Clara Cell Secretory Protein (CCSP or CC10). Despite enticing hypothesis regarding the function of these proteins in the literature, the precise physiological role remained obscure making predictions of a role in PCB-induced respiratory symptoms very speculative. As further steps toward understanding the possible toxicological role of CCSP in PCB poisoning, we embarked on two major projects addressing the structure and function of the protein. First, to obtain solid proof of the association of the methylsulfonyl-PCB with CCSP and to understand the molecular basis of the high affinity and specificity of binding, we determined the structure of the protein in complex with its ligand by NMR. Second, to establish the *in vivo* role of CCSP in PCB bioaccumulation and to obtain an animal model in which to study the physiological function of CCSP as well as the

role in PCB poisoning, mice which are homozygously deficient in CCSP were generated.

2. Materials and Methods

The cDNA for rat CCSP was used to generate an expression plasmid which introduces four histidine codons immediately preceding the stop codon. The protein was expressed in *E. coli* and purified using NiNTA agarose followed by FPLC. ^{13}C, ^{15}N-labelled CCSP was produced by growing bacteria ina medium containing ^{13}C, ^{15}N IsogroTM (Isotec Inc.), $^{15}NH_4Cl$ and ^{13}C-labelled glucose. The purified protein was ether extracted to remove bound impurities. 4,4'-bis([^{13}C]methylsulfonyl)-tetrachlorobiphenyl was synthesized from 2,2',5,5'-tetrachlorobenzidine as described (10). NMR spectroscopy was performed on the CCSP-PCB complex at 30 ^{o}C on a Varian Unity 500 spectrometer using a triple-resonance $^{1}H/^{13}C/^{15}N$ pulsed field gradient probe. NMR assignments and structure determinations were performed as described (10).

The mouse CCSP gene sequences were derived from a genomic clone from a mouse strain 129 genomic library. A targeting vector was constructed in which the gene was disrupted through the insertion of the neo^{r} gene at the 3'-end of exon 1. Targeted ES cells were microinjected into blastocysts and chimeric mice generated. First generation agouti offspring that were heterozygous for the mutant CCSP allele were crossed to establish lines homozygous for either the wild-type or mutant (-/-) alleles. These mice were then investigated with respect to the expression of mRNA for CCSP and other proteins in their lungs by RNAse protection, with respect to Clara cell morphology by electron microscopy and with respect to PCB accumulation by injection of radioactive PCB methyl sulfone (11).

3. Results and Discussion.

By NMR it was possible to determine the solution structure of CCSP in complex with 4,4'-bis([^{13}C]methylsulfonyl)-tetrachlorobiphenyl. CCSP is a dimeric protein where the monomer is composed of four helices. Hydrophobic residues in helices I, II and III of each monomer pack against each other and helices II and III are also packed against helix IV of the other monomer. Each monomer forms an oblate-shaped structure in which surfaces of helices I, III and IV form the dimer interaction surface. The structure reveals the molecular basis for the accumulation certain methylsulfonyl-PCBs by CCSP. The PCB ligand binds in a central cavity formed by side chains and backbone atoms from all eight helices. Ligand-protein interactions are predominantly hydrophobic. The only polar interactions are found between the two Tyr 23 hydroxyl groups and the sulfonyl groups of the PCB metabolite, which involve hydrogen bonds between the hydroxyl protons and one of the sulfonyl oxygen atoms. Interestingly, the structure also shows how ligand binding and release may be controlled by reduction/oxidation of two intermolecular disulfide bonds. Breakage of these bonds induces a local unfolding of the N- and C-termini and a separation of helices creating a channel into the protein. These effects make the ligand binding cavity readily accessible to entry of the ligand. Finally, the entire cavity is not filled by the ligand. A small part of the cavity extends towards the region containing the junction of helices III and IV. Thus, our results not only establish the molecular basis for PCB binding by CCSP but point to an interesting mechanism whereby the entry and exit of ligand can be controlled by oxidation/reduction which possibly could occur through an active mechanism in the lungs. Also, the fact that the ligand binding site could accomodate larger ligands suggest that one should search for additional endogenous ligands.

Mice homozygous for a null allele of the CCSP gene were generated. Measurements of the steady-state levels of CCSP mRNA within total lung RNA demonstrated the

complete absence of normal CCSP transcripts within the lungs of CCSP -/- mice. Transmission electron microscopy of airway cells revealed striking differences between the Clara cells of CCSP -/- and wild-type mice. The Clara cells from CCSP -/- mice completely lacked secretory granules. Challenge studies involving intraperitoneal administration of a radioactively labeled PCB metabolite revealed dramatic differences in the ability of CCSP -/- and wild-type mice to concentrate the compound to target tissues. Essentially, -/- mice were unable to concentrate the PCB metabolite to the lungs and kidneys. Our results provide genetic evidence of a major role for CCSP in the bioaccumulation of certain methylsulfonyl-PCBs and establishes a model in which to study CCSP function and possible role in PCB toxicity.

4. Conclusion

The molecular basis for the binding of certain methylsulfonyl-PCBs to CCSP has been determined and genetic evidence for the importance of CCSP in the bioaccumulation of these compounds has been provided. The establishment of mice homozygously deficient in CCSP now provide a model in which to study CCSP function and possible role in PCB-induced toxicity.

5. References

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