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DETECTION OF A BETA-PARVALBUMIN ISOFORM IN THE MAMMALIAN INNER EAR

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Summary: A small, acidic calcium-binding protein (CBP-15) has been detected in the guinea pig organ of Corti, the auditory receptor organ. The apparent molecular weight (15,000) and very low isoelectric point (pI ≈3.1) suggest that CBP-15 is a β-parvalbumin isoform. Consistent with this hypothesis, CBP-15 exhibits extreme homology to the mammalian oncofetal parvalbumin called oncomodulin. Sequence data have now been obtained for 30 residues in the N-terminal third of CBP-15. Identity with oncomodulin is observed at all 30 positions This finding could necessitate revision of the assumption that postnatal mammals utilize a single α-parvalbumin isoform in muscle and nonmuscle settings alike. © 1995 Academic Press, Inc.

The organ of Corti is the mammalian auditory receptor organ. It is composed exclusively of epithelial cells, the inner and outer hair cells, the receptor cells proper, and several types of supporting cells. The organ of Corti is the site where acoustic messages arriving at the inner ear are transduced into electrical signals transmitted to the central nervous system (1).

We recently identified a small, acidic calcium-binding protein in the organ of Corti (2). This protein (CBP-15) was not detected in any other mammalian tissues, cochlear or otherwise, nor was it found in the avian equivalent of the organ of Corti, the basilar papilla. With an apparent M_r of 15,000 and pI ≈3.1, CBP-15 is a candidate for membership in the β sub-lineage of the parvalbumin family. Although the β-parvalbumin known as oncomodulin (3,4) is expressed

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Abbreviations: ATH, avian thymic hormone; CPV3, chicken parvalbumin 3, an avian thymic parvalbumin; CBP-15, small, acidic calcium-binding protein detected in the organ of Corti from guinea pig; OM, oncomodulin, a mammalian β-parvalbumin expressed in placenta and neoplasms; RPV, rat α-parvalbumin.

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in the fetal placenta and neoplasms (5-10), the protein has not been detected in normal mammalian somatic tissues. It is generally believed that postnatal mammals express a single parvalbumin isoform, which belongs to the α sub-lineage (11). Identification of a β-parvalbumin in the inner ear would necessitate substantial revision of this paradigm. Therefore, we have sought to determine, by direct amino acid sequencing, whether CBP-15 represents a known parvalbumin isoform or in fact constitutes a novel Ca^{2+}-binding protein.

MATERIALS AND METHODS

Reagents were obtained from Sigma Chemical Co. (St. Louis, MO), Fisher Scientific Co. (Pittsburgh, PA), and Bio-Rad (Richmond, CA), unless otherwise stated.

Tissue preparation: Young guinea pigs weighing between 250-350 g were anesthetized to a deep plane of surgical anesthesia with veterinary pentobarbital (33 mg/kg i.p.) and decapitated. Temporal bones were rapidly removed from the skull, cleared of soft tissues, immersed in Freon-12 chilled to its melting point with liquid nitrogen, and freeze-dried at -40° C for five days. The organ of Corti was dissected at room temperature at relative humidity of 40% or less. About 15 μg of freeze-dried organ of Corti were obtained per ear. Details of preparative method and dissection are described elsewhere (12).

Non-denaturing isoelectric focusing: The protocol described in the Hoefer Scientific Instruments (San Francisco, CA) catalog of 1992-1993 was followed with modification in the ampholytes (3.75% of pI 3.5-10, 1.88% of pI 3-5, 0.37% of pI 2.5-4) . For details, see ref. 2.

V8 protease digestion: Limited digestion with *Staphylococcus aureus* V8 protease was carried out by the method of Cleveland et al. (13), as modified by Kennedy et al. (14). The band corresponding to CBP-15 was excised from the gel and transferred to the well of a slab gel (18% polyacrylamide, 1.5 mm thick) to which various amounts of V8 protease were added. The protein mixture was then allowed to run to the stacker/separating gel interface at 50 volts, at which point the current was turned off for 30 minutes. The run was then completed at a constant voltage of 200. For details, consult ref. 15.

Electroblotting: The gel containing the CBP-15 and peptide fragments thereof was electophoretically transferred *in toto* onto Immobilon-P^{SQ} (Millipore, Bedford, MA), employing a Hoefer TE-22 Transfer Electrophoresis unit at a constant voltage of 90 for 120 minutes. The membranes were then briefly stained with Coomassie Blue to allow visualization of the transferred peptide bands.

Amino-terminal sequencing: The bands corresponding to CBP-15 and select fragments were excised from the membrane and subjected directly to sequencing on a Model 470A gas-phase sequenator (Applied Bio-Systems, Foster City, CA), equipped with an on-line phenylthiohydantoin detector model 120A. Because the N-terminus of CBP-15 was blocked, the membrane fragment bearing the intact protein was first subjected to deblocking by treatment with trifluoracetic acid vapor at 60°C for 30 minutes, according to the method of Hirano et al. (16), and resubjected to sequence analysis.

Sequence evaluation: Amino acid sequence data were evaluated for homology with known proteins in the Non-redundant PDB + SwissProt + PIR + SPUdate + GenPept + GPUpdate databases, employing BLAST (Basic Local Alignment Search Tool, ref. 17) comparison, on the World Wide Web BLAST server at the National Center for Biotechnology information (NCBI) (http://www.ncbi.nlm.nih.gov/Recipon/bs_seq.html/).

RESULTS AND DISCUSSION

Parvalbumins are small (M_r 11,500), vertebrate-specific proteins containing two high-affinity Ca^{2+}-binding sites (18-20). Particularly abundant in skeletal muscle, they have also been

detected in select neurons (21), and in kidney, adipose tissue, and testis (11). Parvalbumins are generally regarded as cytosolic Ca^{2+} buffers, with suggested roles in muscle relaxation, neuronal de-excitation, and maintenance of Ca^{2+} homeostasis (22,23). However, there is increasing speculation that specific isoforms may fulfill more specialized biological functions. For example, the mammalian oncofetal parvalbumin called oncomodulin is conjectured to function as a Ca^{2+}-dependent regulatory protein during early embryological development and tumorigenesis (24).

Although the apparent molecular weight determined for CBP-15 by gel electrophoresis (2) appears high for a parvalbumin, it should be noted that several other parvalbumin isoforms have likewise been shown to display anomalously high M_r values. Oncomodulin and the avian parvalbumin called CPV3, for example, both display apparent molecular weights near 15,000 (25,26) when examined by SDS-PAGE.

The parvalbumin family comprises two sub-lineages, α and β, which can be distinguished on the basis of several lineage-specific sequence differences (27). Moreover, β isoforms ($pI < 5$) tend to be more acidic than their α counterparts ($pI \geq 5$). As shown in Figure 1, CBP-15 is clearly the most acidic protein present in the organ of Corti. The apparent isoelectric point is 3.1, considerably lower than that of calmodulin. If CBP-15 is in fact a parvalbumin, it would undoubtedly belong to the β lineage.

The partial amino acid sequence for CBP-15 presented in Figure 2 displays striking similarity to oncomodulin. The internal fragment obtained by V8 digestion exhibits 100% identity with residues 18-34 of the oncofetal protein. Likewise, the 14 residues of the amino-terminal sequence that could be assigned with some degree of certainty are identical to oncomodulin. Homology to other parvalbumins is lower, but nevertheless substantial -- e.g., rat parvalbumin (50%) and the avian parvalbumin CPV3 (73%).

Unfortunately, the available sequence does not include data for either of the Ca^{2+} binding sites (residues 51-62 and 90-101). However, on the basis of physical properties and the observed sequence homology, it is reasonable to conclude that CBP-15 belongs to the parvalbumin family.

There is a distinct phylogenetic trend toward reliance on fewer parvalbumin isoforms. Lower vertebrates express multiple parvalbumins in skeletal muscle (e.g., 30,31). Mammals, by contrast, have retained just one muscle isoform (11). In fact, it is generally accepted that this single parvalbumin, an α isoform, is utilized in muscle and non-muscle tissue settings alike (11). Although mammals also elaborate a β-parvalbumin (oncomodulin), normal expression of the protein is thought to be confined to extra-embryonic tissue of the fetal placenta (5,6). Our results cast uncertainty on this assumption and raise some interesting issues.

Birds, like mammals, express a single muscle parvalbumin (32). However, they express two thymus-specific β-isoforms, ATH (33-35) and CPV3 (26), that are presumably involved in

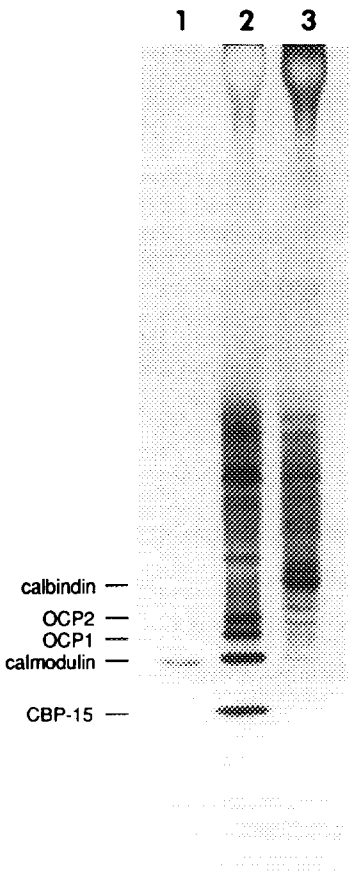

Figure 1. Silver-stained nondenaturing (native) isoelectric focusing gel. Lane 1: calmodulin standard; Lane 2: organ of Corti; Lane 3: basilar papilla. 0.5 μg of standard and 5 μg total protein of tissue were loaded. Reprinted with permission from Hearing Research (2).

some aspect of avian immune function. In this context, it is significant that CBP-15 is not detected in the basilar papilla, the avian auditory receptor organ which in many respects resembles the mammalian organ of Corti (2). The basilar papilla is instead a rich source of calbindin-28K, another calcium binding protein (2). Conversely, calbindin, the major protein in the basilar papilla, is insignificant in the organ of Corti.

It is apparent from the partial sequence data presented herein that a β-parvalbumin is expressed in the organ of Corti. Whether or not the protein is in fact oncomodulin is presently unclear. An unequivocal answer must await acquisition of the entire CBP-15 amino acid sequence.

The organ of Corti is a microscopic structure; a single guinea pig cochlea (4½ turns) yields just 8 μg of total protein, approximately 1% of which (80 ng) is believed to correspond to

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                1                  10                  20                  30
CBP-15     [S I T(D I L S)A X(D)I A X X L Q] [C Q D P D T F E P Q K F F Q T S G]

OM (28)     S I T D I L S A E D I A A A L Q E C Q D P D T F E P Q K F F Q T S G

RPV (29)    S M T D L L S A E D I K K A I G A F T A A D S F D H K K F F Q M V G

CPV3 (26)   S L T D I L S P S D I A A A L R D C Q A P D S F S P K K F F Q I S G
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Figure 2. CBP-15 and rat oncomodulin display extremely high sequence homology. Preliminary amino acid sequence data for CBP-15 is displayed above along with the N-terminal sequences of several relevant parvalbumin isoforms. The sequence information for residues 1-16 was obtained by N-terminal sequencing after deblocking with trifluoracetic acid. Residues 18-34 were obtained by sequencing a peptide fragment released by treatment of intact CBP-15 with V8 protease. X indicates that the residue could not be assigned with any certainty. At several positions, more than one residue was detected. For these cases (italicized and enclosed in parentheses), the major species is reported. Residues shown in bold are identical in CBP-15 and oncomodulin. Data for OM, RPV, and CPV3 were taken from refs. 28, 29, and 26, respectively.

CBP-15. Thus, cDNA cloning and mass spectroscopy, rather than direct amino acid sequencing, are the preferred strategies for obtaining the complete sequence. The coding sequence for OCP2, a protein expressed predominantly in the organ of Corti, was recently isolated from an organ of Corti cDNA library (36). We are hopeful that a similar approach can be used to clone the message for CBP-15.

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