Ontogeny of calbindin-D$_{28K}$ and calbindin-D$_{9K}$ in the mouse kidney, duodenum, cerebellum and placenta

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Summary

The appearance of the calcium-binding proteins (CaBP-D$_{28K}$ and CaBP-D$_{9K}$) in embryonic mouse tissues was determined using a sensitive immunohistochemical assay. CaBP-D$_{28K}$ first appears in myenteric nerve plexuses of the duodenum on day E15, in duodenal villus cells on day E16, in Purkinje cells of the cerebellum on day E19, in cells of the mesonephric duct on day E11 and in the metanephric duct on day E12. CaBP-D$_{9K}$ first appears in enterocytes of the duodenum on day E18, in trophoblastic giant cells (TGC) of the placenta on day E10, and in the metanephric duct on day E15. A differential time of appearance and colocalization of the two CaBPs is demonstrated in the embryonic mouse kidney, suggesting either that vitamin D does not control both CaBPs in the foetus or that the vitamin D control is unequal. The early appearance and location of CaBP-D$_{9K}$ in TGCs may suggest that these cells play an important role in transplacental transfer of calcium.

Key words: calbindins, immunohistochemistry, vitamin D, trophoblastic giant cells, metanephric duct.

Introduction

The group of calcium-binding proteins known as the calbindins (CaBPs) plays a cardinal role in calcium metabolism, acting either as an intracellular facilitator of calcium diffusion or as an intracellular buffer of calcium (Wasserman and Fullmer, 1983; Norman, 1990). The presence of CaBP-D$_{28K}$ in nerve terminals (Jande et al., 1981a,b; Baimbridge and Miller, 1982; Resibois et al., 1988a) suggests a further role for CaBP in neurotransmission. Production of CaBP in a number of tissues is mediated via vitamin D-dependent processes (Haussler et al., 1974; Henry and Norman, 1984). The CaBP group comprises a 28 000 $M_r$ CaBP (CaBP-D$_{28K}$), first found in avian intestine (Wasserman and Taylor, 1966) and a 9 000 $M_r$ CaBP (CaBP-D$_{9K}$), originally found in mammalian intestine (Kallfelz et al., 1967). The presence of CaBPs in numerous tissues of the body has been observed (for review see, Christakos et al., 1989). In most mammals, either CaBP-D$_{28K}$ or CaBP-D$_{9K}$ occur exclusively in a particular tissue (Christakos et al., 1989). Notable exceptions to this generalization are: the presence of both CaBPs in chondrocytes of epiphyseal growth plates (Balmain et al., 1991) and in ameloblasts of developing teeth (Berdal et al., 1991), the transient appearance of both CaBPs in the foetal rat kidney and the presence of both CaBPs in the adult mouse kidney (Rhoten et al., 1985).

Recently, CaBP research has focused on the time at which the CaBPs appear in the various embryonic tissues and reproductive organs, since this can then be correlated to specific events that mark normal development. In the rat reproductive system, CaBP-D$_{9K}$ has been localized in (a) the myometrium, (b) the endometrial stroma of the non-pregnant uterus and the transporting epithelium of the visceral yolk sac, (c) the intraplacental yolk sac (IPYS) (Delorme et al., 1983; Bruns et al., 1985) and the luminal epithelium of the maternal uterus in late gestation, and (d) the epithelial cells lining the fallopian tubes of the mature rat (Mathieu et al., 1989a,b). In the mouse, CaBP-D$_{9K}$ is present in the uterus of pregnant mice and in the endodermal cells of the IPYS (Bruns et al., 1985).

During late gestation in rats and mice, both placental and maternal intestinal levels of CaBP-D$_{9K}$ increase in response to the calcium demands of the developing skeleton (Bruns et al., 1981). First appearance of CaBP-D$_{9K}$ in the yolk sac endoderm of the rat occurs on day E10 (Mathieu et al., 1989a,b), and in the mouse IPYS epithelium on day E17 (Bruns et al., 1985).

Renal CaBP-D$_{28K}$ first appears in the distal convoluted tubules of the rat metanephros on day E19 of gestation (Chandler and Bucci, 1978). This is much later in development than chick renal CaBP-D$_{28K}$ which appears in the mesonephric duct on day E7, and in metanephric tubules on day E12 (Opperman et al., 1990). Duodenal CaBP-D$_{9K}$...
first appears in enterocytes of rat villi on day E17.5 of development (Delorme et al., 1979), and similarly in enterocytes of chick villi on day E21 of development (Opperman et al., 1990).

Enderlin et al. (1987) demonstrated CaBP-D<sub>28K</sub> staining of rat Purkinje cell bodies on day E15 of gestation, which is two days earlier than that demonstrated by Legrand et al. (1983). Similarly, Opperman et al. (1990) have shown chick cerebellar CaBP-D<sub>28K</sub> to be present on day E15 in Purkinje cell bodies prior to neurite extension.

We wish to determine the time of first appearance of CaBP in several tissues (placenta, kidney, duodenum, cerebellum) of the embryonic mouse and to relate this to the functional maturation of these tissues. Furthermore, we wish to establish whether there is either a sequential or simultaneous appearance of CaBP-D<sub>28K</sub> and CaBP-D<sub>9K</sub> in the foetal kidney.

Materials and methods

Animals

Time-mated White laboratory mice of the MF1 strain were used. All animals were housed at 24°C, in an ultraviolet free, 12:12 hour light/dark cycle. Food and water were supplied ad libitum. The day of first appearance of a vaginal plug counted as day E1 of gestation. Six embryos (three from each of two females) were obtained at each stage. Pregnant females were killed by asphyxiation with CO<sub>2</sub> each day from day E11 to two days postnatally. In this strain, parturition occurred on day E19.

Tissue collection

Both kidneys, the duodenum (taken from the pyloro-duodenal junction to a fixed distance 3 mm distal to the junction), cerebellum and placenta from each embryo were fixed in Bouins fluid for 4 hours. Tissues were routinely processed through a graded series of alcohols, cleared in chloroform and embedded in paraplast. Four consecutive 5 µm thick sections were selected at 50 µm intervals throughout the length of each organ.

Immunocytochemistry

Sections were subjected to the indirect peroxidase immunocytochemical technique of Polack and Van Noorden (1978).

Rabbit antisera to rat renal CaBP (28K) and to mouse intestinal CaBP (9K) were a gift of L.Bruns (New Jersey). Anti-rabbit peroxidase-conjugated antiserum was obtained from Dakopatts, Denmark. Sections were reacted with the antisera (CaBP dilutions ranged from 1:250 for first appearance to 1:1000 for later identification) for 18-36 hours at 4°C.

Sections of adult mouse kidney and rat kidney (previously shown to give a positive reaction with antisera) were included as positive controls in each ICC run.

Controls for antibody specificity consisted of replacing the specific antisera with:

1. antiserum preabsorbed overnight at 4°C with purified antigen (the final concentration of antiserum/antigen was the same as the primary antibody used in each run); (2) non-immune rabbit serum at the same concentration as the primary antibody and (3) diluent.

Sections were viewed and photographed under the light microscope using interference microscopy. Consecutive sections were labelled so as to visualize colocalization of both CaBPs within the same cells of the kidney.

Results

Mesonephric kidney

CaBP-D<sub>28K</sub>-immunoreactive cells were first observed in the mesonephric duct and tubules on day E11. At this stage, both the duct and tubules were seen as distinct tubular structures. Immunoreactivity was observed in both the nuclei and cytoplasm of the cells. Not all cells stained positive for CaBP, and the staining intensity of positive cells varied from cell to cell. By day E15, no CaBP-D<sub>28K</sub> staining remained in the mesonephros. CaBP-D<sub>9K</sub> did not appear at all in the mesonephros (Fig. 1).

Metanephric kidney

CaBP-D<sub>28K</sub>-immunoreactive cells were first observed in the cells of the metanephric duct on day E12, one day after the appearance of the duct in the embryo. CaBP-D<sub>9K</sub>-immunoreactive cells were first observed in cells of the metanephric duct and tubules on day E15 (Fig. 1A,B), but not all cells in a tubule stained positively and the intensity of staining varied from cell to cell. Fig. 1 shows CaBP-D<sub>9K</sub> staining the straight collecting tubules and arched collecting tubules of the still developing outer cortex. Absorption controls for CaBP-D<sub>9K</sub> showed no staining of the metanephros (Fig. 2). By day E18 the structure and staining pattern of the metanephric kidney became more like that of the adult. Consecutive sections showed that both CaBPs were present in the same cells of some distal convoluted tubules from day E15 (Fig. 3A,B).

Duodenum

Immunoreactive CaBP-D<sub>28K</sub> first appeared in cells of the short, thick duodenal villi on day E16 (Fig. 4). Immunoreactivity in these cells was cytoplasmic and generally diffuse. However, the staining pattern of two types of cells

Fig. 1. (A) Mesonephros (M) and metanephros on day E15. CaBP-D<sub>9K</sub> immunoreactivity is seen in DCTs, in arched collecting tubules and in straight collecting tubules of the developing outer cortex. No CaBP-D<sub>28K</sub> immunoreactivity is seen in cells of the adjacent mesonephros. ×100. (B) High power of section of metanephros shown in A. CaBP-D<sub>9K</sub> immunoreactivity is present in the cytoplasm of most cells of the metanephric tubules. ×300.

Fig. 2. Adjacent section to Fig. 1A, showing the absence of DAB staining from metanephros after incubation with preabsorbed anti-CaBP-D<sub>9K</sub>. ×100.

Fig. 3. (A,B) Adjacent sections of metanephric tubules (day E15). CaBP-D<sub>28K</sub> (A) immunoreactivity and CaBP-D<sub>9K</sub> (B) immunoreactivity is seen in the same cells of metanephric tubules. Counterstained with haematoxylin. ×300.

Fig. 4. Immature duodenal villus on day E16. CaBP-D<sub>28K</sub> immunoreactivity can be seen extending into the apical cytoplasmic process of an ‘open type’ of gut endocrine cell. ×300.

Fig. 5. Mature duodenal villus on day E18. Immunoreactivity for CaBP-D<sub>28K</sub> is concentrated around the nucleus and the base of the cell. Counterstained with haematoxylin. ×300.
was quite distinct. The first type demonstrated a distinct apical cytoplasmic process, characteristic of the ‘open type’ of gut endocrine cell (Fig. 4). The second cell type demonstrated perinuclear and basal cytoplasmic staining, also characteristic of some gut endocrine cells (Fig. 5). CaBP-D28K also appeared in myenteric ganglion cells and fibres of the duodenum on day E15 (Fig. 6).

By day E17 the villi had lengthened and immunoreac-
tive CaBP-D<sub>9K</sub> first appeared in enterocytes of villi a day later, on day E18 (Fig. 7). Very few cells stained positively and the staining reaction was generally diffuse. On day E19 extensive staining of enterocytes was observed which resembled the staining pattern of the adult duodenum.

**Placenta**

CaBP-D<sub>28K</sub> first appeared in trophoblastic giant cells (TGC), which separated the decidua from Reicherts membrane on day E10 (Fig. 8). Cytoplasmic staining extended into the long cytoplasmic processes of the giant cells (Fig. 8). The intensity of the CaBP staining of the TGCs decreased during gestation, so that by day E18 only a few cells were weakly stained for CaBP. At this stage, the columnar epithelial cells of the intraplacental yolk sac (IPYS) were intensely stained.

**Cerebellum**

Purkinje cell bodies could be seen forming a distinct layer beneath the external germinal layer on day E18, but no visible processes were seen and no positive staining for CaBP-D<sub>28K</sub> was observed. By day E19 a distinct line of Purkinje cell staining was evident (Fig. 9). Staining of this layer was observed to be above the level of the background staining.

**Discussion**

All foetal tissues examined in this study stained positive for CaBP. The time of first appearance was however different in each tissue. Detection of CaBPs first occurred in the TGCs of the placenta on day E10. In the cerebellum however, CaBP first appeared in Purkinje cells shortly before parturition on day E19.

The TGCs appear on day 4.5 of gestation and continue to grow in thickness until they form a loose network of cells attaching embryonic to maternal tissues (Theiler, 1972). These TGCs are believed to be responsible for invading the uterine wall during blastocyst implantation (Rugh, 1968). The early appearance (day E10) of CaBP in TGCs and their morphological distribution between maternal and foetal components of the placenta suggest that the tissue is
involved in the transplacental transfer of calcium to the foetus.

The appearance of CaBP-D_{28K} in yolk sac endoderm on day E10 and in IPYS epithelium are similar to those reported by Bruns et al. (1985). As the IPYS develops and acquires CaBP, there is a concurrent decrease in staining intensity of the TGCs.

CaBP-D_{28K} has only recently been found in the myenteric plexuses of the duodenum (Lee et al., 1987; Resibois et al., 1988b). In the present study, CaBP-D_{28K} was found on day E15 in myenteric ganglion cells and fibres of the duodenum, and in cerebella Purkinje cells on day E19. CaBP-D_{28K}, but not CaBP-D_{9K}, has been found in both the rat and chick CNS (Jande et al., 1981; Baimbridge et al., 1982; Feldman and Christakos, 1983; Legrand et al., 1983) and in nerve plexuses of avian intestine (Resibois et al., 1988a). Recently Resibois et al. (1988a) reported the presence of CaBP-D_{28K} in the myenteric plexus, submucosal plexus and lamina propria of the rat gut. In the cerebellum, CaBPs are present in the Purkinje cells (Enderlin, 1987; Legrand et al., 1983; Opperman et al. 1990) and may also be found in astrocytes of neuroglia (Barbin et al., 1991). Maturation of the cerebellum in the mouse occurs very late in gestation, when compared to that of the rat (day E16; Legrand et al., 1983; Enderlin, 1987) and chick (day E15; Opperman et al., 1990). In the mouse the Purkinje cell layer appears as late as day E17. This is followed by the almost immediate appearance of CaBP. Indeed CaBP appears in Purkinje cells before these cells have developed their characteristic arboreal appearance and its presence is detected as soon as these cells become recognisable. Since transepithelial transport of calcium in nervous tissues is minimal, CaBP is believed to act as an intracellular calcium buffer, regulating calcium levels in neurons with excessive calcium influx (Jande et al., 1981a,b). In addition, CaBP may also play a role in synaptic transport or axoplasmic transport.

CaBP-D_{28K} appears in cells of mouse duodenal villi on day E16. In the adult duodenum, cells containing CaBP-D_{28K} have also been found to contain the neurotransmitters neurotensin (Resibois et al., 1988) and serotonin (Buffa et al., 1989). Furthermore, CaBPs are present in endocrine tissues (e.g., pancreas, thyroid and suprarenal gland; Buffa et al., 1989). A potential relationship thus exists between the various hormones and calcium metabolism and transport.

In the present study, the appearance of duodenal CaBP-D_{9K} in the embryonic mouse begins one day before birth. This suggests that the enterocytes are prepared for active calcium transport. Similar findings have been reported for rat duodenal CaBP, which appears 1.5 days before birth (DeLorme et al., 1979), and for chick duodenal CaBP which first appears on the day of hatching (Opperman et al., 1990). Marche et al. (1980) suggested that CaBP, is an indicator of enterocyte maturation, as it appears in differentiated villus cells with the development of a brush border.

First appearance of renal CaBP in chick mesonephros and metanephros coincides with the functional maturation of these structures (Opperman et al., 1990). The present study has shown a differential time of appearance of the two CaBPs in the foetal mouse kidney. CaBP-D_{28K} appears to play a role in both the mesonephros and the metanephros, for it appears in both these structures as soon as they become discernable. Unlike CaBP-D_{28K}, CaBP-D_{9K} only appears on day E15, one day after the metanephros is said to become functional (Rugh, 1968). Both proteins occur in the mouse embryo and are conserved in the adult. Why both CaBPs are found in the same cells of distal convoluted tubules remains unclear. Since the two CaBPs are also co-localised in cell types involved in vast movements of calcium (i.e. chondrocytes of growth plates and ameloblasts of teeth), presence of both CaBPs in mouse kidney may indicate a need for a much finer control of calcium concentration. The two CaBP systems would thus act separately but in a synchronized manner to achieve this control. The fact that they appear at different times does suggest that vitamin D most likely does not control the production of both CaBPs in the foetal kidney. Alternatively, the vitamin D control of both CaBPs may be unequal as it appears to be in the neonatal mouse kidney (Li and Christakos, 1991).

Vitamin D-dependency of CaBPs has been previously reported in chick intestine (Wasserman and Taylor, 1966) and kidney (Taylor and Wasserman, 1967). In fact, the intestine is the one organ that shows a clear relationship between 1,25(OH)_{2}D_{3}, CaBP production and calcium absorption (Norman, 1990). A recent report has suggested that renal CaBP-D_{28K} in the adult mouse is under the influence of some other substance for its production (Bruns et al., 1987), and is independent of vitamin D. Furthermore, uterine CaBP-D_{9K} (Bruns et al., 1988) and fallopian tube CaBP-D_{9K} (Mathieu et al., 1989) are both responsive to exogenous oestrogen and not to exogenous vitamin D. It appears therefore as if the vitamin D dependency of the CaBPs may be tissue specific and species specific and that the CaBPs may be under the control of other factors.

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References


