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Minireview

Calcyclin – from basic research to clinical implications*

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Discovery of calcyclin

The calcyclin gene (2A9) was isolated as one of the cDNA clones from baby hamster kidney cells stimulated by serum [1, 2]. Gene 2A9 was maximally expressed during the G_0 to S transition phase of the cell cycle and was deregulated in acute myeloid leukemia [2, 3]. Sequence analysis of calcyclin cDNA revealed a high degree of homology to calcium binding proteins from S-100 family [4]. To stress the potential ability of the gene product to bind calcium and its possible involvement in cell cycle, Baserga and his coworkers called it a calcyclin.

In independent studies we purified a novel calcium binding protein from Ehrlich ascites tumor (EAT)[1] cells [5], which we later identified as a calcyclin [6]. Thus we were the first to report that calcyclin is in fact an authentic calcium binding protein. Subsequent studies have generated many interesting data regarding the structure and expression of the calcyclin gene, properties of its protein product, cell and tissue specific distribution under normal and pathological conditions, and possible functions. These observations are summarized below with the emphasis placed on our own studies.

Properties of calcyclin

Calcyclin is a 10 kDa polypeptide which belongs to the EF-hand family of S-100-like calcium binding proteins. The homology between calcyclin and other S-100 proteins is particularly striking in two regions that encode the sequences of calcium binding sites. All S-100 proteins seem to be cell-cycle regulated or induced during differentiation. Moreover, the level of these proteins is changed under a variety of pathological conditions [7] (Table 1).

Mouse calcyclin binds two calcium ions, each with a different affinity, and undergoes a conformational change upon Ca^{2+}-binding [5]. Sequence analysis of mouse calcyclin revealed that it is identical with rat protein and highly homologous to human calcyclin [6, 8]. So far, calcyclin has been isolated to homogeneity from different sources and partially characterized. A protein with properties similar to those EAT calcyclin was isolated from mouse stomach [9] and chicken gizzard [10]. The latter was shown to bind two calcium ions per subunit with K_d values of 10^{-7} M and 10^{-5} M. Recently calcyclin was isolated from human platelets [11], and brain [12]. Calcyclin from EAT cells forms disulfide linked dimers and binds zinc ions, similarly as does S-100β [13]. Calcyclin isolated from rabbit lung does not form covalent dimers [14]. However, the recombinant protein with mutated serine to cysteine gained the ability to form such dimers [15]. The physiological significance of dimerization is not clear, although the dimer seems to have higher affinity for calcium ions than the monomer (U. Wojda, unpublished results). To

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[1]Abbreviations: EAT, Ehrlich ascites tumor; PRA, prolactin receptor associated protein

Table 1
Action of the EF-hand family of S-100-like calcium binding proteins

Name	Cell-cycle regulated or differentiation induced	Pathological context described
Calcyclin, PRA, Caltropin	+	Cancer, metastasis, cirrhosis biliaris
S-100α	+	Kidney and heart diseases
S-100β	+	Cancer and other brain diseases
CF Ag/MRP14	+	Cystic fibrosis, rheumatoid arthritis
ICaBP (calbindin D9K)	?	Intestinal diseases
MRP-8	+	Inflammatory diseases
mts1, 42A, 18A2, P9Ka, pEL68	+	Cancer, metastasis
p11/Calpactin, 42C	+	Cancer

better understand the structure-function relation, two properties of native protein: calcium binding and dimerization were compared with those of calcyclin CNBr-fragments. EAT calcyclin contains only one methionine residue so that cleavage by CNBr yielded two fragments: N-terminal (residues 1 - 56) and C-terminal (residues 57 - 89). Each fragment contains one potential calcium binding site, but only the N-terminal fragment contains a cysteine residue (second amino-acid residue) which can form a dimer by a disulfide bridge. Neither of the fragments bound calcium but both were able to dimerize and aggregate. This indicates that the calcyclin dimer is formed not only by disulfide bridges but also by additional, probably non-covalent, interactions [16]. On the basis of these and other results we suggest that cooperation of both EF-hand domains in calcyclin molecule is required for the high affinity calcium binding and consequently – for calcium-induced conformational changes. The ability of calcyclin to bind sialic acid in a calcium-dependent manner was described by Gabius and coworkers [17, 18]. This finding might be relevant to the suggested role of calcyclin in mucus secretion (see below).

Tissue and cell specific distribution of calcyclin

The distribution of calcyclin was investigated at the mRNA and protein level. Both methods gave similar results and indicated that calcyclin is synthesized only by certain types of cells during specific periods of activity. These observations might help to understand the function of calcyclin.

We conducted an analysis of cell and tissue distribution of calcyclin using polyclonal antibodies. Calcyclin was detected in several mouse tissues including stomach, skeletal muscle, heart, lung, kidney and spleen. We found that in skeletal muscle its expression is limited to fibroblasts [9, 19]. At the same time calcyclin was identified as a prolactin receptor associated protein (PRA) [20] and detected in several human tissues [18]. We have extended these studies to analyze the calcyclin distribution in human and rat tissues. In all organs examined (breast, heart, intestine, kidney, liver, ovary, placenta, skeletal muscle, stomach, thymus, uterus and skin) calcyclin antibodies stained two types of cells: epithelial cells and fibroblasts [21, 22]. However, not all fibroblasts and epithelial cells were stained with calcyclin antibodies in these tissues. On the basis of these observations we suggested that calcyclin expression in the epithelial cells and fibroblasts might be related to their secretory activity [21]. In brain, calcyclin specific antibodies were found to stain subsets of neurones in Ammons horn of hippocamp, granule cells of cerebellum and brain stem. Glial cells which contain large amounts of S-100β, were calcyclin negative [12].

Calcyclin mRNA has been detected in many tissues, cell types and cultured cell lines. Original observations of Baserga and coworkers [1-3] that the gene is activated by serum or growth

factors in fibroblasts have been confirmed and extended by many groups. High levels of calcyclin mRNA were also detected in PC12 cells (rat pheochromocytoma cells) after nerve growth factor treatment [23, 24] and in some human cell lines after transformation [25]. Murphy *et al.* [20] found calcyclin in some breast cancer cell lines, and Guo *et al.* [25] demonstrated the presence of calcyclin mRNA in normal mouse tissues such as epidermis, skin, stomach, uterus of pregnant mouse, placenta, decidua. A high level of calcyclin mRNA was observed in human heart [26]. Several neuroblastoma cell lines of Schwann type morphology also show a high level of calcyclin mRNA [27]. Calcyclin gene expression was stimulated by retinoic acid in the neuroblastoma cell line which does not express calcyclin gene. Comparison of the proteins expressed in normal proliferating cells *versus* virus-transformed cells shows differences in calcyclin level [28, 29]. Thordarson *et al.* [30] found a high level of calcyclin mRNA in decidual tissue of pregnant mouse and Wood *et al.* [31] in hair follicle cells during differentiation. Weterman *et al.* [32] showed that melanoma cell lines with high metastatic activity expressed calcyclin at higher levels than those with low metastatic activity.

The search for calcyclin function

According to theory, as a typical EF-hand calcium binding protein, calcyclin may express its biological activity by several means. It may act as a calcium "buffer" or transporter as it has been shown for other calcium binding proteins. Calcyclin may also act as an activator of calcium-dependent processes and, similarly as calmodulin, interact with some protein targets.

In accordance with this possibility, we have shown that calcyclin changes its conformation exposing hydrophobic domains upon calcium binding. It is also possible that calcyclin may act by binding to non-protein targets in a calcium dependent manner.

Although the function of calcyclin is unknown at present, several speculations and observations have been made. It was suggested that calcyclin may play a role in cell cycle progression [2, 3, 33, 34]. The observations that the calcyclin gene is induced by several factors such as growth factors, oncogenes, phorbol esters or retinoic acid may indicate that calcyclin is required for proliferation of normal and tumor tissues. However, several authors have pointed out that the calcyclin gene is not always expressed during cell cycle progression, and suggested that calcyclin might also be involved in other process(es) [11, 21, 35 - 37]. For example, the recent comprehensive studies on calcyclin mRNA distribution in mouse tissues demonstrated that calcyclin transcripts were restricted to specific cell types within a limited number of mouse organs [37]. High levels of expression were found in the epithelia lining the gastrointestinal, respiratory and urinary tracts, and in the goblet cells of small intestine. This led Timmons and coworkers [37] to suggest a role of calcyclin in the process of mucus secretion. In addition, they observed calcyclin mRNA in the corpus luteum, placenta and nerves within the gut wall, which are also the sites of exocytosis.

There are some reports suggesting that calcyclin might be involved in cell differentiation. The nerve growth factor – or epidermal growth factor – induced differentiation of PC12 cells is accompanied by the activation of several genes, including the calcyclin gene [23, 24]. In neuroblastoma cell lines the calcyclin gene was active in cells of the Schwann type or mixed morphology but not in cells of neuroblastic morphology. When a neuroblastic cell line was induced to differentiate to Schwann type morphology by treatment with retinoic acid, the calcyclin mRNA level was increased [27]. In rat skin the highest level of calcyclin mRNA was found in post-mitotic keratogenous regions of the hair follicle [31], and calcyclin protein was found at the highest level in the hair medulla and sheaths. The presence of calcyclin in platelets (enucleated cells) [11] and in neurons [12] seems to be an additional piece of evidence that the protein might be involved in process(es) related to differentiation or process(es) typical for differentiated cells. Calcyclin was suggested to be a part of the cascade of events leading to cell repair in the corneal epithelium [38].

The presence of calcyclin outside the cells which was demonstrated by Celis *et al.* [28, 29] and Thordarson *et al.* [30] suggests another role for this protein. Thordarson *et al.* showed that calcyclin was secreted from cultured decidual cells into medium and that external calcyclin was able to stimulate secretion of lactogen by

trophoblast cells. Secretion of calcyclin by human epithelial cells of amnion was described by Celis *et al.* [28, 29]. It is possible that the presence of extracellular calcyclin is due to its secretion but it is difficult to explain how it is transported through the cell membrane without a signalling sequence. On the other hand, a similar situation seems to be well documented for S-100β dimer (neurite extension factor) that is secreted from glial cells. Its extracellular activities indicate that it might be considered a mitogenic and neurotrophic factor for nerve cells [39, 40].

The search for calcyclin targets was initiated a few years ago. Using a calcyclin-Sepharose column we identified two 36 kDa proteins which bound to calcyclin in the presence of calcium and were eluted in the absence of this ion [19]. Later, in collaboration with another laboratory, we identified these two proteins as annexin II and glyceraldehyde 3-phosphate dehydrogenase [8]. It remains to be elucidated by future studies whether these interactions have any biological significance. Binding of calcyclin to another annexin (CAP-50) identified in rabbit lung was described by Minami *et al.* [41]. This protein seems to be localized in nuclei of 3Y1 fibroblasts as found by immunohistochemical studies [42]. Also, the observation that calcyclin is associated with prolactin receptors might indicate that there is a calcyclin target in membranes [20]. Calcyclin binding to sialic acid permits to place this protein in the group of carbohydrate binding proteins [17].

Recently, an important discovery was made by Mani *et al.* [43]. They showed that caldesmon (an actin and calmodulin binding protein) binds in a calcium dependent fashion to the 11 kDa smooth muscle calcium binding protein which seems to be an avian calcyclin. The interaction between calcyclin and caldesmon was confirmed by spectroscopic methods and measurement of actomyosin ATPase activity. An avian calcyclin could release the inhibitory effect of caldesmon on actomyosin ATPase activity. In this aspect avian calcyclin (called by authors caltropin) mimics the role played by troponin C in striated muscle and seems to be a calcium-dependent regulator of caldesmon in smooth muscle. The ability of avian calcyclin (kindly provided by Dr. Mani) to interact with caldesmon from chicken gizzard was confirmed in our laboratory. On the other hand, mouse calcyclin slightly reverses the inhibition of actomyosin ATPase by caldesmon (A. Filipek, A. Zasada, R. Makuch, U. Wojda, J. Kuźnicki and R. Dąbrowska, unpublished results).

Calcyclin as a potential marker of human diseases

The cell-specific expression of calcyclin is interesting and useful with regard to basic research but it may also have important clinical implications. Calcyclin seems to be a marker of fibroblasts and epithelial cells and we have suggested that immunohistochemical as well as quantitative analysis of calcyclin might have a diagnostic value in the diseases where proliferation of epithelium or connective tissue takes place [21, 22]. In agreement with this hypothesis we found that, in human liver with cirrhosis biliaris, calcyclin antibodies can visualize the pathological changes in biliary epithelium [21, 44]. We also tried to test this hypothesis using two experimentally induced diseases: rat liver cirrhosis biliaris and feline left ventricular pressure-overload [22]. We found that the increased number of epithelial cells in liver with cirrhosis biliaris (Fig. 1) and of fibroblasts in hypertrophic heart could be detected by immunohistochemistry. Consistently with these observations, the level of calcyclin mRNA and protein was increased in these pathological tissues as shown by Northern blotting, immunoblotting and ELISA. Taken together these data indicate that the calcyclin levels might indirectly show the extent of proliferation of epithelial cells and fibroblasts in the analyzed tissue.

We have also demonstrated the usefulness of calcyclin antisera for monitoring the structural changes of biliary tracts that might occur in transplanted livers. We analyzed sixty liver biopsies obtained from 10 transplanted livers, donors' livers and liver explants [44]. Calcyclin antibodies stained not only biliary ducts in healthy livers but also different types of pseudoductuli, rudimentary ductuli, etc. in the livers that exhibited pathological changes. Also, the single hepatocytes which were probably precursors of biliary epithelial cells, were stained with calcyclin antibodies. Both the proliferation and atrophy of biliary ductuli occurring during acute or chronic rejection episodes could be easily detected using calcyclin stain-

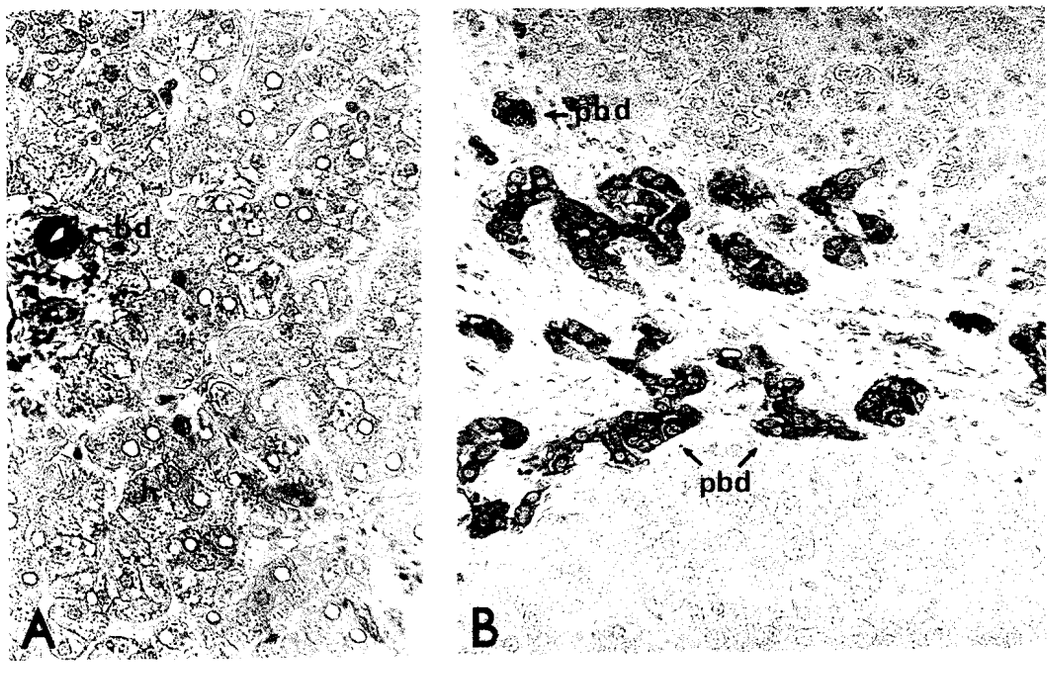

Fig. 1. Sections from rat livers stained with calcyclin antibodies (magnification, 320 ×).
(A) Normal liver: only one bile duct is stained (bd); hepatocytes (h); (B) liver with biliary cirrhosis: reaction with calcyclin antibodies is seen in proliferating bile ducts (pbd).

ing. Thus, this staining permitted to visualize biliary epithelial cells and could be helpful as a prognostic factor after liver transplantation.

As discussed earlier, particularly in the section on calcyclin distribution, several authors have described calcyclin expression in relation to pathological states: leukemia [3], tumors with metastatic ability of *ras*-transformed cells [25] (see also review on *ras*-responsive genes and tumor metastasis by Chambers & Tuck [36]), neuroblastoma cells [27], metastatic human melanoma cell lines [32], transformed cells of colon [45] or liver with cirrhosis biliaris [22]. Cell-specific expression of calcyclin in normal cells and high level of calcyclin in some tumor cells or other pathologically altered tissues indicates that the studies on this protein are of potential clinical significance. We believe that basic research on this and other calcium binding proteins may contribute significantly to understanding of the cause of some human diseases and to development of diagnostic methods.

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