Acta Biochimica Polonica
Vol. 40 No. 1 1993

Biochemical properties of calcyclin – a potential marker of some diseases

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Calcyclin – its gene and protein

Calcyclin is a Ca^{2+}-binding protein that belongs to the S-100 protein family. The calcyclin gene was identified as one of genes induced by growth factors in the cell cycle progression [1]. The level of calcyclin mRNA is increased in several cancer lines [2, 3] and calcyclin expression was correlated with metastatic activity of human melanoma cell lines [4].

The calcyclin protein (10.5 kDa) was isolated for the first time from Ehrlich ascites tumour cells [5]. Some informations on calcyclin were gained by the analysis of its tissue and cell specific distribution. It was found that in sections of a variety of human tissues antibodies against calcyclin stain epithelial cells and fibroblasts [6]. On the basis of these observations we suggest that immunohistological analysis of calcyclin might help to diagnose the diseases where proliferation of epithelium or connective tissue takes place.

Our biochemical investigations on calcyclin showed that, similarly as S-100 β, it has two Ca^{2+}-binding sites of the EF-hand domain structure, a zinc binding site(s), and is able to form dimers [7, 8]. It is known that a covalent dimer form of S-100 exhibits a neurite extension activity [9]. Moreover, dimer formation seems to be a common feature of the S-100 family members [10]. According to these observations it seems important to investigate calcyclin dimer formation as well as the biochemical properties of the dimer and monomer.

Analysis of calcyclin dimer and monomer

Calcyclin monomer and covalent dimer forms are schematically presented in Fig. 1. These forms can be visualised on sodium dodecyl sulphate containing polyacrylamide-gel (SDS-PAGE[1], Fig. 2B, lane 1). The covalent dimer is formed *via* a disulfide bridge, which was confirmed by its reduction with dithiothreitol (Fig. 2B, lane 2). Figure 2 shows preliminary gel filtration results for native calcyclin forms: two peaks were eluted (Fig. 2A) and then analysed on SDS-PAGE (Fig. 2B). Peak II contained the calcyclin monomer (Fig. 2B, lane 5). Peak I consisted of two forms of dimer: disulfide bridge-linked dimer that could be reduced to monomer by dithiothreitol, and a non-covalent dimer, that dissociated to a monomer under denaturing conditions in the SDS-containing gel (Fig. 2, lanes 3, 4). We propose that under native conditions an equlibrium is established between three calcyclin forms: a monomer/a non-covalent dimer/a covalent dimer. The question arises which form(s) could be physiologically active during activation within the cell.

In this aspect we have investigated Ca^{2+}-affinity of calcyclin monomer and of the covalent dimer (Fig. 3). The affinity (expressed as macroscopic binding constant) was determined using flow dialysis techniques under different ionic conditions [11]. Each calcyclin EF-hand domain seems to bind Ca^{2+} ion. The Ca^{2+} affinity of the covalent dimer (about 10^5 M^{-1})

[1]Abbreviations: DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

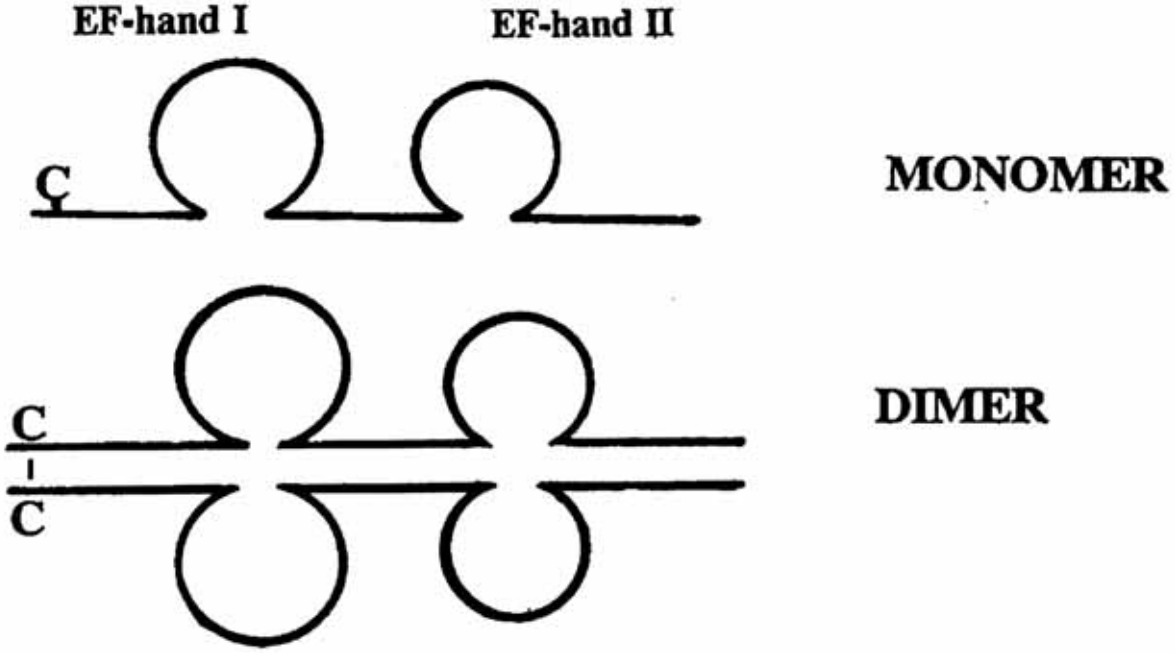

Fig. 1. Schematic structure of calcyclin monomer and dimer.
Monomer of calcyclin contains two EF-hand domains, i.e. potential Ca^{2+}-binding sites drawn as the loops. C, the cysteine residue that participates in dimer formation *via* disulfide bridge

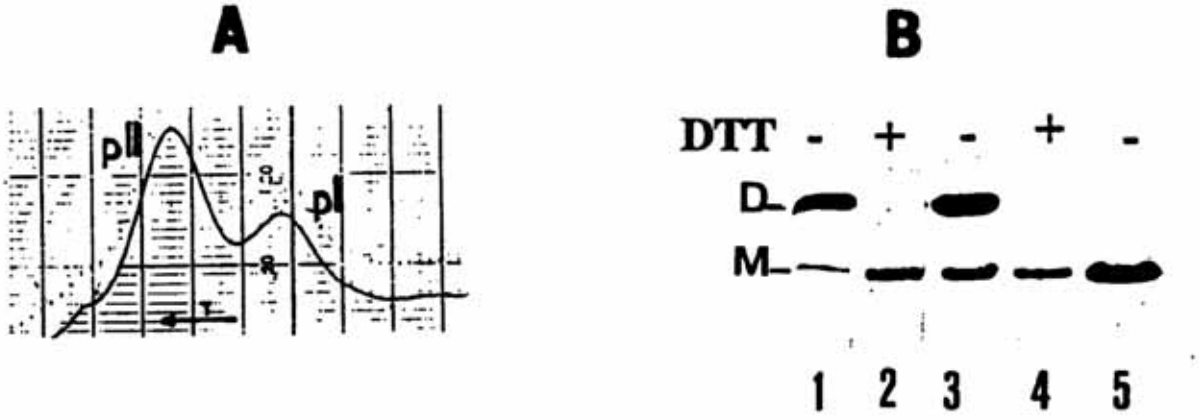

Fig. 2. Analysis of calcyclin monomeric and dimeric forms.
A. Gel filtration analysis. A standard calcyclin sample (a mixture of calcyclin forms) was filtered on Sephadex G-75 column under native conditions (50 mM Tris/HCl, pH 7.5, 100 mM NaCl) and two peaks (pI and pII) were eluted. B. SDS-PAGE analysis of samples before and after gel filtration: 1, a standard mixture of calcyclin forms before filtration; 2, the same mixture with 30 mM dithiothreitol (DTT); samples after gel filtration: 3, from peak I without DTT; 4, from peak II without DTT; 5, from peak I with DTT; M, calcyclin monomer; D, calcyclin dimer

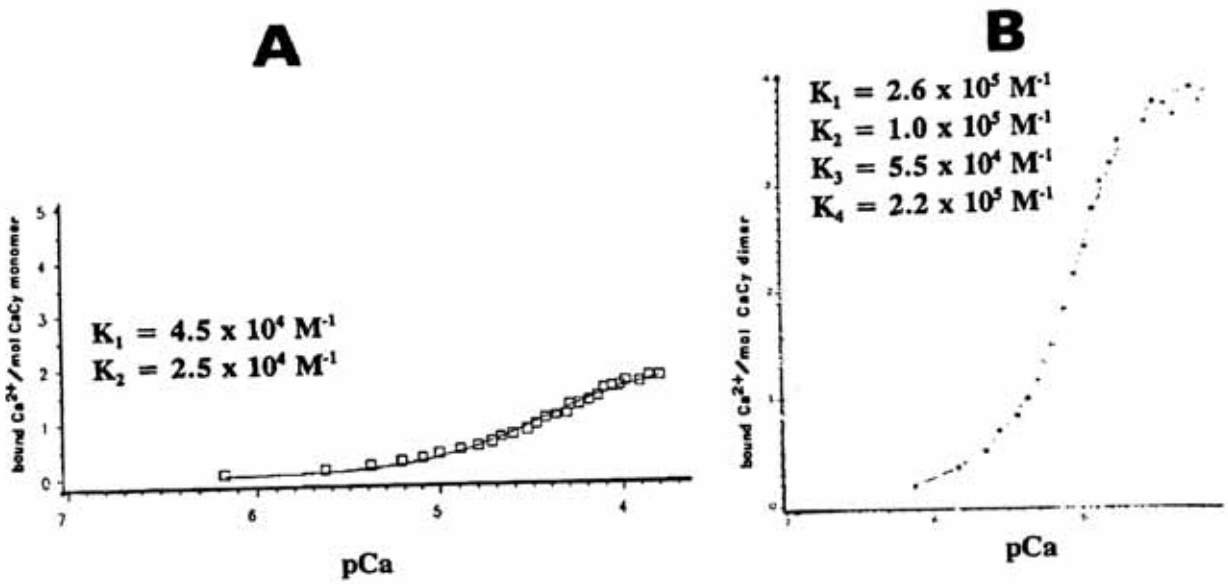

Fig. 3. Affinity of calcyclin forms to Ca^{2+}.
A. Monomer form. B. Covalent dimer form. Ca^{2+}-binding isotherms were obtained using the flow dialysis method. Macroscopic Ca^{2+}-binding constants (K_i) were determined from Adair-Klotz equation. Calcyclin concentrations in binding experiments were 10 - 20 μM in 50 mM Tris, pH 7.5; pCa = –log[free Ca^{2+}]

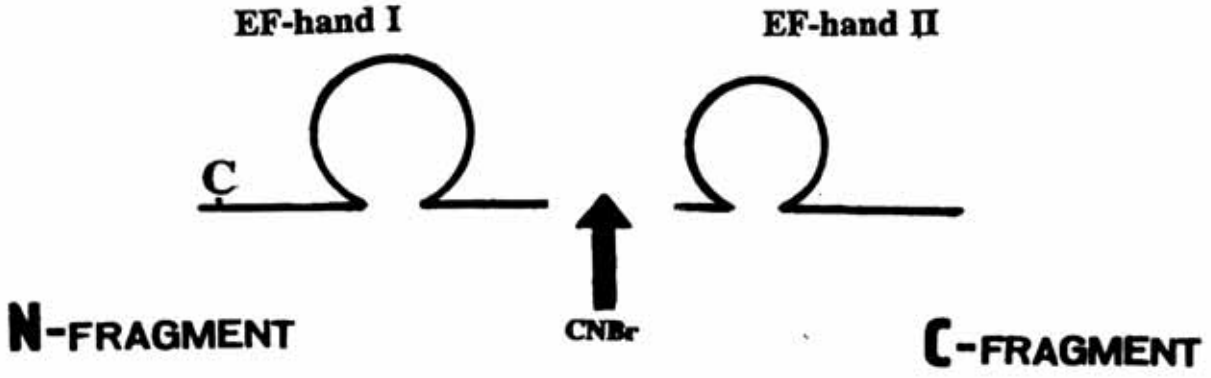

Fig. 4. Schematic structure of calcyclin fragments obtained by CNBr-cleavage.
Each fragment contains a Ca^{2+}-binding site drawn as a loop of EF-hand domain. C, cysteine residue that can form disulfide bridge

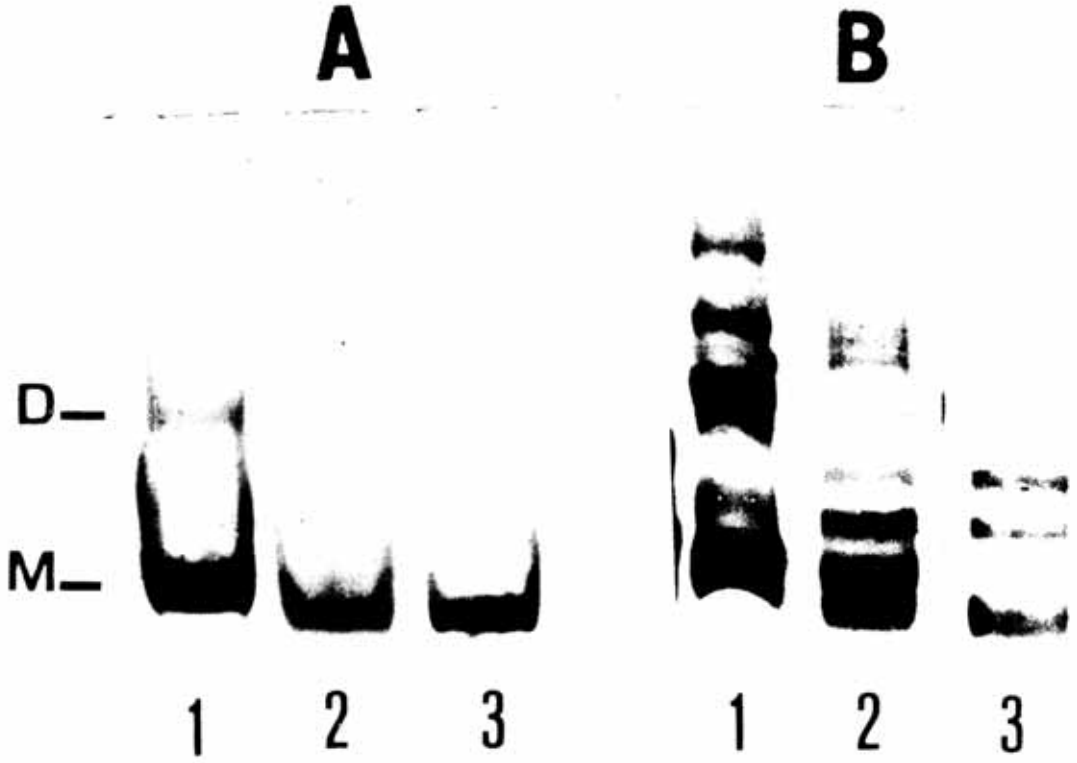

Fig. 5. Aggregations of calcyclin and its CNBr-fragments analysed by SDS-PAGE and stained by silver.
A. Samples were boiled with 2-mercaptoethanol prior electrophoresis. B. Samples were not treated with this agent: 1, intact calcyclin; 2, C-terminal fragment; 3, N-terminal fragment; M, calcyclin monomer; D, calcyclin dimer

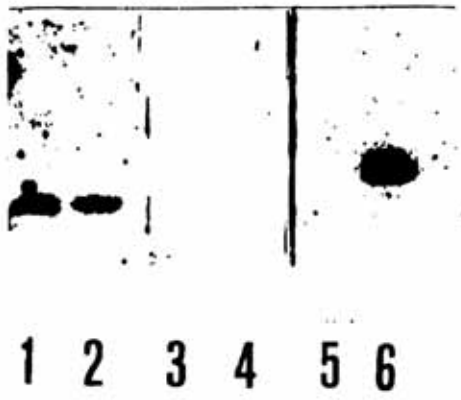

Fig. 6. $^{45}Ca^{2+}$-binding to proteins blotted onto nitrocellulose.
The proteins and calcyclin CNBr-fragments were separated on SDS-gel and transferred to nitrocellulose. The blots were incubated with radioactive $^{45}Ca^{2+}$ at pH 6.8 and autoradiographed for 6 days. 1, S100 protein; 2, calcyclin; 3, N-terminal fragment of calcyclin; 4, C-terminal fragment; 5, bovine serum albumin; 6, calmodulin

appeared to be by one order of magnitude higher than that of the monomer and closer to physiological Ca^{2+} concentration in the cell.

Properties of calcyclin fragments

To learn more about mechanism(s) of dimer formation and properties of Ca^{2+}-binding sites, calcyclin was cleaved by CNBr into two fragments: N-terminal one (N-fragment, residues 1–56) and C-terminal one (C-fragment, residues 57–87). Schematic representation of calcyclin CNBr-fragments is given in Fig. 4.

Each fragment contains one Ca^{2+}-binding site, but only the N-fragment contains a cysteine residue which is necessary for disulfide bridge formation. Fragments were purified to homogeneity by fast protein liquid chromatography, identified by partial sequencing, and their ability for dimerization was analysed (Fig. 5). Under non-reducing conditions both fragments form dimers and other agregates detected by silver staining. The formation of dimer by the C-terminal fragment, that doesn't contain a cysteine residue, seems to support the

hypothesis that a non-covalent dimer might be one of native forms of calcyclin.

We investigated also $^{45}Ca^{2+}$ binding to calcyclin and its CNBr-fragments immobilized on nitrocellulose filters (Fig. 6). None of the fragments was able to bind $^{45}Ca^{2+}$ under conditions in which calcyclin and other Ca^{2+}-binding proteins did bind $^{45}Ca^{2+}$. Thus it appears that after cleavage of calcyclin the Ca^{2+} affinity of a single EF-hand is significantly diminished or destroyed. This suggests that cooperation of the two EF-hand domains plays an important role in formation of calcyclin active Ca^{2+}-binding sites.

We conclude that:

- 1. Under native conditions calcyclin might exist in three forms: a monomer, a non-covalent dimer and a covalent dimer;
- 2. CNBr-fragments of calcyclin are useful for investigations on dimerization mechanisms as well as cooperation of EF-hand domains in Ca^{2+}-binding.

REFERENCES

1. Calabretta, B., Battini, R., Kaczmarek, L., DeRiel, J. K. & Baserga, R. (1986) *J. Biol. Chem.* **261**, 12628 - 12632.
2. Calabretta, B., Kaczmarek, L., Mars, W., Ochoa, D., Gibson, C.W., Hirschhorn, R.H. & Baserga, R. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4463 - 4467.
3. Calabretta, B., Venturelli, D., Kaczmarek, L., Narni, F., Talpaz, M., Anderson, B., Beran, M. & Baserga, R. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1495 - 1498.
4. Weterman, M.A.J., Stoopen, G.M., van Muijen, G.N.P., Kuźnicki, J., Fuiter, D.J. & Bloemers, H.P.J. (1992) *Canc. Res.* **52**, 1291 - 1296.
5. Kuźnicki, J. & Filipek, A. (1987) *Biochem. J.* **247**, 663 - 667.
6. Kuźnicki, J., Kordowska, J., Puzianowska, M. & Woźniewicz, B.M. (1992) *Exp. Cell Res.* **200**, 425 - 430.
7. Kuźnicki, J., Filipek, A., Hunziker, P.E., Huber, S. & Heizmann, C.W. (1989) *Biochem. J.* **263**, 951 - 956.
8. Filipek, A., Heizmann, C.W. & Kuźnicki, J. (1990) *FEBS Lett.* **264**, 263 - 266.
9. Winningham-Major, F., Staecker, J.L., Barger, S.W., Coats, S. & Van Eldik, L.J. (1989) *J. Cell Biol.* **109**, 3063 - 3071.
10. Kligman, K. & Hilt, D.C. (1988) *Trends Biochem. Sci.* **13**, 437 - 443.
11. Wojda, U., Gerard, D., Mely, Y. & Kuźnicki, J. (1992) *Second European Symposium on Calcium Binding Proteins in Normal and Transformed Cells.* Marseille, Abstracts, p. 45.