Volume 254, number 1,2, 141-144 FEB 07529 August 1989

Tissue specific distribution of calcyclin – 10.5 kDa Ca^{2+}-binding protein

Jacek Kuźnicki, Anna Filipek, Peter Heimann*, Leszek Kaczmarek and Bożena Kamińska

Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warsaw, Poland and **University of Bielefeld, Developmental Biology Unit, Postfach 8640, 4800 Bielefeld, FRG*

Received 2 June 1989

Expression of calcyclin in different cell lines and mouse tissues was determined with polyclonal antibodies raised against calcyclin from Ehrlich ascites tumour (EAT) cells. The protein was detected in mouse skeletal and cardiac muscle, in lung, kidney and spleen, and was especially enriched in mouse smooth muscle as well as in rat fibroblasts. No positive immunological reaction was detected in mouse brain, liver and intestine and some tumourigenic cell lines. The level of calcyclin mRNA found in different cells and tissues corresponded well to the calcyclin level estimated by immunoblotting. The calcyclin-like protein was purified from mouse stomach and appeared to be very similar to the EAT protein.

Calcyclin expression; Ca^{2+}-binding protein; Ca^{2+}

1. INTRODUCTION

We have purified to homogeneity a 10.5 kDa calcium-binding protein from Ehrlich ascites tumour (EAT) cells [1], which, on the basis of sequence homologies was called calcyclin-like protein (Kuźnicki, Filipek, Hunziker, Huber and Heizmann, submitted). Originally, calcyclin was a term given to the hypothetical product of a gene (2A9), transcription of which was found inducible by growth factors [2,3]. It has been suggested that calcyclin is involved in the regulation of cell proliferation and, on the basis of its deduced amino acid sequence, that it binds calcium [2,3]. To reveal more information about possible functions of calcyclin we searched for it in a variety of mouse tissues as well as in different cell lines using antibodies raised against the EAT protein. In addition, we purified the calcyclin-like protein from smooth muscle and compared some of its properties with those of the EAT protein.

Correspondence address: J. Kuźnicki, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warsaw, Poland

Abbreviations: EAT, Ehrlich ascites tumour cells; DTE, dithioerythritol; PMSF, phenylmethyl sulfonyl fluoride

2. MATERIALS AND METHODS

2.1. *Proteins*

Calcyclin was isolated from Ehrlich ascites tumour cells as described earlier [1]. Calcium-binding protein from mouse stomach was purified using the same method. The protein concentration was estimated according to Bradford's procedure (BioRad) with bovine γ-globulin as a standard.

2.2. *Cells*

PtK_2 cells (kidney epithelial cells from rat kangaroo) and rat fibroblasts were grown on coverslips in minimal essential medium (MEM) (Gibco, Scotland) supplemented with 7% fetal calf serum (FCS) (Gibco). V2 (rabbit carcinoma) cells were grown in MEM, 15% FCS and Ag8 cells in RPMI 1640 (Gibco), 10% FCS.

2.3. *Tissue and cell extracts*

Three volumes of deionized water were added to the pellet of cultured cells (previously washed with phosphate buffered saline) or to the mouse tissues and the suspensions were homogenized using tissue grind glass-glass homogenizer (B. Braun, Melsungen). To all extracts, 2 mM DTE, 2 mM EGTA and 0.5 mM PMSF was added. The extracts were centrifuged in an Eppendorf centrifuge for 10 min and the supernatants were collected.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2.4. *Antibodies*

The polyclonal antibodies were raised in a female SPF bastard rabbit by injecting 1 mg of purified calcyclin from EAT cells with complete Freund's adjuvant and, after four weeks 1 mg calcyclin emulsified in incomplete Freund's adjuvant. Serum was collected one week after the second injection and antibodies against calcyclin were purified by affinity chromatography.

2.5. *Gel analysis and immunoblotting*

Tissue and cell extracts were subjected to one- or two-dimensional polyacrylamide gel electrophoresis [4,5]. In the latter case, proteins were first separated on the isoelectrofocusing gels in pH 3.5–10 ampholines (LKB Instrument GmbH, Grafelfing, FRG) in capillaries 1.1 mm diameter, and the second dimension was run on 12.5% polyacrylamide minislabs in the presence of SDS. Polyacrylamide gel electrophoresis in the presence of 6 M urea was performed according to Drabikowski et al. [6]. Gels were stained with Coomassie brillant blue. Immunoreactivity was analysed by blotting the proteins from the polyacrylamide gels onto nitrocellulose sheets [7]. The blots were allowed to react with anti-calcyclin followed by sheep-anti-rabbit IgG conjugated with horseradish peroxidase (Nordic, Tilburg, The Netherlands). Staining was developed with chloronaphthol and H_2O_2 [8].

2.6. *RNA isolation, electrophoresis and hybridization*

Total RNA was extracted from cells and tissues as in [9]. Electrophoresis of cellular RNA, blotting onto nitrocellulose and hybridization with full-length human calcyclin gene (2A9) were performed as in [2,3].

2.7. *Light and electron microscopy*

Mouse skeletal muscle (m. vastus from a 2-month-old NMRI mouse) was fixed in Bouin fixative, embedded in paraffin, sectioned (8 μm) and dewaxed. Sections were incubated with calcyclin antibody, washed and subsequently reacted with the second antibody coupled to horseradish peroxidase (HRP). Colour development was performed with diaminobenzidine and the samples were flat-embedded in araldite resin, photographed, ultra-thin sectioned, poststained with uranyl acetate and lead citrate, and inspected with a Zeiss EM 109 electron microscope.

3. RESULTS AND DISCUSSION

3.1. *Tissue specific distribution of calcyclin*

On two-dimensional blots, derived from low ionic strength extracts of EAT cells, polyclonal antibody raised against EAT cells calcyclin reacted only with one protein spot. The spot was corresponding to the monomer of calcyclin (fig.1). The antibody recognized a calcyclin-like polypeptide in immunoblots of a variety of cells and mouse tissues (fig.2). The strongest reaction was observed in mouse stomach and in rat fibroblasts. In spleen, lung and kidney the major staining was observed with a polypeptide at the level of 25 kDa, but a weak reaction with a 10.5 kDa band was also seen.

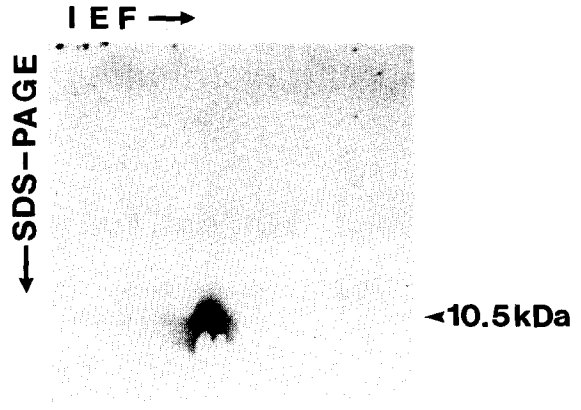

Fig.1. Protein blot analysis using calcyclin antibodies. Low ionic strength extracts of EAT cells were separated electrophoretically on two-dimensional gels. Proteins were transferred to nitrocellulose and the blots were incubated with anti-calcyclin. Immunoreaction was visualized by incubation with peroxidase-coupled sheep anti-rabbit IgG.

The double band of 10.5 kDa protein in the extract of skeletal muscle was not seen in other experiments. No positive immunological reaction with calcyclin antibody was observed in extracts from brain (fig.2), intestine and liver (not shown). The antibody against calcyclin from EAT cells failed to detect any protein in PtK_2 cells, Ag8 cells and V2 rabbit carcinoma cells (not shown). Antibody against calcyclin from EAT cells did not react with other calcium-binding proteins such as calmodulin, parvalbumin, S-100 and oncomodulin (not shown).

3.2. *Purification of calcyclin-like protein from mouse stomach*

The calcium-binding protein was purified from mouse stomach using the procedure developed for EAT calcyclin. This indicates that the smooth muscle protein has chemical and physical properties similar to calcyclin from EAT cells. Moreover, both proteins behaved identically on SDS-PAGE and on urea-PAGE (in the presence and absence of calcium ions), as seen in fig.3, and on two-dimensional gels (not shown). This indicates that they are very similar (if not identical) with respect to their molecular weight, charge and Ca^{2+}-induced conformational states.

3.3. *Northern blot analysis of calcyclin mRNA levels*

Fig.4 presents a Northern blot analysis of

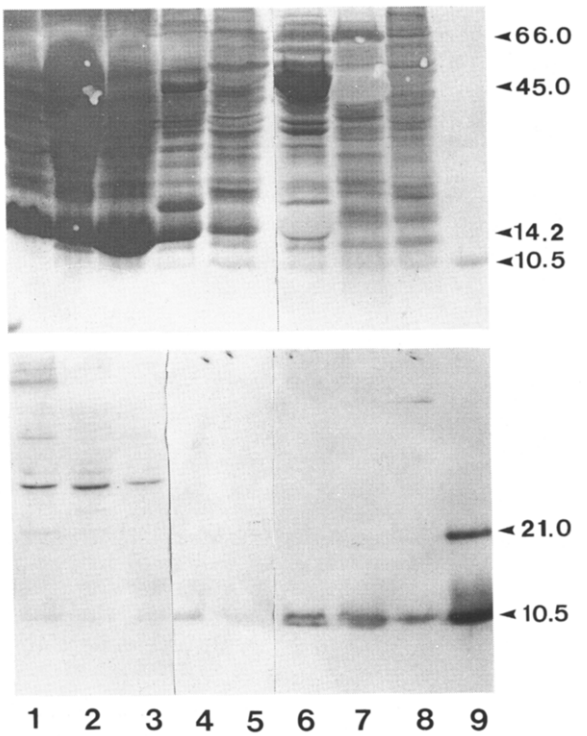

Fig.2. Immunoblot analysis of the tissue distribution of calcyclin. The low ionic strength extracts of different mouse tissues and of rat fibroblasts were separated on SDS gel, blotted to nitrocellulose and stained with Ponceau red (upper part), and anti-calcyclin (lower part). Lanes: 1, lung; 2, kidney; 3, spleen; 4, heart; 5, brain; 6, skeletal muscle; 7, stomach; 8, rat fibroblasts; 9, EAT calcyclin.

calcyclin mRNA levels in EAT cells as well as in different organs of the mouse. The highest level of calcyclin mRNA was observed in EAT cells and a much weaker signal was detected in RNA isolated from stomach. No signal was observed in RNA isolated from liver, brain, kidney, skeletal muscle and spleen. This tissue distribution of calcyclin mRNA is in good agreement with our data on protein distribution and it can be taken as further proof of the identity of calcyclin with the 10.5 kDa calcium-binding protein from EAT cells.

3.4. *Immunocytochemistry*

Cross sections of mouse skeletal muscle after incubation with calcyclin antibodies were inspected in the light and electron microscope (fig.5). It was found that in skeletal muscle of mouse only fibroblasts were stained, but not the muscle fibers.

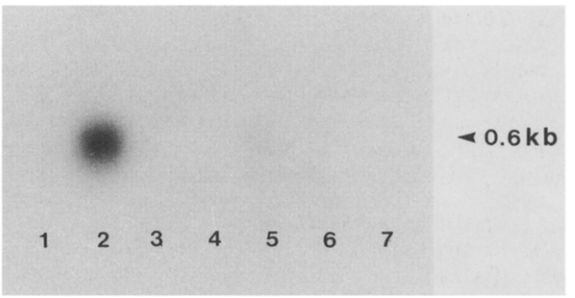

Fig.3. Autoradiogram of Northern blot demonstrating different levels of calcyclin mRNA in mouse tissues. Total RNA isolated from cells and tissues was labeled with ^{32}P-calcyclin probe. Lanes: 1, liver; 2, EAT cells; 3, brain; 4, muscle (m. vastus); 5, stomach; 6, kidney; 7, spleen.

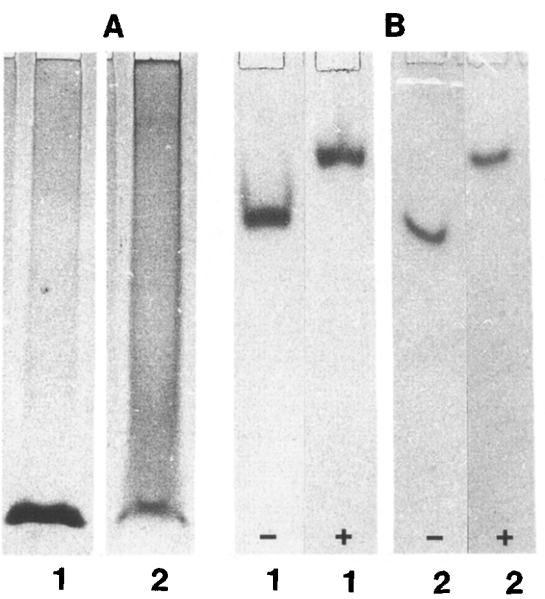

Fig.4. Electrophoretic comparison of calcyclin from EAT cells (1) and a protein from mouse stomach (2). The samples contained either 0.1 mM $CaCl_2$ (+) or 1 mM EGTA (−). (A) 15% PAGE in the presence of SDS; (B) 8% PAGE in the presence of urea.

3.5. *Conclusions*

Calcyclin is a member of the S-100 protein family [10]. It binds calcium and zinc ions and both cations may regulate its possible involvement in the control of cell proliferation activity (Filipek, Heizmann and Kuźnicki, unpublished). The data of this work show that calcyclin is not a tumour specific protein, and that in normal tissues it seems to be expressed in cells capable of proliferation.

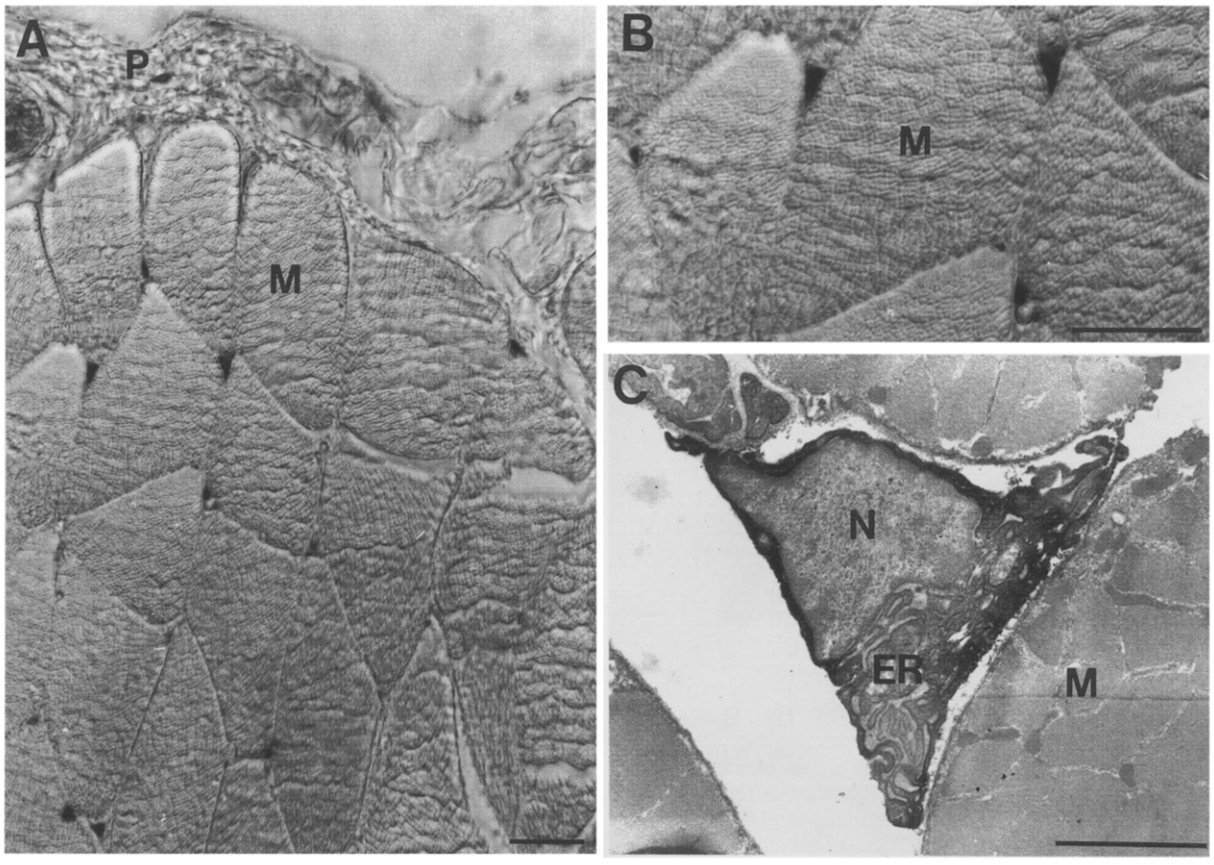

Fig.5. Localization of calcyclin in mouse skeletal muscle. (A,B) Light micrograph; (C) electron micrograph. A and B, Dark HRP-product shows that calcyclin is localized within triangular shaped cells situated between the muscle fibers (M). P, perimysium. C, electron microscopy shows that calcyclin is present within the cytoplasm of fibroblasts excluding the endoplasmic reticulum (ER). N, nucleus. Bar: A,B, 20 μm; C, 2 μm.

Acknowledgements: We express our thanks to Professors B.M. Jockusch and H. Jockusch for their support and advice throughout this work as well as to Dr F.-W. Kluxen for helping us with 2-D gels and to Chr. Wiegand for preparation of the antibodies. Part of this work was performed at the University of Bielefeld by J.K. as a guest scientist of the Deutsche Forschungsgemeinschaft (SFB-223). This work was supported by a grant from the Polish Academy of Sciences CPBP 04.01.

REFERENCES

[1] Kuźnicki, J. and Filipek, A. (1987) Biochem. J. 246, 663–667.
[2] Calabretta, B., Battini, R., Kaczmarek, L., De Riel, J.K. and Baserga, T. (1986) J. Biol. Chem. 261, 12628–12632.
[3] Calabretta, B., Venturelli, D., Kaczmarek, L., Narni, F., Talpaz, M., Anderson, B., Beran, M. and Baserga, R. (1986) Proc. Natl. Acad. Sci. USA 83, 1495–1498.
[4] Lacmmli, U.K. (1970) Nature (London) 227, 680–685.
[5] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[6] Drabikowski, W., Kuźnicki, J. and Grabarek, Z. (1977) Biochim. Biophys. Acta 485, 124–133.
[7] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[8] Hawkes, R., Niday, E. and Gordon, J. (1982) Anal. Biochem. 119, 142–147.
[9] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[10] Kligman, D. and Hilt, D.C. (1988) Trends Biochem. Sci. 13, 437–443.