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Calcium-binding protein from mouse Ehrlich ascites-tumour cells is homologous to human calcyclin

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A Ca^{2+}-binding protein was purified from mouse Ehrlich ascites-tumour cells. The protein forms monomers and disulphide-linked dimers, which can be separated by reverse-phase h.p.l.c. A partial amino acid sequence analysis demonstrated that the protein has an EF-hand structure. A striking homology was found to rat and human calcyclin (a member of the S-100 protein family), which is possibly involved in cell-cycle regulation.

INTRODUCTION

The Ca^{2+} signal in eukaryotic cells is transduced by a variety of Ca^{2+}-binding proteins characterized by an EF-hand structure (Kretsinger, 1980), e.g. calmodulin (Cohen & Klee, 1988), parvalbumin (Heizmann, 1984; Heizmann & Hunziker, 1989; Heizmann & Braun, 1989), S-100 proteins (Baudier, 1988; Kligman & Hilt, 1988), oncomodulin (MacManus *et al.*, 1988) or calbindins (Wassermann & Fullmer, 1985; Heizmann & Hunziker, 1989).

We have recently purified a Ca^{2+}-binding protein from mouse Ehrlich ascites-tumour cells which appeared to be different from calmodulin, parvalbumin, S-100 protein, oncomodulin and calbindin (Kuźnicki & Filipek, 1987). This protein binds two Ca^{2+} ions, each with a different affinity, and undergoes a conformational change upon Ca^{2+} binding. Its amino acid composition, Ca^{2+}-binding parameters and Ca^{2+}-induced conformational change suggested that the protein from Ehrlich ascites-tumour cells may belong to the family of EF-hand proteins.

In the present work, we improved the isolation procedure, purified large amounts of this protein and carried out a partial amino acid sequence analysis. When we searched in a protein databank (PIR, Protein Identification Resource) for proteins with related sequences, a striking homology was found to rat and human calcyclin, a protein accumulating in a cell-cycle-dependent manner (Calabretta *et al.*, 1986*a*,*b*; Ferrari *et al.*, 1987).

MATERIALS AND METHODS

Protein purification

The Ca^{2+}-binding protein was purified from Ehrlich ascites-tumour cells (200 μg/g wet wt. of cells), as previously described (Kuźnicki & Filipek, 1987). Final purification was performed by h.p.l.c. using a reverse-phase (C_8) column (Aquapore RP-300 cartridge, particle size 10 μm, pore size 30 nm; Brownlee Laboratories, Santa Clara, CA. U.S.A.). The buffer systems were buffer A (25 mM-Tris/HCl, pH 7.5, 0.1 mM-EGTA in water) and buffer B [25 mM-Tris/HCl, pH 7.5, 0.1 mM-EGTA, 60% (v/v) acetonitrile in water]. A linear gradient from 0 to 100% B in 45 min was used, with a flow rate of 2 ml/min; 30 μg of protein was injected in each run. The chromatogram was monitored at 220 nm. Protein peaks were pooled separately, dialysed and freeze-dried. Calmodulin and S-100 protein were purified from bovine brain as described by Drabikowski *et al.* (1977). Rat muscle parvalbumin was isolated as described by Berchtold *et al.* (1982). Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Polyacrylamide-gel electrophoresis

Proteins were separated by Tricine/SDS/polyacrylamide (15%)-gel electrophoresis as described by Schägger & von Jagow (1987) in the presence or absence of 2-mercaptoethanol. Gels were stained with Coomassie Blue R 250. For molecular-mass determinations the calibration protein mixture (MW-SDS-17) from Sigma was used.

Electrophoretic transfer

Proteins were electrophoretically transferred to nitrocellulose (Schleicher und Schüll, Dassel, Germany) in a Bio-Rad Trans-Blot apparatus at 70 mA for 12–16 h at room temperature with a transfer buffer containing 192 mM-glycine, 25 mM-Tris, pH 8.3, and 20% (v/v) methanol (Towbin *et al.*, 1979).

^{45}Ca-transblot electrophoresis

After the transfer of the proteins, the nitrocellulose was washed for 3 × 20 min in a buffer containing 60 mM-KCl, 10 mM-imidazole/HCl and 5 mM-$MgCl_2$, pH 6.8 (Maruyama *et al.*, 1984). Then the nitrocellulose was incubated for 10 min with 0.1 mCi of $^{45}CaCl_2$ (sp. radioactivity 41 mCi/mg; New England Nuclear) in 100 ml of

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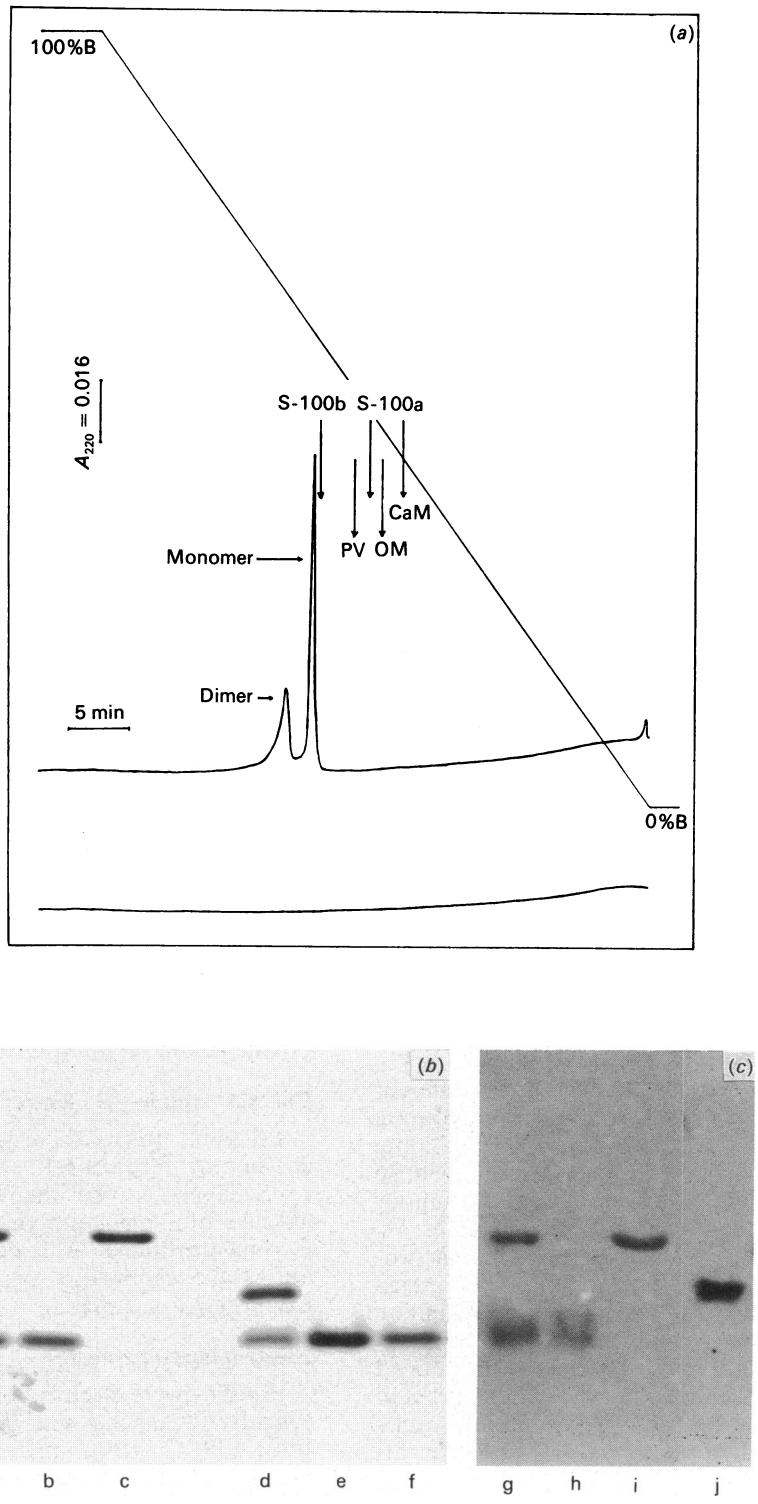


Fig. 1. Separation and analysis of the Ca^{2+}-binding protein from Ehrlich ascites-tumour cells

(*a*) On reverse-phase h.p.l.c., the monomeric protein was eluted at 62%, and the dimeric form at 66% buffer B. Reference proteins: S-100a; S-100b; PV, parvalbumin; OM, oncomodulin; CaM, calmodulin. A 70 μg portion of Ca^{2+}-binding protein was applied to the column. (*b*) One-dimensional SDS/polyacrylamide gel in the absence (a–c) or presence (d–f) of 2-mercaptoethanol. Lanes: a, Ca^{2+}-binding protein (16 μg) before h.p.l.c. (mixture of monomer and dimer); b and e, monomeric protein (8 μg) eluted at 62% buffer B; c and f, dimeric protein (9 μg) eluted at 66% buffer B; d, mixture of S-100 proteins (5 μg, 10 kDa, lower band) and parvalbumin (6 μg, 12 kDa, upper band). Proteins are stained with Coomassie Blue. (*c*) ^{45}Ca-transblot electrophoresis. Proteins (a–c) from Fig. 1(*b*) were blotted on to nitrocellulose (g–i) and incubated with ^{45}Ca. Lanes: g, mixture of monomer and dimer; h, monomer; i, dimer; j, parvalbumin.

the above buffer. Subsequently the nitrocellulose was washed with water for 5 min and dried. Autoradiographs were produced by exposure to Kodak X-Omat films for 48–72 h at −70 °C.

Immunoblotting

Polyclonal antibodies against the homogeneous mouse protein were raised in rabbits, and affinity-purified (Kuźnicki *et al.* 1989). Purified Ca^{2+}-binding protein (monomer and dimer) from Ehrlich ascites-tumour cells, bovine brain calmodulin, bovine brain S-100 proteins, rat muscle parvalbumin and Ehrlich ascites-tumour cell extracts [heat-treated (15 min at 95 °C) or non-treated] were checked for cross-reactivity with these antibodies by immunoblotting (Towbin *et al.*, 1979).

Amino acid analysis

Monomeric and dimeric protein were hydrolysed in the gas phase under argon with 6 M-HCl at 110 °C for 22–24 h. Amino acid analyses were performed on a Chromakon 500 amino acid analyser (Kontron, Zurich, Switzerland), which was equipped with an *o*-phthalaldehyde fluorescence-detection system for high sensitivity (Böhlen & Schröder, 1982).

Tryptic digestion of the purified protein

Protein dimers were digested with trypsin (Sigma) in 0.2 M-NH_4HCO_3/0.1 mM-$CaCl_2$, pH 8.2, with an enzyme/substrate ratio of 1:10 (w/w) for 3 h at 37 °C. The digestion was stopped by adding a drop of acetic acid. The tryptic peptides were separated by reverse-phase chromatography on a 4 mm × 250 mm C_8 column (Nucleosil 300-7; Macherey–Nagel, Düren, Germany). Buffer A consisted of 0.1 % trifluoroacetic acid in water, and buffer B was 0.1 % trifluoroacetic acid in 90 % (v/v) acetonitrile. A linear gradient was run from 0 to 100 % B in 180 min, with a flow rate of 0.5 ml/min. The eluate was monitored at 220 nm.

Amino acid sequence analysis

Automated Edman degradation of the tryptic peptides was carried out on a gas/liquid-phase micro-sequenator (model 470A) equipped with an on-line PTH-Analyzer (model 120A) and a model 900A computer (Applied Biosystems, Foster City, CA, U.S.A.). The experimental protocols for sequencing and h.p.l.c. identification of phenylthiohydantoin derivatives of amino acids were as specified by the manufacturer.

The two peptide sequences were compared with known amino acid sequences by computer search (PIR databank, protein sequence database, release 38, March 1989, accession number BCHUY89).

RESULTS

Purification by h.p.l.c.

The Ca^{2+}-binding protein from Ehrlich ascites-tumour cells was isolated as described by Kuźnicki & Filipek (1987) and finally purified by h.p.l.c. using the EGTA buffer system (Fig. 1*a*). Two protein peaks were eluted, at 62 % and at 66 % buffer B respectively. Other Ca^{2+}-binding proteins, such as bovine brain calmodulin (45 % buffer B) and oncomodulin (48–49 %), S-100a (52 %), parvalbumin (54 %) and S-100b (61 %) from rat, were eluted at different positions. Both h.p.l.c. peaks were subjected to SDS/polyacrylamide-gel electrophoresis (Fig. 1*b*). In the absence of 2-mercaptoethanol, the protein eluted at 62 % buffer B migrated as a 10 kDa band, and the protein at 66 % buffer B gave rise to a 20 kDa band (Fig. 1*b*, lanes b and c). In the presence of 2-mercaptoethanol both peaks (62 and 66 % B) migrated as 10 kDa bands (Fig. 1*b*, lanes e and f), indicating the presence of a disulphide-linked dimeric form of the protein. The monomeric form co-migrates on SDS/polyacrylamide gels with S-100 proteins (Fig. 1*b*, lane d).

The amino acid composition of the monomer and dimer is given in Table 1. Both compositions were identical, suggesting that the larger protein is a dimer of the smaller protein.

When the monomeric and dimeric forms of the protein were subjected to h.p.l.c. in the presence of Ca^{2+} (instead of EGTA), they were eluted as a single peak at 49 % buffer B (results not shown).

^{45}Ca autoradiography

The monomers and dimers of the protein from Ehrlich ascites-tumour cells were blotted from SDS/polyacrylamide gels on to nitrocellulose, which was then incubated with ^{45}Ca. Both forms showed a strong Ca signal, comparable with that of parvalbumin (Fig. 1*c*).

Table 1. Amino acid composition of the calcium-binding protein from Ehrlich ascites-tumour cells, human calcyclin and S-100b protein from bovine brain

	Proposed no. of residues per molecule		
Amino acid	Monomer and dimer of Ca^{2+}-binding protein from mouse Ehrlich ascites tumour cells	Human calcyclin*	Bovine brain S-100b protein†
Aspartate	9	9	9
Threonine	2	3	3
Serine	4	3	5
Glutamate	13	14	19
Proline	1	1	0
Glycine	10	6	4
Alanine	8	8	5
Cysteine	1	1	0
Valine	3	3	6
Methionine	1	1	3
Isoleucine	5	5	4
Leucine	12	14	8
Tyrosine	3	3	1
Phenylalanine	2	3	7
Histidine	1–2	3	7
Lysine	7	9	8
Arginine	3	3	1
Total no. of residues	86	89	90

* Calabrette *et al.* (1986*a*).
† Marshak *et al.* (1981).

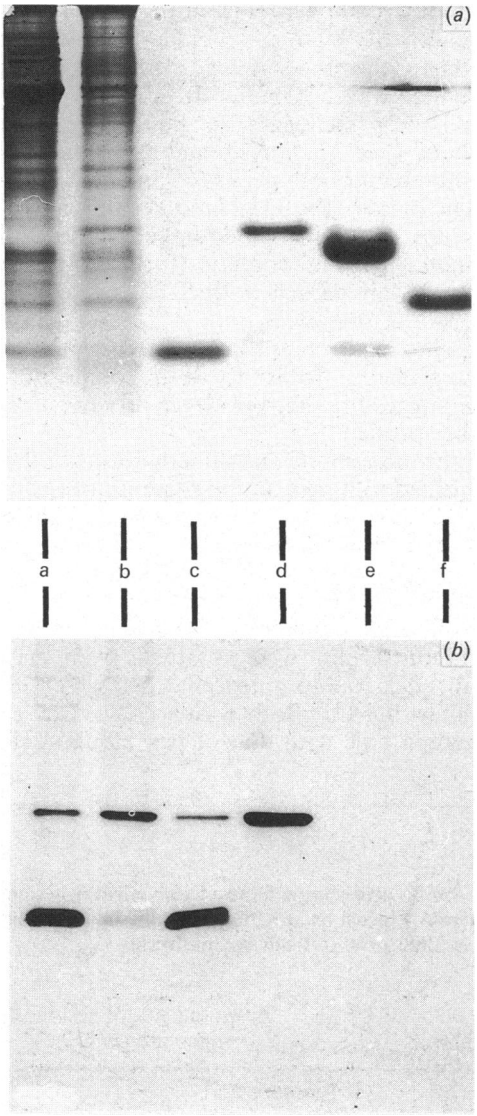


Fig. 2. Characterization of polyclonal antibodies against the Ca^{2+}-binding protein by immunoblotting

(*a*) One-dimensional SDS/polyacrylamide gel without 2-mercaptoethanol; (*b*) the corresponding immunoblot. Lanes: a, low-ionic-strength extract of Ehrlich ascites-tumour cells (42 μg); b, the supernatant after heat treatment (45 μg); c, monomeric protein with trace amounts of dimer (8 μg on gel and 1 μg on immunoblot); d, dimer (8 μg and 1 μg respectively); e, calmodulin (4 μg) and S-100 protein (4 μg); f, parvalbumin (4 μg). Proteins (*a*) were stained with Coomassie Blue. The corresponding immunoblot was developed with chloronaphthol and H_2O_2.

Immunoreactivity

Affinity-purified antibodies raised against the Ca^{2+}-binding protein from Ehrlich ascites-tumour cells were tested by immunoblotting for cross-reaction against calmodulin, parvalbumin or S-100 proteins (Fig. 2, lanes e and f). There was no cross-reactivity with these components. When total Ehrlich-ascites-tumour-cell extracts were tested, the antibody reacted solely with the monomeric and dimeric forms of the antigen, but with no other protein components, demonstrating its specificity (Fig. 2, lanes a–d). In Ehrlich-ascites-tumour-cell extracts the protein exists as monomer (major form) and dimer (minor form) (Fig. 2, lane a). In heat-treated extracts the dimeric form is prominent (Fig. 2, lane b).

Tryptic digestion of the purified protein

The Ca^{2+}-binding protein from Ehrlich ascites-tumour cells is highly resistant to proteolysis by chymotrypsin, papain or submaxillaris-gland protease regardless of the Ca^{2+} concentration (results not shown). However, with trypsin, at an enzyme/substrate ratio of 1:10 (w/w), cleavage of the protein could be achieved, although incomplete. The h.p.l.c. elution profile of the tryptic peptides is shown in Fig. 3. One of these peptides (a in Fig. 3), eluted at 49 % buffer B, was taken for amino acid sequence analysis.

Amino acid sequence analysis of the tryptic peptide

The amino acid sequence of the aforementioned peptide a is shown in Fig. 4(*a*). The sequencing was terminated after cycle 14, owing to the small amount of the peptide. A computer homology search of this sequence revealed a high similarity to human calcyclin. The peptide is located in the second Ca^{2+}-binding site of human calcyclin, demonstrating that it is an EF-hand protein (Fig. 4*b*). The mouse peptide a sequence is identical with the rat calcyclin sequence (Fig. 4*a*). There is one conservative amino acid exchange, aspartic acid for glutamic acid, in this region between the human and rat species, compared with four differences in the total sequence.

DISCUSSION

Previously a Ca^{2+}-binding protein was purified from mouse Ehrlich ascites-tumour cells (Kuźnicki & Filipek, 1987), but its identity or possible relation to other Ca^{2+}-binding proteins has not been established. Here we demonstrate that this protein exists in a monomeric and a dimeric form, which could be separated by reverse-phase h.p.l.c. in the presence of EGTA. Both forms may be physiologically active. A similar result has been found for the tumour-specific oncomodulin. Mutus *et al.* (1988) suggested that the dimeric form of oncomodulin, which may resemble the calmodulin molecule, may also have calmodulin-like regulating activity.

The amino acid sequence analysis of a tryptic peptide of the Ca^{2+}-binding protein from Ehrlich ascites-tumour cells showed identity with rat calcyclin (Murphy *et al.*, 1988) and a high homology to human calcyclin (Calabretta *et al.*, 1986*a*). The molecular mass found for our protein (10 kDa) is supported by the cDNA-derived amino acid sequence of calcyclin.

The putative Ca^{2+}-binding properties of calcyclin were predicted from the sequence data (Calabretta *et al.*, 1986*a*). Our results confirm this suggestion by Ca^{2+}-binding data from our previous paper (Kuźnicki & Filipek, 1987). The monomer and dimer of this protein bind Ca^{2+} in the ^{45}Ca-transblot electrophoresis.

The 2A9 gene encoding human calcyclin is considered to be a promising candidate among the genes which may play a role in cell-cycle control (Calabretta *et al.*, 1986*a*,*b*). Some help in understanding the role of calcyclin may come from studies of its distribution. Preliminary immunological data showed that the Ca^{2+}-binding pro-

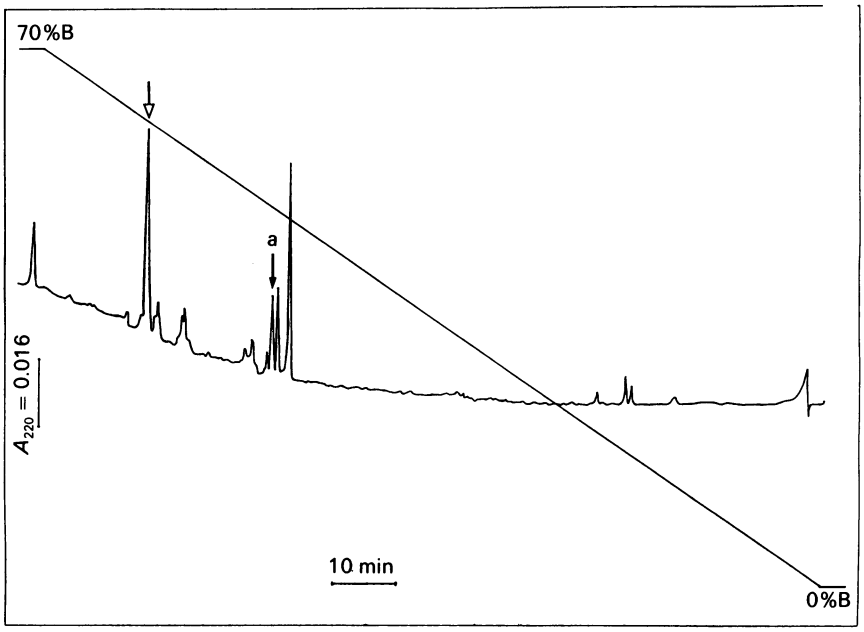
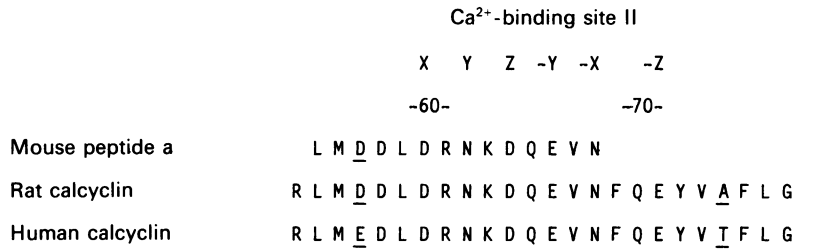


Fig. 3. Tryptic-peptide profile of the purified Ca^{2+}-binding protein

The conditions are described in the Materials and methods section. Peptide a (eluted at 49% buffer B) was subjected to amino acid sequence analysis. The undigested protein (dimer) was eluted at 60% buffer B (↓).

(*a*)



(*b*)

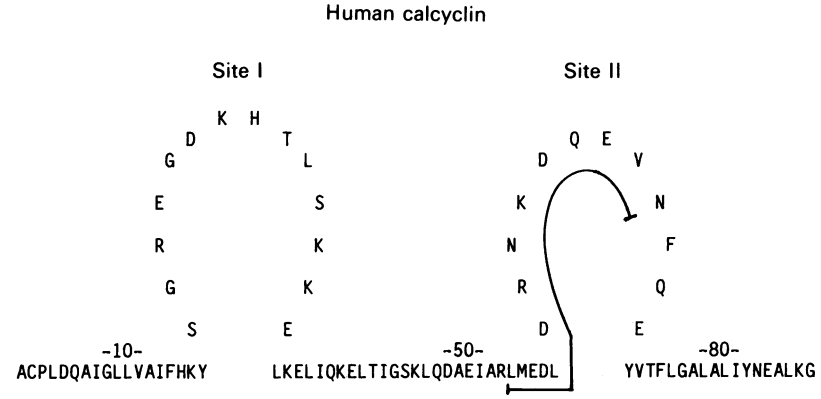


Fig. 4. Sequence comparison of calcyclin with peptide a obtained by tryptic digestion of the Ca^{2+}-binding protein

(*a*) The amino acid sequence of peptide a is aligned with the corresponding sequences of rat and human calcyclin. X, Y, Z, −Y, −X and −Z indicate potential Ca^{2+} ligands according to Kretsinger (1980). Amino acid exchanges are underlined. (*b*) The cDNA-derived protein sequence of human calcyclin (taken from Calabretta *et al.*, 1986*a*). The Ca^{2+}-binding sites I and II are indicated by the loop. The position of the mouse peptide a is marked by a bar.

tein from Ehrlich ascites-tumour cells is present in a variety of normal tissues (Kuźnicki *et al.*, 1989), and is therefore not tumour-specific, as is the Ca^{2+}-binding protein oncomodulin.

Calcyclin, which is also identical with the prolactin-receptor-associated protein (Murphy *et al.*, 1988), belongs to the family of S-100 proteins. These proteins, probably also including our protein, are a group of small Ca^{2+}-binding proteins involved in various functions such as cell-cycle regulation, cell differentiation and cytoskeletal–membrane interaction. Recently, new members of the S-100 family have been described (Kligman & Hilt, 1988; Heizmann & Hunziker, 1989).

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