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p30, a novel protein target of mouse calcyclin (S100A6)

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A novel protein target of mouse calcyclin (S100A6) was detected by a gel overlay method with ^{125}I-labelled calcyclin. Interaction of calcyclin with its 30 kDa target protein (p30) present in Ehrlich ascites tumour (EAT) cells depended on the presence of Ca^{2+} ions. The binding of p30, evidenced by the reaction with ^{125}I-labelled calcyclin, was found to be of higher affinity than the binding between mouse calcyclin and annexin II or glyceraldehyde-3-phosphate dehydrogenase. Examination of tissue extracts by the gel overlay method has shown that p30 is present not only in the EAT cells but also in mouse brain and spleen. This novel target protein of mouse calcyclin was purified to homogeneity from EAT cells by means of Phenyl-Sepharose chromatography, affinity chromatography and CM-cellulose chromatography. Purified p30 was digested with α-chymotrypsin and a partial amino acid sequence of one of the resulting peptides was established. A database search analysis revealed that the sequence is unique, with a similarity of less than 55 % to any other known protein sequence.

INTRODUCTION

Calcyclin is a Ca^{2+}-binding protein belonging to the S-100 family [1]. So far 16 different S-100 proteins have been isolated and characterized from various tissues [2,3]. All members of the S-100 family contain two 'EF-hand' motifs and have very high similarities in their amino acid sequences, not only at the Ca^{2+}-binding sites but also in other regions of the molecules. S-100 proteins are distributed in a cell- and tissue-specific manner and they have been shown to be involved in many cellular processes such as cell cycle progression or differentiation.

The biochemical properties of calcyclin have been established [4–6] and its structure is now quite well known [7]. The function of calcyclin is, however, not yet understood although there are many data in the literature concerning this problem, as reviewed in [8]. For example, a regulatory function of calcyclin has been suggested on the basis of its Ca^{2+}-dependent binding to target molecules. By affinity chromatography, annexin II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were found to bind to mouse calcyclin in the presence of Ca^{2+} [9]. Although these proteins have been identified, the functional implications of their interactions with calcyclin have not been elucidated. It is thus reasonable to study the Ca^{2+}-dependent interaction of mouse calcyclin with its targets in more detail and to define its physiological relevance. In the current work, with the gel overlay method, we have found that mouse calcyclin interacted in a Ca^{2+}-dependent manner with a novel target, a 30 kDa protein present in Ehrlich ascites tumour (EAT) cells and in some other mouse tissues. The aim of this work was to purify the 30 kDa protein from EAT cells and to identify it on the basis of its amino acid sequence.

MATERIALS AND METHODS

Proteins

Calcyclin target protein (p30) was purified from mouse EAT cells. All purification steps were performed at 4 °C. EAT cells (50 g) were washed three times with 0.9 % NaCl and homogenized with a Polytron homogenizer (6000 rev./min) in a buffer containing 20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, 10 mg/l leupeptin, 5 mg/l aprotinin and 20 mg/l soybean trypsin inhibitor. The homogenate was centrifuged for 1 h at 30000 ***g***, and $CaCl_2$ was added to the supernatant to a final concentration of 1 mM. The supernatant was applied to a Phenyl-Sepharose column (2 cm × 15 cm) equilibrated with buffer A [20 mM Tris (pH 7.5)/1 mM DTT/0.25 mM PMSF/0.5 M NaCl/1 mM $CaCl_2$]. The unbound fraction was than applied to a calcyclin affinity column equilibrated with buffer A. The column was washed extensively and the protein fraction containing p30 was eluted with buffer A containing 2 mM EGTA instead of $CaCl_2$. The fraction eluted with EGTA was concentrated with Amicon PM 10 membrane, dialysed against buffer A, incubated with phosphatidylserine liposomes for 20 min at room temperature and centrifuged for 20 min at 100000 ***g***. The liposomes were washed twice in the same buffer. The supernatants were combined and dialysed against buffer B [10 mM imidazole (pH 7.0)/0.2 mM EGTA/0.25 mM PMSF/1 mM DTT]. After dialysis this fraction was applied to a CM-cellulose column (1.5 cm × 20 cm) equilibrated with buffer B. The proteins were eluted with a linear gradient of the above buffer containing 0.25 M NaCl (0–100 %) at a flow rate of 0.5 ml/min. The protein of molecular mass 30 kDa was eluted at 120–150 mM NaCl. The detection of p30 at each step of purification was performed by SDS/PAGE [15 % (w/v) gel] with low-molecular-mass standards from Bio-Rad followed by gel overlay with ^{125}I-labelled calcyclin.

Calcyclin from mouse EAT cells was purified as described by Filipek et al. [10]. A homogeneous mouse EAT annexin II preparation was obtained as described previously [9]. Rabbit skeletal muscle GAPDH was purchased from Sigma.

Tissue extracts

Various mouse tissues were homogenized with a Polytron homogenizer (6000 rev./min) in 20 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 mM EGTA/1 mM DTT/1 mM PMSF/10 mg/l leupeptin/5 mg/l aprotinin/20 mg/l soybean trypsin inhibitor, and then centrifuged for 1 h at 4 °C and 30000 ***g***. The supernatants were subjected to SDS/PAGE [15 % (w/v) gel]. Calcyclin

Abbreviations used: DTT, dithiothreitol; EAT, Ehrlich ascites tumour; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PVDF, poly(vinylidene difluoride).

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binding was assessed by means of the gel overlay method with ^{125}I-labelled calcyclin.

Gel overlay experiments

Mouse EAT calcyclin was ^{125}I-labelled with Amersham reagent. Gel overlay experiments were done as follows. The examined fractions were electrophoretically transferred from the SDS/gel to nitrocellulose. The blots were washed three times for 10 min in 50 mM Tris/HCl, pH 7.5, and blocked for 2 h in 50 mM Tris/HCl (pH 7.5)/4 % BSA/1 mM $CaCl_2$ (or 2 mM EGTA). The nitrocellulose was then incubated for 1 h in 50 mM Tris/HCl (pH 7.5)/200 mM NaCl/1 % (w/v) BSA/1 mM $CaCl_2$ (or 2 mM EGTA) supplemented with 10 μM ^{125}I-labelled calcyclin (specific radioactivity 0.45 mCi/mmol). Subsequently the blots were washed three times for 20 min in the incubation buffer. After being dried the nitrocellulose was exposed to Rentgen-XS film (Organica Foton, Warsaw, Poland).

Partial amino acid sequence

Purified p30 (50 μg) was digested with 0.5 μg of α-chymotrypsin in 100 μl of 20 mM Tris/HCl buffer, pH 7.5, at room temperature for 30 min. The reaction was stopped with SDS-sample buffer. Samples were boiled for 5 min and applied to a 15 % (w/v) SDS gel. Peptides were transferred to a poly(vinylidene difluoride) (PVDF) membrane in a buffer containing 50 mM borate and 20 % (v/v) methanol, pH 9.0. The membrane was then stained with 0.1 % Coomassie Brilliant Blue R-250/40 % (v/v) methanol/10 % (v/v) acetic acid and destained with 40 % methanol/10 % acetic acid. Sequencing of the peptide of approx. 17 kDa was performed by WITA GmbH (Wittmann Institute of Technology and Analysis of Biomolecules, Technologiezentrum, Teltow, Germany).

Electrophoresis and immunoblotting

Gel electrophoresis with 15 % (w/v) polyacrylamide containing 0.1 % SDS was performed by the method of Laemmli [11]. Gels were stained with Coomassie Brilliant Blue. After separation by SDS/PAGE, proteins were transferred electrophoretically to nitrocellulose and p30 was detected with ^{125}I-labelled calcyclin.

Other methods

Phosphatidylserine (5 mg/ml) liposomes were prepared in 20 mM Tris/HCl (pH 7.5)/50 mM NaCl/0.1 mM EDTA/0.05 % sodium azide by sonication in Ultrasonic Disintegrator, Model UD-11 (Techpan, Warsaw, Poland). Mouse EAT calcyclin was coupled with cyanogen bromide-activated Sepharose 4B (Pharmacia) by the procedure outlined by the manufacturer. Protein concentration was estimated by the method of Lowry et al. [12] with BSA as a standard.

RESULTS

Detection of p30 and its purification

When the EAT cell supernatant after centrifugation at 30000 ***g*** was examined by the gel overlay method, autoradiography revealed a single ^{125}I-labelled calcyclin-binding protein with a molecular mass of approx. 30 kDa (Figure 1, lane 2). Therefore we performed a purification of p30. The initial step of purification of p30 from EAT cells was achieved by means of hydrophobic chromatography on a Phenyl-Sepharose column. Supernatant after centrifugation at 30000 ***g*** was supplemented with $CaCl_2$ to a final concentration of 1 mM and applied to a Phenyl-Sepharose column. This chromatographic step was used to obtain p30 free of calcyclin. The 30 kDa protein was found in the flow-through fraction whereas calcyclin remained bound to the Phenyl-Sepharose in the presence of Ca^{2+}. The second purification step led to the separation of p30 from the bulk of the proteins by affinity chromatography with calcyclin coupled to CNBr-Sepharose. The 30 kDa protein was eluted in the buffer containing EGTA, together with annexin II and GAPDH. The third purification step was performed to remove annexin II (and other annexins). Unlike annexins the 30 kDa protein did not bind to liposomes in the presence of Ca^{2+}. The final purification of p30 was achieved by ion-exchange chromatography on a CM-cellulose column. Approx. 50 μg of p30 was obtained from 50 g wet weight of cells, indicating that the concentration of this protein in EAT cells is rather low. Purified p30 migrated as a single band with the same mobility on SDS/PAGE (15 % gel) as bovine carbonic anhydrase (a component of the low molecular mass standards); thus its molecular mass was approx. 30 kDa (Figure 1, lane 3). When equal amounts of purified p30, annexin II and GAPDH were incubated with ^{125}I-labelled calcyclin in the presence of Ca^{2+}, the signal of iodinated calcyclin for p30 was the most intense (Figure 2). None of the proteins was labelled by ^{125}I-labelled calcyclin in the presence of 2 mM EGTA (results not shown).

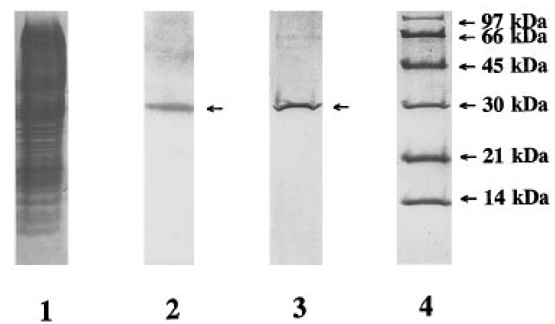

Figure 1 SDS/PAGE and autoradiogram of proteins from EAT cell supernatant

SDS/PAGE of the proteins from EAT cell supernatant (100 μg) after centrifugation at 30000 ***g*** (lane 1) and an autoradiogram of these proteins blotted from the SDS/gel on nitrocellulose and incubated with ^{125}I-labelled calcyclin in the presence of Ca^{2+} (lane 2); SDS/PAGE of purified p30 (2 μg) from the EAT cells (lane 3) and of molecular mass standards (lane 4).

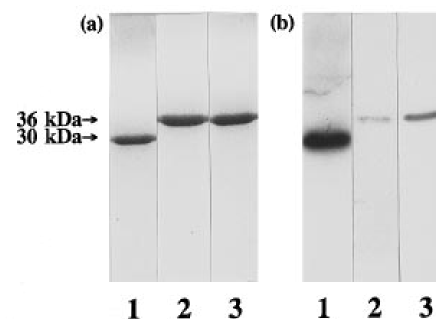

Figure 2 SDS/PAGE (a) and autoradiogram of proteins (5 μg of each) incubated with ^{125}I-labelled calcyclin in the presence of Ca^{2+} (b)

Lane 1, p30 from EAT cells; lane 2, annexin II; lane 3, GAPDH.

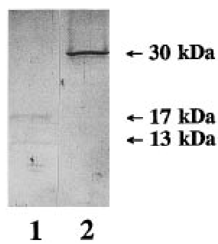

Figure 3 SDS/PAGE of the proteolytic fragments derived from p30

p30 was digested with α-chymotrypsin as described in the Materials and methods section. Lane 1, 13 and 17 kDa fragments of p30 (2 μg); lane 2, p30 (2 μg) not treated with α-chymotrypsin.

Partial amino acid sequence of p30

Figure 3 shows the SDS/PAGE of the peptides derived from p30 after enzymic digestion with α-chymotrypsin. Peptides of p30 were separated by SDS/PAGE (15% gel), transferred to a PVDF membrane and the partial amino acid sequence of a peptide of approx. 17 kDa was established directly from the PVDF membrane. This amino acid sequence was shown to be: TVKISNYGRDQSD. A computer search analysis of this sequence revealed no similarity higher than 55% to any protein from the Swissprot database.

Tissue distribution of p30 in the mouse

Various mouse tissues were analysed for the presence of p30 by using ^{125}I-labelled calcyclin. In the presence of Ca^{2+} the most intense signal of ^{125}I-labelled calcyclin bound to 30 kDa protein was observed in brain and spleen. A less intense signal was seen in the supernatant fraction of stomach, and a protein band of approx. 28 kDa was labelled with iodinated calcyclin in the supernatant fraction of heart. No calcyclin binding was detected in the supernatant fraction of liver, kidney, lung and skeletal muscle (results not shown).

DISCUSSION

Using the gel overlay method we have identified in the present study a novel target protein of mouse calcyclin in EAT cells. This protein was named p30 in accordance with its molecular mass estimated by SDS gel electrophoresis. Previously the use of ^{125}I-labelled calcyclin gel overlay allowed the detection of several proteins interacting with calcyclin in the EAT cell membrane fraction [13]. In the present study the EAT cell supernatant from centrifugation at 30000 ***g*** was examined and p30 was the only protein detected in this fraction by ^{125}I-labelled calcyclin. After purification, the p30 revealed the most intense ^{125}I-labelled calcyclin signal on the autoradiogram when compared with annexin II and GAPDH, the calcyclin targets earlier identified by affinity chromatography [9]. Although the interaction of ^{125}I-labelled calcyclin with annexin II was recently shown to be weak [10], the relatively weak signal of GAPDH (not corresponding to its high-affinity calcyclin binding evidenced by other methods [10,14]) is probably due to the destruction of its tetrameric structure during electrophoresis in the presence of SDS. The results of this work indicate that p30 might represent, in addition to GAPDH, a calcyclin target in EAT cells.

Some potential targets were also identified for calcyclin isolated from other species. The interaction was shown for bovine heart calcyclin with annexin II, annexin VI and GAPDH [14], for chicken gizzard calcyclin with caldesmon [15] and for rabbit lung calcyclin with annexin XI [6,16,17]. Along with annexin XI, the binding of a protein of approx. 30 kDa to rabbit ^{125}I-labelled calcyclin in the lung extract was reported [6]. However, this 30 kDa protein was not further studied or identified.

The p30 was detected in EAT cells and the gel overlay method showed that it is also present in other mouse tissues such as brain and spleen. This protein was purified from EAT cells, and an analysis of its partial amino acid sequence indicated that it might not only represent a novel calcyclin target in the mouse but could also be a novel protein. The susceptibility of p30 to proteolysis and its small amount in EAT cells made it impossible to perform a biochemical characterization of this protein but further studies of p30 should yield a more profound understanding of calcyclin function within the cell. The determination of the full amino acid sequence of p30 and purification of the recombinant protein for biochemical studies are within the scope of our future work.

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