Calcyclin is a calcium and zinc binding protein

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Calcyclin, a cell cycle regulated protein, was recently purified from Ehrlich ascites tumour (EAT) cells and shown to be a calcium binding protein. Here we show that calcyclin monomer and dimer also bind zinc ions. Zinc binding sites seem to be different from calcium binding sites since: preincubation with Ca^{2+} lacks effect on the binding of Zn^{2+}, and Ca^{2+} (but not Zn^{2+}) increases tyrosine fluorescence intensity. Binding of Zn^{2+} reduces the extent of the conformational changes induced by Ca^{2+}, and seems to affect Ca^{2+}-binding. The data suggest that Ca^{2+} and Zn^{2+} might trigger the biological activity of calcyclin.

Calcyclin; Calcium-binding protein; Zinc-binding protein

1. INTRODUCTION

Calcyclin is the name that was given to a gene originally identified as a cDNA clone (2A9) whose cognate RNA is growth regulated [1,2]. The steady-state levels of cytoplasmic mRNA recognized by a calcyclin probe increase when quiescent fibroblasts are stimulated to proliferate by either serum, platelet-derived growth factor or epidermal growth factor. The level of calcyclin mRNA also increases in human acute myeloid leukemia and it was suggested that calcyclin is involved in the control of cell proliferation [3]. Also, it was deduced from cDNA sequence that calcyclin contains the EF-hand structural elements, which suggested calcium binding. This suggestion was confirmed when calcyclin was purified from EAT cells and directly shown to bind calcium ions [4,5]. It was also shown that the expression of calcyclin is tissue- and cell-specific [6]. The calcyclin cDNA sequence has strong homology to the sequences of the S-100 proteins and to the p11 (p11 is a subunit of the complex that serves as a cellular substrate for tyrosine kinase (reviewed in [7]). We showed earlier, that calcyclin, similarly to S-100 protein, formed dimers [5]. Since the β subunit of S-100 binds Zn^{2+} and the binding sites are different from the Ca^{2+}-binding sites [8,9] we investigated if calcyclin has similar properties. We found that calcyclin is a Zn^{2+}-binding protein and demonstrated that Zn^{2+}- and Ca^{2+}-binding sites are located differently at the calcyclin molecule.

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Abbreviations: EAT cells, Ehrlich ascites tumour cells; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-*O,O'*-bis(2-aminoethyl)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

2.1. *Proteins*

Calcyclin was isolated from EAT cells and monomers and dimers were separated by HPLC [4,5]. Calmodulin and S-100 proteins (a mixture of α and β subunits) were purified from bovine brain [10] and parvalbumin from rat muscle [11]. Protein concentration was estimated as described in [12].

2.2. *Polyacrylamide gel electrophoresis and blotting*

The details on tricine-SDS-polyacrylamide (15%) gel electrophoresis [13] and on electrophoretic transfer [14] are given in our earlier paper [5].

2.3. *^{65}Zinc- and ^{45}calcium-transblot electrophoresis*

After the transfer of proteins onto nitrocellulose, the filters were washed in a buffer containing 50 mM NaCl, 100 mM Tris-HCl, pH 7.5, for at least 1 h [15]. The nitrocellulose filters were incubated for 15 min with 10 μCi of $^{65}ZnCl_2$ (2 Ci/g, Dupont de Nemours, FRG) in 20 ml of the above buffer. The filter was washed with the same buffer containing no Zn^{2+} for 15 min with two changes. In competition experiments, divalent metal ions were included in the buffer during all steps. Autoradiographs were exposed to Rentgen-XS films (Organika-Foton, Poland) for 7–14 days at −70°C. ^{45}Calcium-transblot was done as in [16].

2.4. *Fluorescence measurements*

The effect of Ca^{2+} and Zn^{2+} on tyrosine fluorescence intensity of calcyclin was examined using a Perkin-Elmer MPF-2L spectrofluorimeter, at 20°C. Tyrosine fluorescence was monitored at 304 nm, with excitation at 276 nm. Calcyclin was decalcified as described in [4].

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3. RESULTS

Fig. 1 shows the autoradiographs of the blots incubated with either radioactive Ca^{2+} (Fig. 1A) or Zn^{2+} (Fig. 1B,C). All proteins tested (calmodulin, parvalbumin, calcyclin and S-100) bound Ca^{2+}, but only calcyclin and S-100 bound Zn^{2+}. There is more $^{65}Zn^{2+}$ in the S-100 track than in the calcyclin track, because different amounts of the proteins were present in the gel and because there is slightly lower binding of Zn^{2+} by calcyclin than by S-100 protein (see Fig. 2, where similar amount of both proteins was used).

Calcyclin forms dimers by S–S bridges that are visible on SDS-polyacrylamide gels without 2-mercaptoethanol. Fig. 1C shows that calcyclin monomer as well as its dimers bind Zn^{2+}.

The ability of Zn^{2+} to bind to the proteins immobilized on nitrocellulose depends on the pH of the binding buffer. Therefore we examined the Zn^{2+} binding to calcyclin, calmodulin and S-100 protein at pH 6.8, 7.5 and 8.0. Autoradiography of the probed filters revealed that calcyclin and S-100 bound Zn^{2+} not only at pH 7.5 (Fig. 1), but also at pH 6.8 and pH 8.0 (not shown). The intensity of Zn^{2+} signal was higher at pH 8.0 than at pH 6.8, but calmodulin did not bind Zn^{2+} at any pH (shown at pH 7.5; Fig. 1B).

Divalent metal ions such as Cd^{2+} can replace Zn^{2+} ions in several zinc binding proteins (reviewed in [17]). Therefore we examined the ability of cadmium ions to compete with $^{65}Zn^{2+}$ in the zinc overlay assay (Fig. 2). Nitrocellulose filters containing calcyclin and S-100 protein were equilibrated for 1 h with a buffer containing 0.1 mM $CdCl_2$. The filters were then incubated with $^{65}ZnCl_2$ plus competitor and washed in a buffer that contained the competitor alone. The autoradiographs from such experiments showed that Cd^{2+} significantly decreased binding of Zn^{2+} to both proteins, but Zn^{2+}-binding by S-100 protein was more efficiently inhibited by Cd^{2+} (Fig. 2B). In contrast, Ca^{2+} at the same concentration (0.1 mM), did not compete with $^{65}Zn^{2+}$ in binding to calcyclin or S-100 (not shown), and at 10 mM had only small effect, probably due to the non-specific binding (Fig. 2C).

Ca^{2+} binding to calcyclin induces conformational changes that can be measured by an increase of the intrinsic tyrosine fluorescence [4]. Calcyclin contains 3 tyrosine residues (Fig. 3). Tyrosine 18 and 72 are near the first and second Ca^{2+} binding site, respectively, and both may be responsible for the Ca^{2+}-induced changes in fluorescence intensity. To check if Zn^{2+} binds to different sites in the calcyclin molecule, we studied the effect of each cation on the tyrosine fluorescence intensity. When Zn^{2+} was added to the apo-protein there was no increase in the fluorescence intensity unless Ca^{2+} was added (Fig. 4). Upon Ca^{2+} saturation in the presence of Zn^{2+} there was 17% increase of the fluorescence intensity. When Ca^{2+} was added in the absence of Zn^{2+} the fluorescence intensity increased up to 26%. Subsequent addition of Zn^{2+} partly (about 1/3 of the increase) decreased the level of fluorescence intensity (Fig. 4). These results and those in Fig. 3 suggested that Ca^{2+} and Zn^{2+} may bind to the different sites at the molecule.

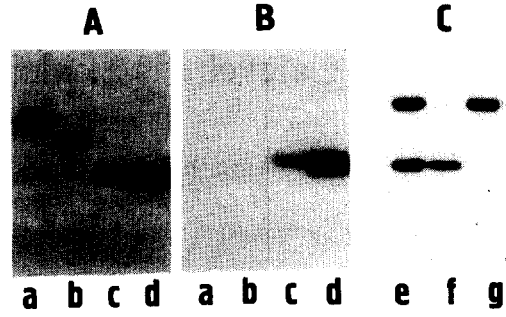

Fig. 1. $^{65}Zn^{2+}$ and $^{45}Ca^{2+}$ binding on nitrocellulose. The proteins were separated on polyacrylamide gels and transferred to nitrocellulose. The blots were incubated with either radioactive calcium (A) or zinc (B, C) (at pH 7.5), and autoradiographed for 5 days (A) or 14 days (B, C). A, B: (a) 8 μg of bovine brain calmodulin; (b) 6 μg of rat muscle parvalbumin; (c) 7.5 μg of EAT cells calcyclin; (d) 19 μg of bovine brain S-100 protein. C: (e) 18 μg of calcyclin dimer and monomer; (f) 9 μg of calcyclin monomer; (g) 9 μg of calcyclin dimer.

Ca^{2+} binding parameters were measured in the absence or presence of 0.1 mM $ZnCl_2$ by titration of the fluorescence intensity of calcyclin with increasing concentrations of Ca^{2+} (see [4] for details). Both titration curves exhibited an end-point at a Ca^{2+}/protein ratio of about 1.7 suggesting the binding of 2 calcium ions (not shown).

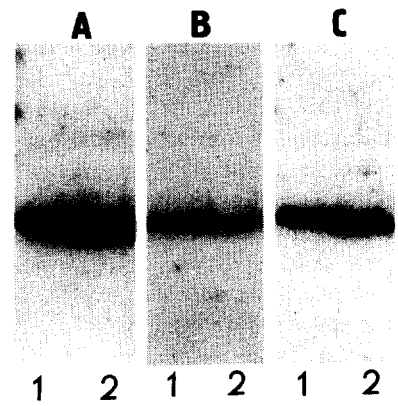

Fig. 2. Zn^{2+} binding in the presence of Cd^{2+} or Ca^{2+}. Autoradiographs (14 days) of the nitrocellulose filters containing 7.5 μg of calcyclin (1) and 8.5 μg of S-100 protein (2) after incubation with $^{65}ZnCl_2$. The control filters (A) were preincubated in a buffer containing no cations added, incubated in a buffer containing $^{65}Zn^{2+}$ and washed with a buffer containing no cations. (B, C) The filters were preincubated in buffers containing competitive metal ion (B, 0.1 mM $CdCl_2$; C, 10 mM $CaCl_2$), incubated in a buffer containing $^{65}ZnCl_2$ plus the same concentration of competing ion, and next washed with buffers containing $CdCl_2$ or $CaCl_2$, respectively.

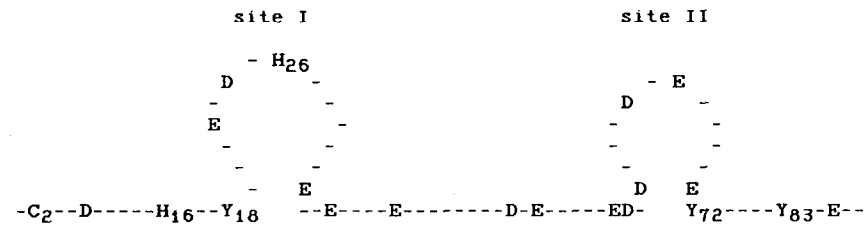

Fig. 3. Schematic structure of calcyclin. The plausible Ca^{2+} binding sites (I and II) are drawn as the loops. Only tyrosine residues (Y) and the amino acids that could participate in Zn^{2+}-binding are shown by the letters: C, cysteine; D, aspartate; E, glutamate; H, histidine. Other amino acids are shown by dashes. The numbers refer to the position of amino acids in cDNA-derived protein sequence of human calcyclin [2].

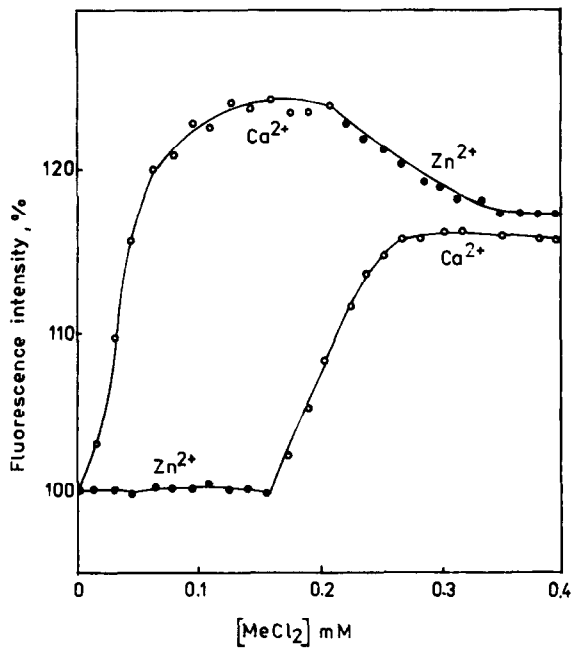

Fig. 4. Changes in tyrosine fluorescence intensity of calcyclin in the presence of Ca^{2+}, Zn^{2+} or both. Tyrosine fluorescence intensity of decalcified calcyclin was monitored: upon addition of $CaCl_2$ (open circles) and subsequent addition of $ZnCl_2$ (closed circles) (one line); or (second line) after addition of both cations in the opposite order. The [$MeCl_2$] shows the total concentration of $CaCl_2$ and $ZnCl_2$ added.

4. DISCUSSION

In this report we showed that calcyclin, similarly to S-100 protein, binds Zn^{2+}. The binding is specific, since other calcium binding proteins such as calmodulin or parvalbumin did not bind Zn^{2+} under the same conditions. Calcyclin seems to have lower affinity for Zn^{2+}, and/or lower number of Zn^{2+}-binding sites than S-100 protein. Because Cd^{2+} displaces Zn^{2+} less efficiently from calcyclin, than from S-100 protein, we suggest that these proteins have different affinities for cadmium ions.

Competition binding experiments on nitrocellulose (Fig. 2C) and the observation that Zn^{2+} binding to apoprotein does not induce the conformational changes characteristic for Ca^{2+} (Fig. 4) seems to suggest that Zn^{2+} binds to the sites different from those engaged in Ca^{2+} binding. Since the extent of Ca^{2+}-induced changes of tyrosine fluorescence intensity depends on the presence of Zn^{2+}, it might be that Ca^{2+}–calcyclin complex has different conformation than that of Ca^{2+}–Zn^{2+}–calcyclin. The results also suggest that Zn^{2+}-binding may affect Ca^{2+}-binding. We calculated from the titration curves that, despite Zn^{2+} presence, the number of calcium ions bound to calcyclin (2 ions) and the calculated product (8×10^{-12} M^2) of both dissociation constants are the same. It is impossible to estimate from this value the dissociation constant for each site since they have different affinities for Ca^{2+}. The results in Fig. 4 also may suggest that apparent cooperativity of Ca^{2+}-binding site(s) changes upon Zn^{2+}-binding to another scheme of binding.

The question arises where the zinc binding sites are located in the calcyclin molecule and what their structure is? There are several different ways in which Zn^{2+} could interact with proteins (reviewed in [17]). The first one involves interaction of the cation with residues located in a short segment of a protein that contains pairs of closely spaced cysteine and histidine residues or clusters of cysteine residues forming so called 'zinc fingers'. Human calcyclin contains only one cysteine (position 2) and two histidine residues (16 and 26) and therefore it is unlikely that they could form a 'finger' (Fig. 3). The second type of structure to bind Zn^{2+} has been described as the clusters of 4–12 acidic amino acid residues [18], but there are no such clusters in the calcyclin molecule (Fig. 3). A third possibility is an interaction of Zn^{2+} with residues distantly located from each other in the primary sequence that form a structural element called a 'conformation' site (reviewed in [19]). It is possible that such a site may be responsible for binding of Zn^{2+} to calcyclin, but this must be further investigated.

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