Calmodulin binding to human spectrin

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Calmodulin is shown to interact with human spectrin dimer. The binding was highly calcium-dependent and observed in two different kinds of experiments. Firstly, affinity chromatography of calmodulin on a Sepharose 4B column with immobilized spectrin, and secondly, partition in aqueous two-phase polymer systems. In the column experiments stoichiometric amounts of calmodulin were retained on the spectrin–Sepharose column when micromolar concentrations of calcium were present. The calmodulin bound could be eluted with EGTA. The partition coefficient of calmodulin in an aqueous two-phase polymer system containing calcium was changed upon addition of spectrin, indicating an association between the two proteins. In the absence of calcium, spectrin did not cause any change in the partition behaviour of calmodulin, thus showing that the association requires calcium.

Calmodulin *Human spectrin* *Protein–protein interaction* *Erythrocyte* *Aqueous two-phase partition*

1. INTRODUCTION

Calcium is known to be involved in the control of many important cellular functions [1,2]. It has been suggested, for example, that the organization and function of cell cytoskeletons are influenced by calcium, since the assembly and disassembly of both microtubules [3] and actin-based microfilaments [4] have been shown to be calcium-dependent. One of the most important receptors for calcium in cells is calmodulin, a low-M_r protein which seems to be present in all eukaryotic cells [5,6]. By undergoing a conformational change on binding calcium, calmodulin mediates the effect of calcium on various enzyme activities [7] and on different cellular phenomena, such as motility, secretion and possibly also mitosis [8].

The observation that calmodulin inhibitors induce stomatocytosis has led to the suggestion that calmodulin is also involved in the maintenance of the normal biconcave shape of human erythrocytes [9]. Since the shape of erythrocytes is controlled by the cytoskeleton it was surmised that calmodulin interacts with the erythrocyte cytoskeleton. This is supported by the finding that spectrin, the main protein of the erythrocyte cytoskeleton, is phosphorylated by a calmodulin-dependent kinase [10] even though the effect of this phosphorylation on cell shape is still under discussion [11].

It was recently demonstrated that erythrocyte membranes can bind ^{125}I-labeled calmodulin [12]. Although the main calmodulin-binding activity was found to be connected to the $(Ca^{2+} + Mg^{2+})$-ATPase and to two polypeptides of 8 and 40 kDa, respectively, calmodulin binding to spectrin was implied. Direct binding of calmodulin to spectrin or spectrin-like proteins has also been reported. However, in the case of mammalian spectrin, the interaction with calmodulin has not actually been proved. So far, results showing an interaction between calmodulin and human spectrin have been obtained only in the presence of 6 M urea [13,14] which indicates that the binding site observed under those conditions might not be exposed normally. Other investigations where an interaction between calmodulin and avian spectrin has been demonstrated have failed to detect such a binding to human or rat spectrin [15,16]. The cytoskeletal proteins, brain fodrin and intestinal TW 260/240 protein, which are in structure and general properties similar to spectrin, have shown calmodulin-binding activity [17]. In both fodrin and TW 260/240, as well as in avian spectrin, a 240-kDa subunit has been identified as the binding site for

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calmodulin. A similar 240-kDa subunit which cross-reacts with antibodies raised against avian spectrin [18] is a component of mammalian spectrin. These results indicate that an interaction between calmodulin and mammalian spectrin is likely to occur. The importance of confirming whether calmodulin binds to human spectrin, and thereby can be involved in the control of the erythrocyte cytoskeleton, has led to this work.

By using two different methods, affinity chromatography on immobilized spectrin and partition in aqueous two-phase systems [19], we have here investigated whether human spectrin interacts with calmodulin.

2. MATERIALS AND METHODS

Calmodulin was prepared from bovine brain as in [20] and lyophilized after addition of 1.5 mM $CaCl_2$. The identity of calmodulin was determined by its mobility on SDS-polyacrylamide gels as compared to commercially available calmodulin (Boehringer Mannheim GmbH). Human spectrin dimer was prepared by conventional methods [21], stored on ice and used within 2 weeks after preparation. Spectrin was coupled to CNBr-activated Sepharose (Pharmacia, Sweden), using the method recommended by the manufacturer, except that the amount of protein added to the gel was 1.8 mg/g dry gel. The gel was packed in a column (1 × 8 cm) and equilibrated in buffer A (10 mM Tris–HCl, 75 mM KCl, 0.1 mM $MgCl_2$, 1 mM EGTA (pH 7.3) at a flow rate of 13 ml/h. Calmodulin was dissolved in buffer A or buffer A containing 1–5 mM $CaCl_2$ and applied to the column. Elution was carried out with the same buffer used for solubilizing the sample, and 0.5-ml fractions were collected. After 10 vols buffer had passed through the column, the elution medium was changed to buffer A containing 10 mM EGTA.

Aqueous two-phase polymer systems [19] containing 7.4% (w/w) dextran T500 (Pharmacia), 8.4% (w/w) polyethylene glycol 4000 (Union Carbide, USA), 135 mM KCl, 12.5 mM Pipes (pH 7.0), 0.125 mM $MgCl_2$, 1.25 mM EGTA, and in some cases 1.25 mM $CaCl_2$, were prepared in 160-g batches. This should give 0.12 mM free Mg^{2+} and, when $CaCl_2$ was added, 0.125 mM free Mg^{2+} and 20 μM free Ca^{2+}. The phase systems were left to separate overnight. Aliquots of 0.2 ml from the lower phase and 0.6 ml from the upper phase were taken and mixed in test tubes and thereafter 0.2 ml sample was added to each test tube. The calmodulin used in the partition experiments was labeled with ^{14}C using reductive methylation [22]. The proteins were incubated for 10 min in the phase systems, under constant end-over-end rotation at room temperature. After centrifugation of the phase systems, the partition of calmodulin was determined by counting 0.2 ml upper phase and 0.15 ml lower phase in 1.5 ml distilled water plus 2 ml Luma-Gel scintillation fluid (Lumac, The Netherlands). The partition of spectrin in the absence of calmodulin was determined by measuring the protein concentration in each phase.

The purity of the proteins was determined by SDS-polyacrylamide gel electrophoresis in the discontinuous buffer system in [23].

Protein concentration was measured by the Coomassie blue method [24].

3. RESULTS AND DISCUSSION

Both the calmodulin and spectrin preparations were essentially pure as determined by SDS-gel electrophoresis. The calmodulin was considered to be active as long as addition of calcium caused a conformational change. Binding of calcium and a subsequent change in the conformation of calmodulin was demonstrated since a difference in its binding behaviour was observed upon addition of calcium, not only to the phenyl–Sepharose during preparation but also to the spectrin–Sepharose column.

When calmodulin was applied to the spectrin–Sepharose column and eluted in the absence of calcium, all of the added protein eluted at the expected position, i.e., close to the void volume. In the presence of calcium, however, calmodulin was retained within the column. When the eluent buffer was changed to one containing either EGTA or the calmodulin inhibitor chlorpromazine [25], the bound calmodulin was eluted. A typical elution profile is shown in fig.1. The small portion of protein that passed right through the column in the presence of calcium was observed when calmodulin was added in more than stoichiometric amounts to the spectrin bound. Calculation of the maximal amount of calmodulin

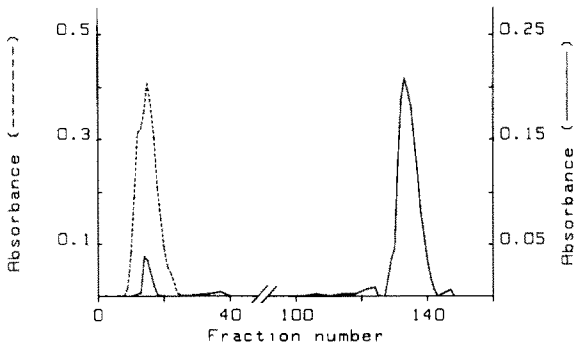

Fig.1. Affinity chromatography of calmodulin on a spectrin–Sepharose column. (- - -) Elution with buffer A. (——) Elution with buffer A containing 5 mM $CaCl_2$. At fraction number 100 the elution medium was changed to buffer A containing 10 mM EGTA. Conditions as described in section 2.

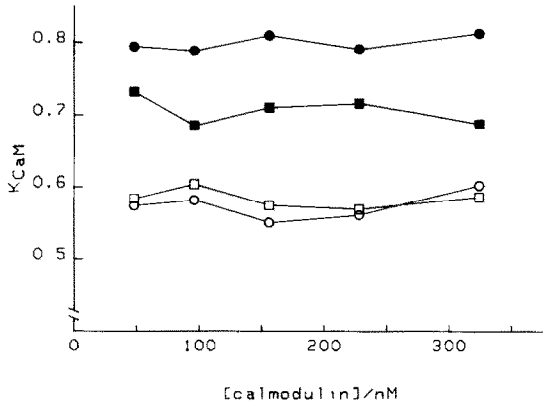

Fig.2. Effect of calcium and spectrin on the partition coefficient of calmodulin, K_{CaM}, in an aqueous two-phase polymer system. The partition of calmodulin was measured in the presence (●, ■) or absence (○, □) of calcium, with spectrin added to a concentration of 0.85 μM (■, □) or with no spectrin added (● ,○). The concentrations of calmodulin and spectrin are calculated from the amount of protein added and from the total volume of the phase-system. In the series where calcium is present each point represents a mean value of 4–5 experiments. The partition coefficients of calmodulin alone differ from those of calmodulin partitioned in the presence of spectrin at 4 of the 5 calmodulin concentrations tested, as calculated by Student's t-test using 99% confidence level. At the lowest calmodulin concentration the uncertainties in the determination of K are large due to few counts in the sample and therefore this difference cannot be statistically ensured.

bound to the column indicates a 1:1 binding on a molar basis.

In a control experiment, calmodulin was added to a Sepharose column which was prepared in exactly the same way as the spectrin–Sepharose column, but with no spectrin added. Neither in the absence nor in the presence of calcium did calmodulin interact with the column. This proves that our results reflect a true interaction between calmodulin and spectrin, and not an unspecific binding to the column.

The immobilization of spectrin on CNBr–Sepharose might, however, alter the properties of spectrin and therefore we tried to detect this interaction by another method which would not involve any covalent modification of the spectrin molecule. Partitioning in aqueous two-phase polymer systems has proved to be very useful [19] for studying protein associations and with this method we could confirm our results from the affinity chromatography experiments. When the proteins were partitioned separately in the calcium-containing phase system, spectrin was almost entirely found in the lower phase, whereas calmodulin had a partition coefficient of 0.8, but when they were partitioned together in the same phase system, the partition coefficient for calmodulin was lowered to around 0.7 (fig.2). In the absence of calcium the partition coefficient reached a value of 0.6 independently of the presence of spectrin. This effect can only be explained by a calcium-dependent interaction between the two proteins. Assuming a stoichiometry of 1:1 and that the partition of the formed complex is the same as that of spectrin, the results yield a dissociation constant of 15–25 μM. This value need not necessarily be the same as in the absence of dextran and polyethylene glycol since the presence of neutral polymers might strengthen the association, presumably due to the excluded volume effect [26]. Previous binding studies using aqueous two-phase partition [27–30] have, however, given dissociation constants similar to or slightly lower than those determined by other techniques. The higher partition coefficient of calmodulin in the presence of calcium is a consequence of the increased amount of hydrophobic surfaces on the protein resulting from the calcium-induced conformational change. A higher partition coefficient achieved upon increased hydrophobicity of the partitioned molecule can be ex-

plained by the fact that the upper polyethylene glycol-rich phase is more hydrophobic than the lower dextran-rich phase [31].

Our results might be a step towards understanding the molecular mechanism behind the effects of calmodulin on erythrocyte shape that has been reported [9]. The hypothesis that calmodulin acts as a mediator of calcium in the control of protein–protein interactions within the erythrocyte cytoskeleton [9,32] has, however, at least one serious drawback. The calcium concentration needed for half-maximal binding of calmodulin to other proteins ranges from 2 to 11 μM free calcium [33–35]. This should be compared with the estimated free calcium concentration in human erythrocytes of 0.1–1 μM [36]. Thus the available calcium is perhaps not sufficient to activate calmodulin. Nevertheless, other proteins may interact with calmodulin and thereby affect its calcium binding properties and thus also the saturation level. It has been shown that addition of stoichiometric concentrations of troponin I to calmodulin decreases the half-maximum calcium concentration from 11 to 1.6 μM [33]. Alternatively, compartmentation of calcium might occur which would subsequently lead to higher calcium concentrations locally.

To establish the physiological significance of the interaction between calmodulin and spectrin it is necessary to pursue this issue further. Special emphasis should be given to factors affecting the calcium concentration in the erythrocyte and to proteins that might affect the binding characteristics of calmodulin.

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