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Interaction of calmodulin with lactoferrin

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Calmodulin, as a major intracellular calcium-binding protein, regulates many Ca^{2+}-dependent enzymes and plays an important role in a wide spectrum of cellular functions of the eukaryotes. Interaction between calmodulin and human lactoferrin, a 78 kDa protein with antibacterial properties, was found in the presence of Ca^{2+} using (i) a method for the detection of calmodulin binding proteins with biotinylated calmodulin, (ii) affinity chromatography on an agarose–calmodulin column with subsequent detection by an enzyme-linked immunosorbent assay (ELISA). The binding of calmodulin to lactoferrin blocked the ability of lactoferrin to agglutinate *Micrococcus lysodeikticus*.

Calmodulin; Lactoferrin; Ca^{2+}-binding protein; Lactotransferrin

1. INTRODUCTION

Calmodulin is an intracellular, calcium-binding protein involved in the regulation of several calcium-dependent signalling pathways and essential cellular processes [1]. This protein is present in virtually all eukaryotic cells [2] and a calmodulin-like protein has been well characterized in prokaryotes [3]. Interactions between calmodulin and several peptides and proteins have been previously described and viewed as models for the study of calmodulin-binding domains of the proteins with calmodulin-regulated functions [4–8]. Lactoferrin is a 78 kDa iron-binding glycoprotein present in most mammalian fluids and in secondary granules of neutrophilic leukocytes. Physiological activities proposed for this protein include defence against mucosal infection and its function as a regulator of granulopoiesis [9]. This protein is one of the most important antibacterial components of mucosal surfaces and is present at high concentrations in mucosal secretions [10]. Lactoferrin inhibits the growth of many bacteria through simple iron deprivation [11,12] and by a still unclear direct bactericidal activity [13]. In the present work, the interaction of calmodulin with lactoferrin is described. Direct evidence of this binding comes from the observations carried out using two different methods. The binding between both proteins was Ca^{2+}-dependent and did not depend on the iron content of lactoferrin itself. Other members of the transferrin family, such as transferrin and ovotransferrin, did not show this ability. Finally, indirect evidence was provided by the calmodulin blocking effect of lactoferrin-induced agglutination of bacterial protoplasts upon lysozyme action.

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2. MATERIALS AND METHODS

2.1. *Detection of calmodulin binding*

Samples of iron-saturated and iron-free proteins (human lactoferrin, human trasferrin and ovotransferrin from Sigma), hen egg-white lysozyme (Boehringer-Mannheim) and molecular weight standards (Pharmacia, Sweden) were applied on SDS slab gels (12%) and electrophoresis was carried out by the method of Laemmli [14]. After electrophoresis, the proteins were transferred to nitrocellulose membranes for 3 h at 400 mA using the method of Towbin et al. [15] followed by the detection of calmodulin binding proteins as described by Billingsley et al. [16]. The membranes were incubated in a blocking solution (50 mM Tris–HCl, pH 7.4, 250 mM NaCl, 1 mM $CaCl_2$) containing 1.5% bovine serum albumin (BSA). After blocking and washing, the membranes were incubated with biotinylated calmodulin (1 μg/ml) for 1 h at room temperature and transferred to an avidin–alkaline phosphatase (Sigma) solution followed by addition of substrates for colour development. To detect calcium-dependent binding of biotinylated calmodulin, incubations and washes of parallel membrane strips were processed with 5 mM EGTA or 100 μM trifluoperazine (Boehringer-Mannheim) which substituted $CaCl_2$. Lactoferrin was lysozyme free as determined by transimmunoblot using an antibody anti-human lysozyme (Dakopatts, Denmark).

2.2. *Affinity chromatography on an agarose–calmodulin column*

Affinity chromatography was performed as described previously [17] on an Affigel–calmodulin (Bio-Rad) column of 1 ml (2.5 × 0.5 cm) which was equilibrated before washing three times with a solution containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl and 1 mM $CaCl_2$. The human lactoferrin sample (500 μl from a solution of 1.2 mg/ml) was loaded and left for 30 min to allow maximal binding. The chromatography was performed at 4°C with a flow rate of 1 ml/h. Lactoferrin was eluted in the presence of a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 5 mM EGTA. Fractions (300 μl) were tested for the presence of lactoferrin using a 'sandwich' enzyme-linked immunosorbent assay (see section 2.3).

2.3. *Determination of lactoferrin by ELISA*

Detection of lactoferrin was performed by using an enzyme-linked immunosorbent assay (ELISA) described by Gutteberg et al. [16]. Appropriate dilutions of coating antibody and conjugated antibody were chosen to give the best spread in optic densities for lactoferrin in the range 1–1,000 μg/l. Microtitre wells (Costar, Cambridge, MA) were each coated with 0.1 ml of affinity antibody anti-human lactoferrin (Sigma) diluted 1:20,000 in coating buffer (0.05 M $NaHCO_3$, pH 9.6) for 13 h at 4°C. Wells were then blocked with 0.25 ml of phosphate-buffered saline (PBS) containing 1% BSA and 0.1% NaN_3 for 1 h at 37°C before 0.1 ml of eluted fraction was added and left for 1 h at 37°C. The peroxidase-conjugated anti-human lactoferrin (Jackson Immunoresearch Labs., Avondale, PA) diluted at 1:100,000 in PBS containing 1% BSA and 0.01% thimerosal was added and left for 1 h at 37°C. A solution of *o*-phenylenediamine dihidrochloride (1 mg/ml) in substrate buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 5.0) plus 0.003% H_2O_2 was added to each well and colour was allowed to develop for 30 min at 37°C before being stopped with 0.1 ml of 3 N H_2SO_4. Absorbances were read at 490 nm on a microtitre plate reader (Organon Teknika). Human lactoferrin diluted in PBS (2–1,000 μg/l) was used as standard.

2.4. *Lactoferrin-induced agglutination*

The enzymatic activity of lysozyme (EC 3.2.1.17) was determined from the rate of lysis of a fresh suspension of lyophilized cells of *Micrococcus lysodeikticus* ATCC 4698 (Sigma) in 0.066 M sodium phosphate buffer, pH 5.5/100 mM NaCl, previously prepared to give an absorbance of 0.8 at 450 nm. Hen egg-white lysozyme (5 μg/100 μl) was mixed in the spectrophotometric cuvette, with 2.4 ml of the cell suspension and the decrease of absorbance was recorded at 20°C. The effect of lactoferrin on the lysis of *M. lysodeikticus* by lysozyme was performed as described by Perraudin et al. [19]. Human lactoferrin solution (100 μl from a solution of 10 mg/ml) was added to the cell suspension prior to lysozyme and the extent of agglutination was followed in the conditions described above. Calmodulin was added in order to reach different concentrations in the presence of lactoferrin and lysozyme.

2.5. *Calmodulin binding assays to bacterial membranes*

Calmodulin binding to the immobilized cellular membranes of *M. lysodeikticus* was determined on nitrocellulose sheets, using the method described by Schryvers [20] and modified by using biotinylated calmodulin and the avidin–alkaline phosphatase system described in section 2.1.

3. RESULTS

3.1. *Detection of lactoferrin on transfer membranes by using biotinylated calmodulin*

After electrophoresis in SDS slab gels the proteins were immobilized on nitrocellulose membranes by electroblotting. Calmodulin binding proteins were recognized by incubation with biotinylated calmodulin followed by detection with avidin–alkaline phosphatase chromogens. Sectioned membranes were incubated with biotinylated calmodulin in the presence of Ca^{2+} (1 mM), EGTA (5 mM) or trifluoperazine (100 μM). A coloured band corresponding to lactoferrin was seen on the membrane in the presence of Ca^{2+} after 20 min of colour development (Fig. 1). When EGTA was included in the biotinylated calmodulin incubation a signal was not detected on the membrane. Similar results were obtained when trifluoperazine, a calmodulin antagonist, was added to the incubation mixture. Non-specific binding

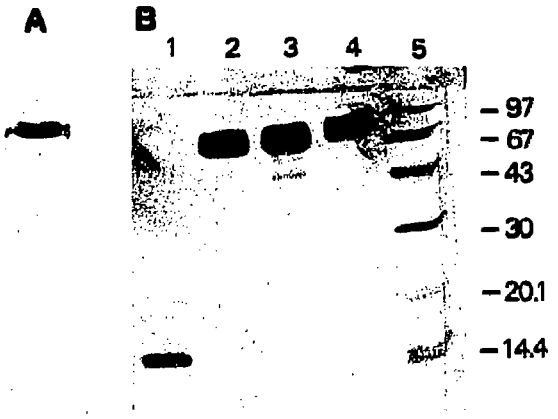

Fig. 1. Binding of biotinylated calmodulin to human lactoferrin. SDS-PAGE of iron-free proteins was carried out in duplicate and the polyacrylamide gel was electroblotted. (A) Electroblotted section exposed to biotinylated calmodulin and the avidin–alkaline phosphatase system. Lane corresponds to human lactoferrin. (B) Coomassie blue-stained portion of the blot. (Lane 1) hen egg lysozyme; (lane 2) ovotransferrin; (lane 3) human transferrin; (lane 4) human lactoferrin; (lane 5) molecular weight markers (sizes given in kilodaltons).

of avidin–alkaline phosphatase to lactoferrin and to other proteins (transferrin, ovotransferrin and lysozyme) was not observed. Calmodulin was unable to bind to human transferrin or ovotransferrin in the assayed conditions but calmodulin binding was observed when lysozyme was used as a positive control [17].

3.2. *Binding of lactoferrin to an Affigel–calmodulin column*

Binding of lactoferrin to calmodulin was determined by affinity chromatography in a calmodulin–agarose column. Preparations of lactoferrin in buffer containing 1 mM Ca^{2+} were chromatographed through a column of Affigel–calmodulin. Affinity chromatography studies demonstrated that the direct interaction between calmodulin and lactoferrin is dependent on Ca^{2+}. Lactoferrin was eluted only in the absence of this metal ion and in the presence of 5 mM of EGTA, a Ca^{2+}-chelating agent. Lactoferrin was detected in the eluted fractions using an enzyme-linked immunoassay (Fig. 2). At least 43% of the applied protein was recovered from the column.

3.3. *Inhibition of lactoferrin agglutination for calmodulin*

Lactoferrin agglutination of *M. lysodeikticus* (formerly named *M. luteus*) has been described as a model system to study the interaction of lactoferrin with bacterial cells [19]. In this study the possible effect of calmodulin binding to lactoferrin on the lactoferrin-induced agglutination was determined. The lactoferrin agglutination assays on *M. lysodeikticus* in the presence of different concentrations of calmodulin (0.17, 0.35, 3.5 mg/ml) were performed at 20°C. A loss in the ability of lactoferrin to agglutinate the bacterial cells was ob-

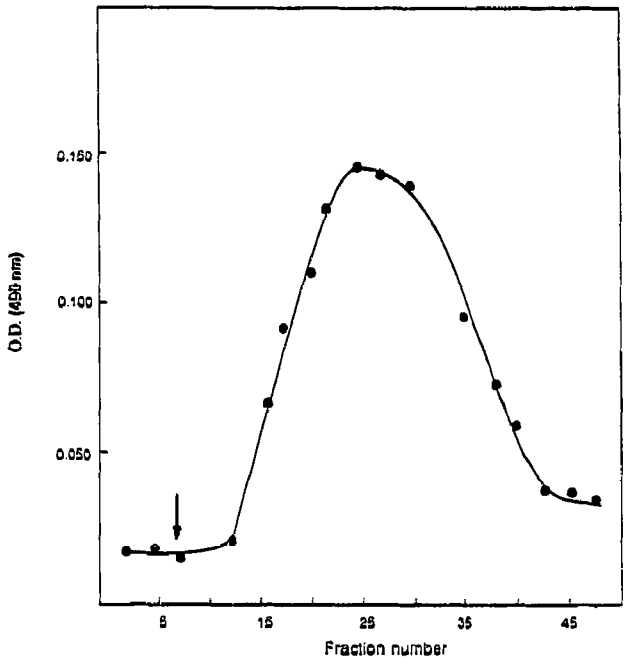

Fig. 2. Ca^{2+}-dependent binding of lactoferrin to an agarose–calmodulin column. Lactoferrin (0.6 mg in 500 μl) was applied on a 1 ml Affigel-calmodulin column. The column was equilibrated with a buffer containing 1 mM $CaCl_2$. Elution was carried out by running a buffer with 5 mM EGTA. Fractions were taken and lactoferrin was detected by ELISA. The arrow indicates EGTA addition for elution of lactoferrin.

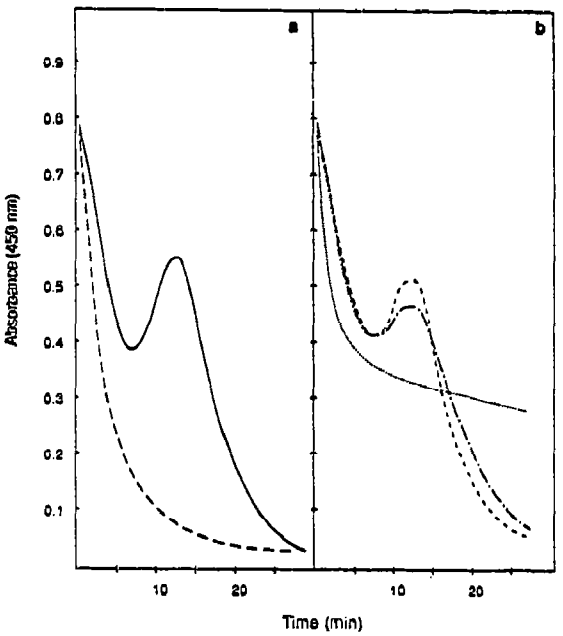

Fig. 3. Blocking effect of calmodulin on lactoferrin-induced agglutination. (a) Lytic effect of (5 μg/ml) lysozyme on a suspension of *M. lysodeikticus* (– – –). Bacterial agglutination in the presence of lysozyme (5 μg/ml) plus 1 mg of lactoferrin (—). (b) Lactoferrin-induced agglutination in the presence of different concentrations of calmodulin: (---) 0.17 mg/ml; (–·–) 0.35 mg/ml; and (····) 3.5 mg/ml. The curves are representative of three separate experiments.

served (Fig. 3). The calmodulin blocking was dependent on the concentration of calmodulin present in the sample. Control assays with higher concentrations of BSA (6 mg/ml) were unable to block the agglutination. Using the method described in section 2.5, calmodulin binding to outer membrane of *M. lysodeikticus* was not detected.

4. DISCUSSION

Several basic proteins and small polypeptides have been described that bind to the acidic Ca^{2+}-binding protein, calmodulin [4–8]. However, it has been suggested that other physical and chemical properties besides the basic nature of these proteins should be considered when explaining this interaction [7]. The present study shows that calmodulin interacts with human lactoferrin in vitro and that this interaction occurs in a Ca^{2+}-dependent manner. These findings are supported by the results obtained using a method for the detection of calmodulin binding proteins. Biotinylated calmodulin was able to bind to immobilized lactoferrin in the presence of Ca^{2+}, unlike calmodulin binding of membranes which was not observed when EGTA was present. Affinity chromatography on an agarose-calmodulin column also showed a Ca^{2+}-dependent binding between both proteins, and lactoferrin was eluted from the affinity column only in the presence of the above Ca^{2+}-chelating agent. Lactoferrin is a member of the transferrin family of proteins, which includes serum transferrin, ovotransferrin and melanotransferrin [21]. Although these iron-binding proteins have a 40–50% amino acid sequence identity between them [22], and the presence of Fe^{3+} on lactoferrin induces a conformational change on the molecule [23], under our experimental conditions, lactoferrin was the only iron-binding protein studied which was able to bind calmodulin. The presence of Fe^{3+} on lactoferrin did not modify the calmodulin–lactoferrin interaction.

A possible explanation for the binding between these molecules could be the hydrophobic nature of both proteins. As has been previously reported, Ca^{2+} induces exposure of two hydrophobic domains on calmodulin [24] and lactoferrin has been shown to interact with bacterial lipopolysaccharides of the cellular membrane [25]. Although the physiological role is unknown, other antibacterial proteins such as lysozyme and seminalplasmin bind to calmodulin in the presence of Ca^{2+} [17,26]. In this sense our results could be helpful in trying to understand how calmodulin recognizes its target proteins. Also, the ability of calmodulin to block the bacterial lactoferrin-induced agglutination is indeed interesting. A similar blocking effect was described previously for the antibacterial protein, seminalplasmin, which in the presence of Ca^{2+} forms biologically inactive complexes with calmodulin [25]. In view of the lack of

interaction of calmodulin with the bacterial membrane, the blocking effect observed suggests a competition between calmodulin and the lactoferrin receptor on the cell membrane. The blocking property of calmodulin on lactoferrin-induced agglutination could help to understand the lactoferrin-recognizing domains that bind to receptors on *M. lysodeikticus*. Studies are in progress in the belief that this model will permit other approaches to study the antimicrobial action of lactoferrin.

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