Calumenin interacts with serum amyloid P component

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Abstract We recently reported the identification of human calumenin, a novel Ca^{2+} binding, transformation-sensitive and secreted protein [Vorum et al. (1998) Biochim. Biophys. Acta 1386, 121–131; Vorum et al. (1999) Exp. Cell Res. 248, 473–481] belonging to the family of multiple EF-hand proteins of the secretory pathway that include reticulocalbin, ERC-55, Cab45 and crocalbin. In order to further investigate the extracellular functions of calumenin we immobilized the recombinant protein to a column. After application of a placental tissue extract we were able to elute one protein that interacts with calumenin in the presence of Ca^{2+}. Amino acid sequencing identified this protein as serum amyloid P component (SAP). Furthermore, we verified and characterized the calumenin-SAP interaction by the surface plasmon resonance technique. The findings indicate that calumenin may participate in the immunological defense system and could be involved in the pathological process of amyloidosis that leads to formation of amyloid deposits seen in different types of tissues.

Key words: Calumenin; Serum amyloid P component; Ca^{2+}-dependent interaction; Low-affinity EF-hand; CREC protein family

1. Introduction

The endoplasmic reticulum (ER) harbors a number of proteins responsible for binding and storage of Ca^{2+}, such as calreticulin [1], endoplasmin [2], BiP/immunoglobulin heavy chain-binding protein [3], PDI/protein disulfide-isomerase [4] and calnexin [5,6] which all coordinate the Ca^{2+} ion in stretches of highly acidic amino acids [7]. The function of these proteins is thought to be storage for calreticulin [8] whereas endoplasmin, BiP, PDI and calnexin belong to the molecular chaperones involved in the folding and maturation of newly synthesized proteins [9]. Recent reports show that, besides the above mentioned proteins, the ER also harbors proteins coordinating Ca^{2+} to multiple EF-hand helix-loop-helix motifs [10]. These include reticulocalbin [11], ERC-55 [12], calumenin [13,14] and crocalbin [15]. Much less is known about the function of the latter family. So far, ERC-55 and crocalbin have been reported to interact with other proteins. ERC-55 interacts with the vitamin D receptor modulating its function [16] and with the transforming protein, E6, from papillomavirus [17]. Furthermore, ERC-55 and crocalbin interact with the snake venom toxins taipoxin [18] and crotoxin [19], respectively.

Among the EF-hand family members of the secretory pathway we have recently found that calumenin is not restricted to the ER but also found in the Golgi complex as well as being secreted [20]. Furthermore, we have found that calumenin binds Ca^{2+} with a rather low affinity around 1.6×10^3 M^{-1} corresponding to a K_d around 0.6 mM for each of the EF-hands [14]. However, this rather low affinity may be ideal for Ca^{2+}-regulated interactions under conditions of relatively high Ca^{2+} concentrations as present in the secretory pathway and extracellularly, i.e. the locations where calumenin is found.

In the present report we have searched for Ca^{2+}-dependent protein interactions with calumenin by immobilizing recombinant calumenin to a column. After application of a placental tissue extract and washing with a high concentration of salt we were able to elute one protein that interacts with calumenin in the presence of Ca^{2+}. Sequencing of a tryptic internal peptide identified this protein as serum amyloid P component (SAP). The binding of calumenin to SAP was further verified and characterized by the surface plasmon resonance technique.

2. Materials and methods

2.1. Expression of recombinant calumenin in Escherichia coli

Expression of recombinant calumenin in *E. coli* and subsequent chromatographic purification procedures were done as previously described in detail [14].

2.2. Affinity purification and identification of a calumenin interacting protein from human placenta

Placentas from normal human afterbirths were handled as described [21]. In brief, pieces of villous tissue were washed in PBS, homogenized at 0°C in 0.25 M sucrose, 10 mM HEPES, 5 mM EDTA, 0.1 mM PMSF, pH 7.4 and centrifuged at $600\times g$ for 3 min. After removal of the supernatant, the pellet was homogenized and centrifuged at $600\times g$. The supernatants were combined and centrifuged again at $3000\times g$ for 5 min. The new supernatant was then centrifuged at $48\,000\times g$ for 40 min and the pellet homogenized in 140 mM NaCl, 0.6 mM $CaCl_2$, 10 mM sodium phosphate, 0.1 mM PMSF, pH 7.8. The proteins were solubilized in the same buffer with 0.5% CHAPS. This suspension was stirred for 10 min at 0°C and then centrifuged for 40 min at $48\,000\times g$.

The final supernatant was applied to a column of immobilized recombinant calumenin [14] equilibrated in MB-buffer (2 mM $CaCl_2$, 1 mM $MgCl_2$, 140 mM NaCl, 10 mM HEPES, pH 7.4) and to a control column equilibrated in MB-buffer without calumenin immobilized. The columns were washed in MB-buffer with 1 M NaCl and 0.1% CHAPS and eluted with MB-buffer containing 10 mM EDTA and 0.1% CHAPS. The eluate was analyzed for potential ligands by gel electrophoresis.

Eluted proteins from several purifications were pooled, upconcentrated, electrophoresed, Western blotted onto PVDF membranes and amido black stained. Two protein bands were cut from the membrane and internal sequenced after tryptic digestion at the Protein Structure Core Facility, University of Nebraska Medical Center (Omaha, NE, USA).

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Abbreviations: CRP, C-reactive protein; ER, endoplasmic reticulum; SAP, serum amyloid P component

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After protein identification the column was also used to check its ability to capture 75 μg of pure SAP from a solution in MB-buffer. Pure SAP was dissolved in MB-buffer applied to the calumenin column equilibrated in MB-buffer. The column was then washed in MB-buffer with 1 M NaCl and 0.1% CHAPS and eluted with MB-buffer containing 10 mM EDTA and 0.1% CHAPS.

2.3. Analyses of calumenin-SAP interaction by surface plasmon resonance

Surface plasmon resonance measurements were performed on a BIAcore 2000 instrument (Biacore, Uppsala, Sweden). This technology relies on the phenomenon of the surface plasmon resonance that occurs when surface plasmon waves are excited at a metal/liquid interface. Light is directed at, and reflected from, the side of the surface not in contact with sample, and surface plasmon resonance causes a reduction in the reflected light intensity at a specific combination of angle and wavelength. Biomolecular binding events cause changes in the refractive index at the surface layer, which are detected as changes in the surface plasmon resonance signal. In general, the refractive index change is proportional to change in mass concentration at the surface layer.

Coupling of calumenin was performed by using the amine coupling method [22]. A continuous flow of HBS buffer (10 mM HEPES pH 7.4, 3.4 mM EDTA, 150 mM NaCl, 0.005% surfactant P20) passing over the sensor surface of a CM5 sensor chip was maintained at 5 μl min^{-1}. The carboxylated dextran matrix of the sensor chip flow cell 1 and 2 was activated by the injection of 60 μl of a solution containing 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccimide in water. Calumenin was then at a concentration of 175 μg ml^{-1} in 10 mM sodium formate pH 3.5, injected over flow cell 1 with a flow rate of 5 μl min^{-1}. Totally 60 μl was injected. The remaining binding sites in both flow cells were blocked by injection of 35 μl 1 M ethanolamine pH 8.5. The surface plasmon resonance signal from immobilized calumenin generated 2431 BIAcore response units (RU) equivalent to 66 fmol calumenin/mm^2.

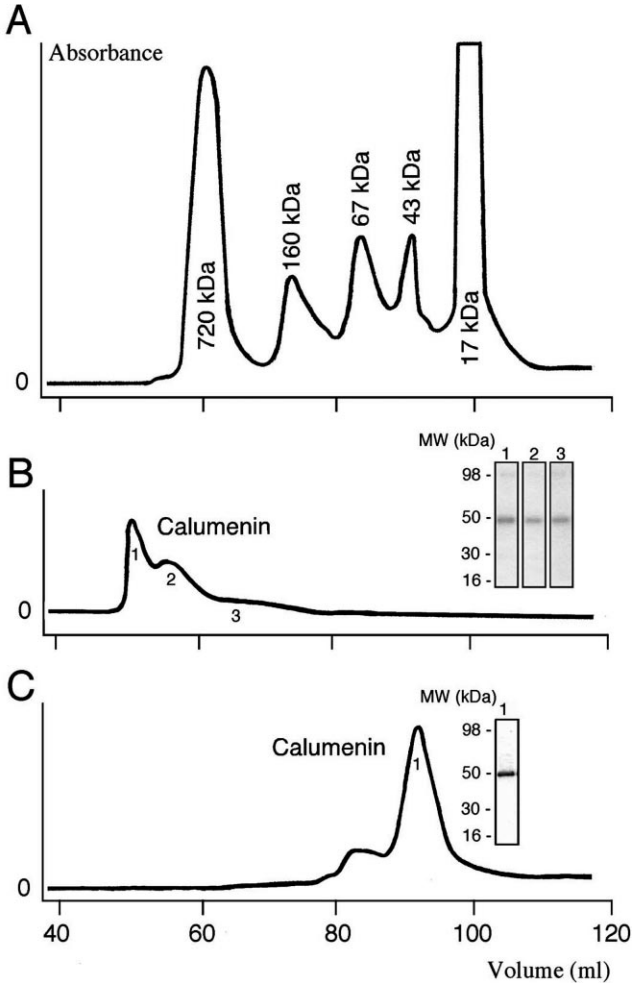

Fig. 1. Gel filtration of calumenin on a Superdex 200 column. A: A chromatographic separation of α2-macroglobulin (720 kDa), IgG (160 kDa), albumin (67 kDa), ovalbumin (43 kDa), and myoglobin (17 kDa). B: A chromatographic elution of calumenin in 150 mM NaCl, 20 mM HEPES, pH 7.4. C: A chromatographic elution of calumenin in 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl, pH 8.0, 100 mM DTT.

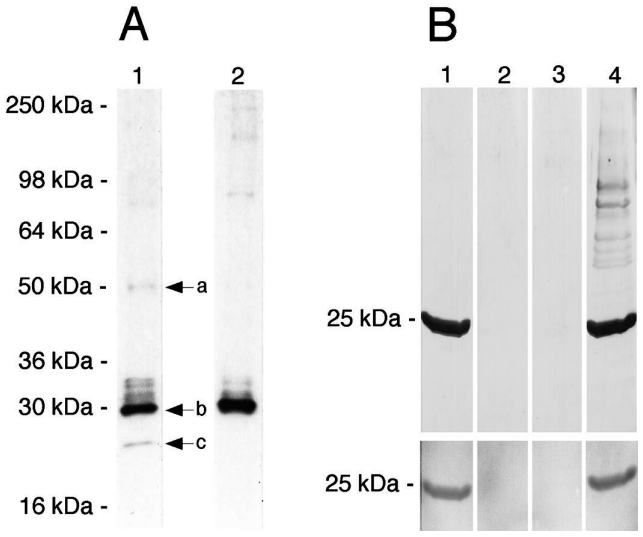

Fig. 2. A: Gel electrophoresis of calumenin affinity-purified placenta proteins. Silver stained gel of placenta proteins upconcentrated on a column with immobilized calumenin (lane 1) and a reference column without calumenin (lane 2) both eluted with MB-buffer containing 10 mM EDTA and 0.1% CHAPS. The bands termed a and c are only present in the calumenin containing column. The c band was identified by internal peptide sequencing as SAP. B: Gel electrophoresis analysis of eluted fractions from affinity chromatography of pure SAP dissolved in MB-buffer. Upper part: silver staining and lower part: Western blot reacted with anti-SAP. Lane 1: Pure SAP in MB-buffer before application to column. Lane 2: Flow-through in MB-buffer. Lane 3: Wash (MB-buffer with 1 M NaCl and 0.1% CHAPS). Lane 4: Elution (MB-buffer containing 10 mM EDTA and 0.1% CHAPS). The eluted fractions were upconcentrated to similar volumes and comparable amounts were applied to each lane.

Commercially available SAP obtained from Calbiochem (Bad Soden, Germany) was dissolved in 10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM EGTA, pH 7.4 at a concentration of 50 nM and analyzed by injecting 40 μl onto the derivatized sensor chip and the control flow channel. In order to further investigate the calumenin-SAP interaction in a serum-like medium, human serum was fractionated through serially arranged Superdex 75 and Superdex 200 columns (Pharmacia). About 200 fractions each containing approximately 1 ml were analyzed for the presence of SAP and SAP depleted fractions were analyzed by injecting 40 μl onto the derivatized sensor chip and the control flow channel. The samples were dissolved in 10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM EGTA, pH 7.4. Running buffer consisted of 10 mM HEPES, 150 mM NaCl, 1.5 mM $CaCl_2$, 1 mM EGTA, pH 7.4. The BIAcore response is expressed in relative RU, i.e. the difference in response between flow cell with immobilized calumenin and the control flow channel. Injecting two times 10 μl 1.6 M glycine-HCl buffer, pH 3.0, performed regeneration of the sensor chip after each analysis cycle.

3. Results

3.1. Calumenin forms high molecular mass aggregates in solution

The deduced molecular mass of calumenin is 37 kDa and it migrates with an apparent molecular mass around 50 kDa in

SDS gels, apparently due to its acidic p*I* [14]. During the purification procedure of the recombinant protein we noticed that the protein in solution forms aggregates with much higher molecular mass than the monomer protein. Fig. 1 shows an eluate from a gel chromatographic separation of the recombinant protein on a Pharmacia Superdex 200 column. This column is suitable for separation of proteins with elution volumes between 60 and 100 ml corresponding to molecular masses in an approximate range between 17 and 720 kDa as observed by the chromatographic separation of α2-macroglobulin (720 kDa), IgG (160 kDa), albumin (67 kDa), ovalbumin (43 kDa) and myoglobin (17 kDa) (Fig. 1A). The elution volume of calumenin is spread out between around 50 ml tailing off to around 80 ml corresponding to aggregates with molecular masses from about 300 kDa to beyond that of α2-macroglobulin at 720 kDa (Fig. 1B). Separate analyses by gel electrophoresis of samples from the eluate showed that the three peaks all contained the recombinant protein migrating as expected in SDS gels around 50 kDa (Fig. 1B, inset). It is obvious that the concentration of monomer protein at 37 kDa, with an elution volume around 90–95 ml, is negligible. Thus evidently, calumenin forms higher molecular mass aggregates in solution without denaturing agents present. When urea was added to the elution buffer together with dithiothreitol the protein peak could be isolated with an elution volume that was in agreement with the molecular mass of the monomer protein at 37 kDa (Fig. 1C).

3.2. *Affinity purification of SAP by immobilized calumenin*

The observation that calumenin is secreted as well as being localized in the secretory pathway indicates that the protein may have functions extracellularly as well as intracellularly. In order to address this question we immobilized calumenin to a column with the purpose of purifying putative interacting proteins from a placental tissue extract. Proteins that were captured by the immobilized calumenin in the presence of Ca^{2+} were eluted again with EDTA. Several liters of tissue extract were affinity purified on the column, gel electrophoresed, and blotted onto a PVDF membrane. After amido black staining several bands could be detected. A representative silver stained gel of the preparation is shown in Fig. 2A (lane 1). A strong band migrating around 30 kDa was found

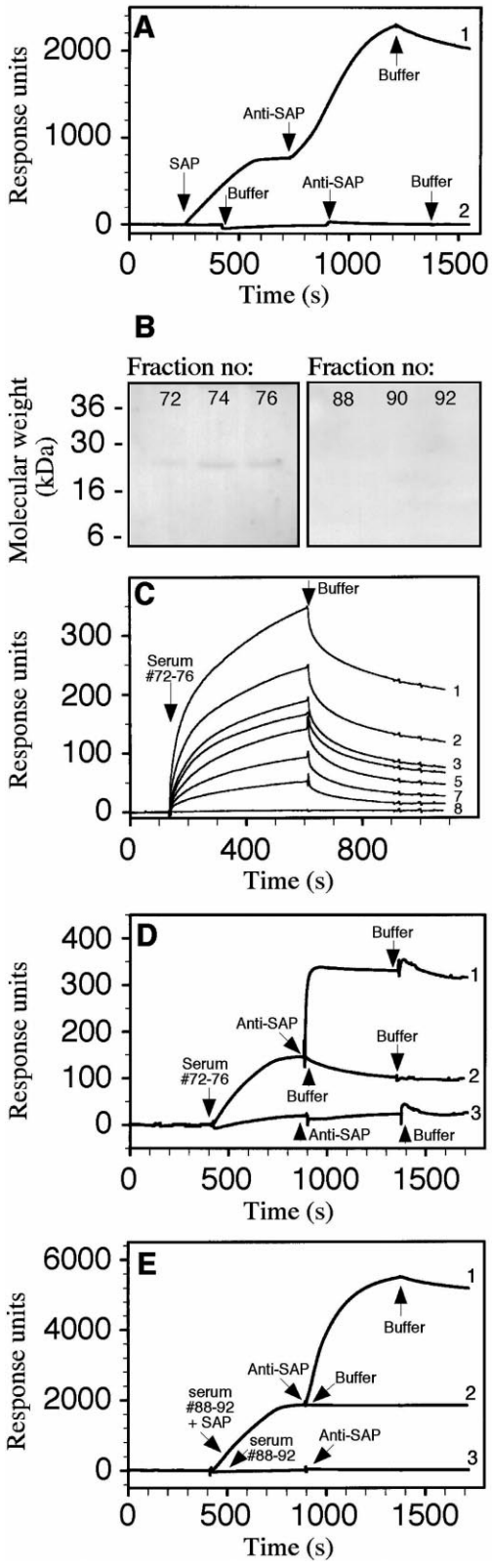

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Fig. 3. Surface plasmon resonance analysis of the binding of calumenin to SAP. Calumenin was immobilized to a sensor chip, and the on and off rates for ligand binding were recorded on a BIAcore 2000. The arrows indicate start of injection of ligand (association phase) or the start of injection of buffer alone (dissociation phase). The recorded sensorgrams in (A) show binding of SAP to immobilized calumenin in 10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM EGTA, pH 7.4 (curve 1). The interaction was verified by the additional binding of anti-SAP antibody. Curve 2 is a control with calumenin replaced with buffer showing no binding of anti-SAP to calumenin. B: Western blots reacted with anti-SAP antibody of fractions of a chromatographic separation of serum proteins. Fractions #72–76 contain SAP whereas fractions #88–92 were devoid of SAP. C: Binding of pooled SAP-enriched fractions (#72–76) to immobilized calumenin in 10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM EGTA, pH 7.4. Curves 1–7 show 1, 2, 3, 4, 5, 10 and 20× diluted fractions. Curve 8 shows absence of binding with the addition of 3.4 mM EDTA. D: Verification of SAP binding to immobilized calumenin by the additional binding of anti-SAP antibody (curve 1). SAP dilution corresponds to curve 3 in Fig. 3C. Curve 2 is a control with anti-SAP antibody replaced with buffer. Curve 3 is a control demonstrating that anti-SAP antibody does not react with immobilized calumenin alone. E: Binding of pooled SAP-depleted fractions (#88–92) with the addition of commercial SAP verified by the addition of anti-SAP antibody (curve 1). Curve 2 is a control with anti-SAP replaced with buffer. Curve 3 is a control showing no binding of pooled SAP-depleted fractions without the addition of commercial SAP.

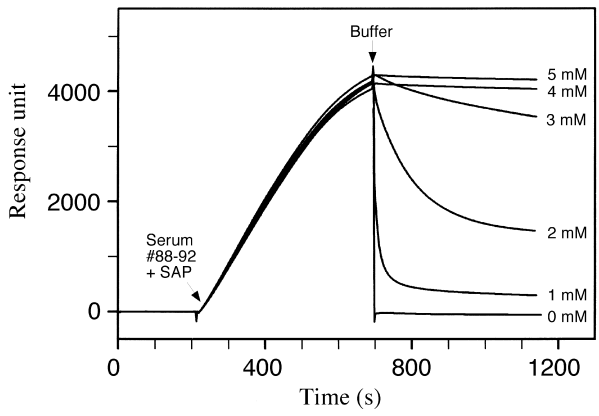

Fig. 4. Surface plasmon resonance analysis of the influence of Ca^{2+} on the interaction of SAP with calumenin. In each cycle commercial SAP (50 nM) in SAP depleated serum fractions (#88–92) was injected onto the calumenin chip in 10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM EGTA, pH 7.4. Next, a buffer (10 mM HEPES, 150 mM NaCl, 1 mM EGTA, pH 7.4) containing decreasing amounts of free Ca^{2+} as given was injected in the dissociation phase. The initial dissociation rate constants were determined as 8.8×10^{-5} s^{-1}, 8.7×10^{-5} s^{-1}, 76.1×10^{-5} s^{-1}, 516×10^{-5} s^{-1}, 1820×10^{-5} s^{-1}, and 10^{-1} s^{-1}, respectively.

in the eluate from the column with immobilized calumenin (marked b in lane 1) as well as from the reference column without immobilized calumenin (lane 2). This interaction was therefore considered to be artifactual. However, two other bands, one around 50 kDa (marked a in lane 1) and one around 25 kDa (marked c in lane 1), were found in the eluate from the calumenin column but not in the eluate from the reference column (lane 2). Both of these bands were cut out for internal peptide sequencing. Unfortunately, only the c band was present in sufficient amounts giving the peptide sequence; (Q)GYFVEAQPK which unequivocally identified c as SAP.

The very low amounts of SAP purified from the extract might indicate that the SAP calumenin interaction is of low affinity and that only small amounts of the SAP present were purified. Therefore, we Western blotted a sample of the placenta extract before application to the column. The blot was exposed to anti-SAP and revealed that only a very small amount was present in the sample (not shown). We then analyzed the affinity of calumenin for SAP by applying a pure solution of SAP to the column (Fig. 2B, lane 1). Analysis of the flow-through fraction revealed that all the calumenin applied to the column was bound to it (Fig. 2B, lane 2). The interaction was resistant to a wash in 1 M NaCl (Fig. 2B, lane 3) indicating that the interaction is strong and thereby putatively of physiological significance. However, the strong interaction was inhibited and SAP could be eluded when EDTA was applied to the column (Fig. 2B, lane 4).

3.3. Characterization of the calumenin-SAP interaction

The binding of calumenin to SAP was verified and characterized by the surface plasmon resonance technique described in detail in Section 2.3. Human recombinant calumenin was immobilized to a sensor chip, and the on and off rates for SAP binding were recorded as seen in Fig. 3A,C,D,E. Arrows indicate start of sample injection (association phase) or start of injection of buffer alone (dissociation phase). In Fig. 3A (curve 1) an increase in response was seen when the sensor chip with immobilized calumenin was exposed to a flow with pure commercial SAP in a buffered solution. In order to confirm that calumenin in fact interacted with SAP, the presence of SAP complexing with calumenin was demonstrated by adding an anti-SAP antibody. A prompt increase in response (curve 1) was seen when the sensor chip with calumenin-SAP complexes was exposed to a flow with anti-SAP reflecting the association phase of anti-SAP with the calumenin-SAP complex. Curve 2 demonstrates that the anti-SAP antibody did not react with immobilized calumenin alone. Fig. 3A represents one among several experiments. Occasionally, binding of SAP in buffered solution fails or is unstable. This could be due to the fact that SAP binds several proteins in a Ca^{2+}-dependent way [23–26] that might stabilize the interaction. In order to circumvent these problems we chose to perform the experiments in a serum-like medium. We therefore gelfractionated human serum.

About 200 fractions were analyzed for the presence of SAP by Western blotting with anti-SAP antibody (Fig. 3B). SAP enriched fractions (pooled fractions 72–76) and SAP depleted fractions (pooled fractions 88–92) were subsequently analyzed for calumenin binding activity. In Fig. 3C a prompt increase in response was seen when the sensor chip with immobilized calumenin was exposed to a flow with SAP enriched serum fractions diluted 1 to 20 times (curves 1–7) reflecting the association phase of the calumenin-SAP interaction. The bound SAP dissociated slowly as detected by a slow decrease in the response by injecting buffer alone. The dissociation phase (Fig. 3C) is apparently not monophasic but bi- or multiphasic suggesting molecular heterogeneity of the SAP-calumenin complexes formed. The self aggregation of SAP and of calumenin and the presence of other components in serum that may participate in the complex formation may all take part in the dissociation phase. However, further dissection of the molecular nature of the heterogeneous complexes must await more detailed studies. The specificity and the Ca^{2+}-dependence of the SAP-calumenin interaction were verified by monitoring Ca^{2+}-depleted samples. Curve 8 shows that the binding is abolished when 3.4 mM EDTA was added to the sample with the highest SAP content.

In Fig. 3D (curve 2) an increase in response was seen when the sensor chip was exposed to a flow with three times diluted SAP enriched serum fractions (equivalent to curve 3 in Fig. 3C). In order to confirm that immobilized calumenin in fact interacted with SAP, present among several other components in the SAP enriched serum fractions, the presence of SAP complexing with calumenin was also here demonstrated by adding an anti-SAP antibody. A prompt increase in response (curve 1) was seen when the sensor chip with calumenin-SAP complexes was exposed to a flow with anti-SAP reflecting the association phase of the anti-SAP antibody with the calumenin-SAP complex. Curve 3 demonstrates that the anti-SAP antibody did not react with immobilized calumenin alone.

Fig. 3E (curve 2) shows binding activity of SAP-depleted fractions to which commercially available SAP has been added. The presence of commercial SAP complexing with the calumenin was again demonstrated by adding anti-SAP (curve 1). Curve 3 shows absence of binding activity of SAP-depleted fractions with no addition of commercial SAP. The apparent horizontal curve seen after the wash (Fig. 3E, curve 2) reflects a low dissociation constant and thereby a strong affinity of SAP to the column as previously

concluded (Fig. 2B). However, the interaction is still reversible as seen by the release of SAP by EDTA in buffer (Fig. 2B) as well as in the serum-like medium (Fig. 3C). It should be noted that there may be differences in the binding strength of the pure SAP in buffered solution and the SAP in serum, since the latter may contain complexes of SAP with other ligands present in serum known to bind to SAP.

Fig. 4 shows a quantitative assessment of the Ca^{2+} dependence of the SAP-calumenin interaction. The association phase was performed in presence of 4 mM free Ca^{2+}. The Ca^{2+} concentration in the dissociation phase was varied stepwise between 0 and 5 mM. At a free Ca^{2+} concentration of 4 or 5 mM (Fig. 4) we found a low dissociation constant at 9×10^{-5} s^{-1} corresponding to a strong affinity of SAP to calumenin. As the Ca^{2+} concentration decreases we find a corresponding increasing dissociation rate, i.e. 76×10^{-5} s^{-1} at 3 mM, 516×10^{-5} s^{-1} at 2 mM, 1820×10^{-5} s^{-1} at 1 mM and 10^{-1} s^{-1} at 0 mM free Ca^{2+} concentration. We thus find a marked change in affinity between SAP and calumenin by changing the free Ca^{2+} concentration in the mM range.

In conclusion, the surface plasmon resonance analysis clearly verifies and demonstrates a Ca^{2+}-dependent interaction between calumenin and SAP in the mM range of free Ca^{2+}.

4. Discussion

Calumenin belongs to a subset of the EF-hand superfamily in the ER and Golgi apparatus consisting of reticulocalbin [11], ERC-55 [12], Cab45 [27], crocalbin [15] and calumenin [14,28]. Due to structural similarities among the members of this family they are likely to share some functional properties. However, marked differences also exist between them. Especially, calumenin is distinct from the others. Intracellularly, it localizes to the whole secretory pathway whereas reticulocalbin and ERC-55 both are localized to the ER, and Cab45 is exclusively found in the Golgi compartment. Furthermore, calumenin is the first member of this family reported to be secreted [20].

In order to further investigate the function of calumenin we immobilized the recombinant calumenin to a column. After application of a placental tissue extract and after washing with a high concentration of salt we were able to elute one protein that interacts with calumenin in the presence of Ca^{2+}. Sequencing of a tryptic internal peptide identified this protein as SAP. SAP, which is mainly synthesized in the liver and secreted to the blood stream, belongs to the pentraxin family of plasma proteins and serves a function in non-specific immunity [29]. It is a glycoprotein composed of 10 identical, non-covalently linked 25-kDa subunits, which are arranged in two parallel cyclic pentagonal structures interacting face-to-face [30]. Each subunit may bind two Ca^{2+}-ions and this complex may bind several ligands including DNA and chromatin, fibronectin, complement component 4-binding protein, glycosaminoglycans, heparan and phosphorylethanolamine [23–26]. All these interactions are Ca^{2+}-dependent and appear to involve a unique binding site in the SAP molecule [31,32].

SAP is related in structure to C-reactive protein (CRP), which also belongs to the pentraxin protein family [33,34]. In humans CRP is a major acute phase reactant that is massively induced by inflammation and provides enhanced protection against invading micro-organisms, limit tissue damage and promote a rapid return to homeostasis [29]. Although SAP is not a major acute phase reactant in humans it may still be induced locally or systemically by different combinations of cytokines [29]. Calumenin, with its ubiquitous presence [14], might thus in some way participate in this host defence system.

Although the physiological function of SAP is largely unknown, SAP has received considerable medical interest as it associates with all types of amyloid deposits, including those found in Alzheimer's disease [33,35]. SAP has a high tendency to undergo Ca^{2+}-dependent self-aggregation, which may be important for its deposition in amyloid. Interestingly, our study shows that calumenin also in solution forms high molecular mass aggregates with about 10 or more molecules. Thus, the tendency of both molecules to form high molecular mass aggregates together with the fact that they bind each other in the presence of Ca^{2+} might play a role in the pathogenesis of amyloidosis.

We have previously determined that each of the EF-hands of calumenin binds the Ca^{2+} ion with a dissociation constant of 0.6 mM [14]. The free Ca^{2+} concentration extracellularly is in the mM range but also intracellularly in the ER the free Ca^{2+} concentration may vary up to the 3–5 mM area [36]. The Ca^{2+} dissociation constants of the calumenin EF-hands are thus close to the level of the free Ca^{2+} concentration where calumenin is localized, i.e. in the secretory pathway and extracellularly suggesting that the EF-hands of calumenin may well serve a regulatory role rather than a structural role.

In summary, we were able to identify SAP as the first ligand known to interact with calumenin. The calumenin-SAP interaction was verified and characterized by the surface plasmon resonance technique. The findings may indicate that calumenin could participate in the immunological defense system and be involved in the pathological process of amyloidosis that leads to formation of amyloid deposits.

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