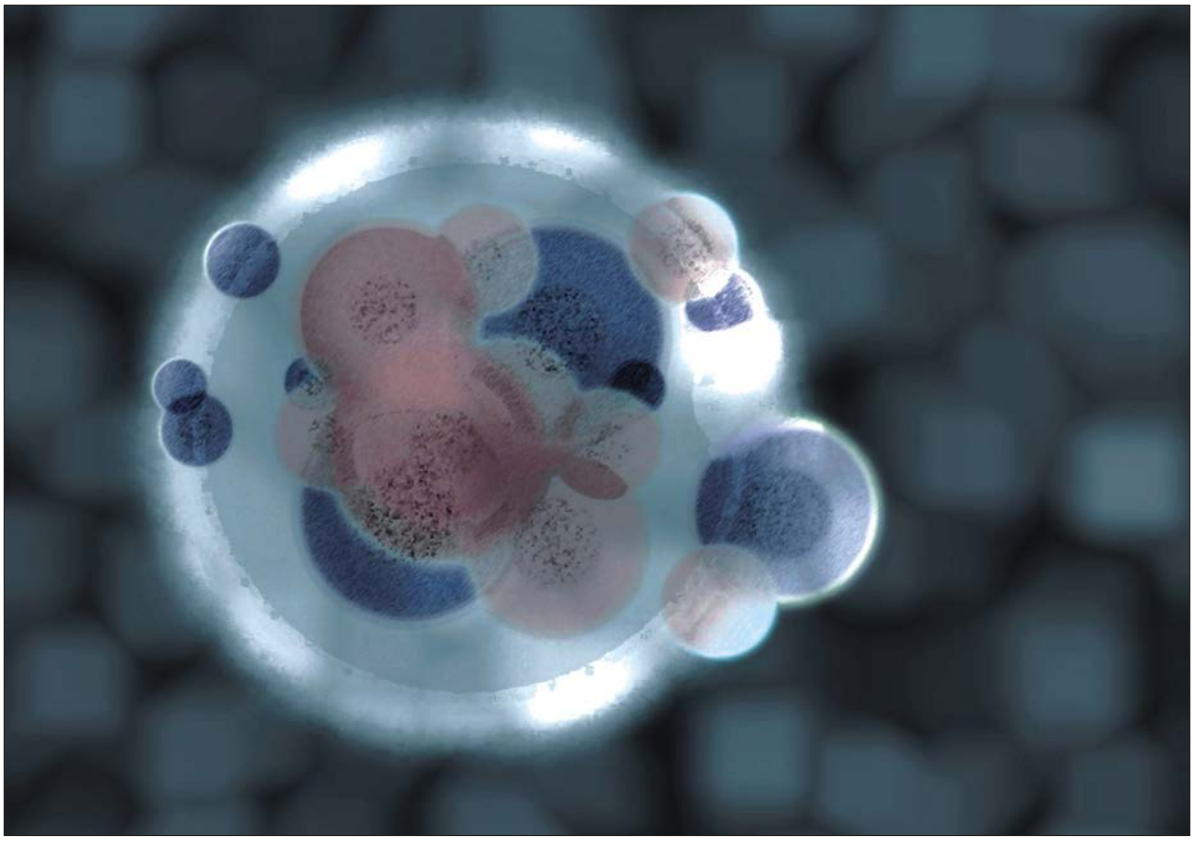

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Calumenin and Reticulocalbin are Associated with the Protein Translocase of the Mammalian Endoplasmic Reticulum

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Abstract: Transport of precursor proteins into the Endoplasmic Reticulum (ER) requires signal peptides in the precursor proteins and a protein translocase in the ER membrane. Recent findings about the protein translocase of the mammalian ER suggest that the membrane is permeable to small molecules during protein translocation/insertion. Since the ER also plays a central role in calcium homeostasis the question arises of how these two activities of the ER are reconciled. Here two EF-hand proteins of the ER were characterized as associates of protein translocase. A model for the activity of these proteins in protein transport and calcium homeostasis is proposed.

Key words: EF-hand proteins, mammalian ER, protein translocase, Sec63p, calumenin

INTRODUCTION

The decisive initial step in secretion of most mammalian proteins is their transport into the lumen of the rough Endoplasmic Reticulum (ER)[1]. Typically, transport of presecretory proteins into the ER involves cleavable signal peptides at the amino terminus of the precursor proteins and a protein translocase in the ER membrane, comprising Sec61αp, Sec61βp, and Sec61γp as the core components[2,3]. Cryo- and freeze fracture-electron microscopic analysis of the Sec61p complexes as present in intact membranes or in artificial membranes or detergent micelles suggested that between three and four heterotrimeric Sec61p complexes form the core unit of the protein translocase with a pore of 2 nm and an overall diameter of approximately 9 nm[4-7]. In addition, Hsp70 protein family members of the ER lumen (BiP/Grp78 and Grp170) are part of the protein translocase and facilitate insertion of presecretory proteins into the Sec61p complex as well as completion of translocation[8-11]. Based on the analogy to the situation in yeast[12,13] these Hsp70 protein family members of the mammalian ER can be expected to be recruited to the Sec61p complex by the membrane integrated Hsp40 protein family member, Sec63p[14,15].

Biochemical analysis of presecretory polypeptides in transit through the ER membrane first demonstrated a hydrophilic environment for the transport substrates[16]. Subsequently, the mammalian ER was found by electrophysiological techniques to contain ion channels that are activated by the release of nascent polypeptides in transit[17,18]. Thus these ion channels were proposed to be involved in protein translocation and termed protein-conducting channels. More recently, the protein-conducting channels or pores were shown by fluorescence quenching experiments to have a diameter of between 4 and 6 nm in the active or open state[19] and to be sealed by translating ribosomes and the lumenal chaperone BiP[20,21]. In all these studies the nature of the protein-conducting channel remained obscure. Moreover, no generally accepted conclusive concepts were available which could explain how during and in the absence of transport, the ER membrane barrier is maintained and how the large variety of smaller peptides and large proteins can be transported while keeping a tightly sealed channel for small ions like Ca^{2+}. Recently, we characterized the purified Sec61p complex as the ion-conducting channel that was previously observed in the membrane of the mammalian ER[22]. We showed that under protein transport conditions the interaction of presequences of precursor polypeptides with the Sec61p complex is required and sufficient to induce the formation of open ion channels. The Sec61p channel, as present in intact membranes or in purified form in proteoliposomes, was characterized as a highly dynamic pore structure with a multitude of open channel

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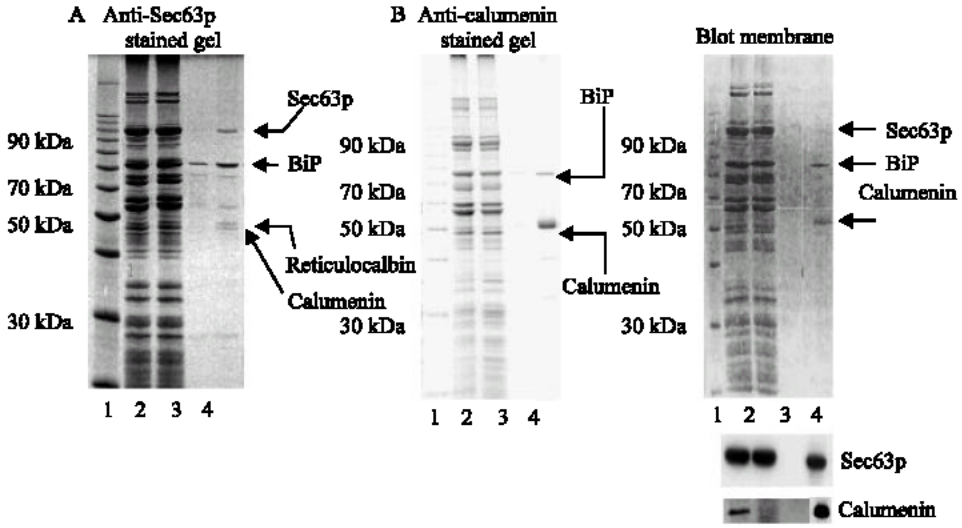

Fig. 1: Immunoaffinity purification of canine Sec63p and calumenin from pancreatic microsomes
Dog pancreas microsomes were prepared and treated with nuclease and EDTA as described[15]. The absorbance at 280 nm of the final microsomal suspension was 50, as measured in 2% SDS. The microsomes were stripped with respect to ribosomes according to published procedures[3]. After reisolation of the stripped microsomes by centrifugation the pellets were resuspended in extraction buffer (20 mM Hepes-KOH pH 7.5, 400 mM KCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 15% glycerol, 0.65% CHAPS) at a concentration of 0.66 eq μL^{-1}. The unsolubilized material was pelleted by centrifugation for 30 min at 2°C and 424,000xg. The detergent extracts were incubated with affinity purified and immobilized peptide antibodies (2 eq μL^{-1} of antibody resin) that were directed against Sec63p (A, lanes 1-4) or calumenin (B, lanes 1-4) for 3 h at 4°C. The suspension was subsequently transferred into a column. After collecting the flow through, the column was washed five times with 1.6 volumes of loading buffer per volume of antibody resin, before eluting the protein that had bound to the peptide antibodies with two volumes of the corresponding peptide at 1 mg mL^{-1} in loading buffer per volume of antibody resin. Aliquots of the extract (lanes 1) and the pass-through (lanes 2) as well as 50 times larger aliquots of the last washing step (lanes 3) and the eluate (lanes 4) were subjected to protein precipitation. The precipitates were analyzed by electrophoresis in 10 or 15% polyacrylamide gels and subsequent staining with Coomassie Brilliant Blue (stained gel) or subsequent electroblotting to PVDF membranes, followed by staining (blot membrane, upper part) or by incubation with specific antibodies and peroxidase conjugate of anti-rabbit IgG-goat antibodies (blot membrane, lower parts). The antibodies were visualized by incubation of the blots in ECL (Amersham Biosciences, Freiburg, Germany) and subsequent exposure to X-ray film (Kodak, Rochester, USA). The protein ladder (10-200 kDa) was run on the same gel (Invitrogen life technologies, Carlsbad, USA). We note that different protein ladders were used for the different gels.

Antibodies were made against synthetic peptides plus an additional carboxy-terminal cysteine (Sec63p, MAGQQFQYDDSGNC; Calumenin, KPTEKKDRVHC). These peptides were coupled to keyhole limpet hemocyanine that had been activated with N-Succinimidyl-3-maleinimido-propionate (Fluka, Buchs, Switzerland). To obtain antibodies for use in immunoaffinity chromatography, 2 mg of coupled peptide for each immunization were mixed with 200 μg of adjuvant peptide and incomplete Freund´s adjuvant and injected into rabbit. Antibodies were affinity purified on immobilized peptides (Sulfolink; Pierce, Rockford, USA). Both purified antibodies react only with the expected proteins in immunoblots. The acid eluted antibodies were coupled to a mixture of Protein A- and Protein G-Sepharose (fast flow; Amersham Biosciences, Freiburg, Germany) with dimethyl pimelimidate.

Fig. 2: The sequences of the precursors of human proteins reticulocalbin and calumenin and partial sequences of the corresponding mature canine proteins (shown below the human sequences)
The canine sequences were derived from amino terminal sequencing (A, upper band of doublet in Fig. 1A, lane 4; B, lower band of doublet in Fig. 1A, lane 4). Note that: (I) the two dots represent an identical amino acid residue present in the canine protein and the respective human protein; (ii) X represents an ambiguous result in the amino acid analysis; (iii) the underlined human sequences represent the EF-hand motifs; (iv) the putative ER retention motif is printed in bold face. Amino acid sequencing by Edman degradation in a Procise 491 Protein sequencer (Applied Biosystems, Foster City, USA) was carried out as described previously[9].

states ranging from 0.6 to 6 nm. The permeability of the Sec61p channel was found to be regulated by precursors, by the ribosome (i.e. in co-translational transport) and by the lumenal chaperone BiP. The activity of the latter two modulators depended on their simultaneous interaction with a precursor polypeptide in transit.

Immunoaffinity purification of Sec63p from microsomal detergent extracts led to the co-purification of Sec62p and subunits of the Sec61p complex, respectively and vice versa[15]. These observations contributed to the conclusion that Sec62p and Sec63p are subunits of the protein translocase that is present in the mammalian endoplasmic reticulum and contains the heterotrimeric Sec61p complex as the core component. At the time we were unable to identify two additional microsomal proteins that were co-purified with Sec63p[15]. Recently, amino terminal sequence analysis of these two proteins identified these proteins as reticulocalbin (Fig. 1A, upper band of doublet and 2A) and calumenin (Fig. 1A, lower band of doublet and 2B), respectively two EF-hand calcium-binding proteins that are resident proteins of the endoplasmic reticulum[23,24]. In order to confirm the interaction of Sec63p with calumenin and reticulocalbin, respectively, We raised anti-peptide antibodies against the amino terminus of calumenin and carried out the reciprocal immunoaffinity purification as compared to above. Dog pancreas microsomes were solubilized in detergent at high salt concentration and the extract was applied to immobilized anti-peptide-antibodies that were directed against calumenin. The immunoaffinity resin was washed and the proteins were eluted with the respective peptide. The extract, pass-through, wash and the specific

Table 1: Calcium-binding proteins of the canine pancreatic rER (calcium concentration: up to 3 mM; note that the cytosolic calcium concentration may be as low as 0.1 μM). The concentrations of the various proteins in the ER lumen were estimated on the basis of the approximate internal volume of the microsomes as compared to the volume of the microsomal suspension and, in the case of soluble proteins of the ER lumen, on the amount of the respective protein that was found in the cytosolic fraction after homogenization of the pancreatic tissue and subsequent differential centrifugation, too. The data on calcium-binding sites were taken from references 23-28 (Bold face) or extrapolated from these references on the basis of sequence identities

	Concentration in RM suspension (μM)	Concentration in ER lumen (mM)	Calcium-binding	
			Sites number	Affinities (μM)
BiP (Grp78)	5.0	2	**30**	**10**
Grp170 (ABP150)	0.6	0.24	30	**10**
Calreticulin (CaBP3, CRP55)	9.0	3.6	**1-5, 30**	**1-5, 10**
Grp94 (CaBP4)	4.0	1.6	**1-5, 30**	**1-5, 10**
Calnexin (IP90, p88)	not determined		**1-2**	1-5
PDI	10.0	4.0	1-5	1-5
PDIp	4.0	1.6	1-5	1-5
Grp58 (ER-60)	6.5	2.6	1-5	1-5
ERp72 (CaBP2)	0.9	0.36	**1-5**	**1-5**
P5 (CaBP1)	2.5	1	**1-5**	**1-5**
Calumenin	0.5	0.2	**4-7**	**100-1000**
Reticulocalbin	not determined		**4-6**	**100-1000**

eluate were collected and analyzed by SDS/PAGE and Western blot (Fig. 1B). The two dominant products of the anti-calumenin-resin, were identified as BiP and calumenin. The identities of calumenin and BiP, respectively, were confirmed by amino terminal sequence analysis (data not shown). In addition, Sec63p was observed in the peptide eluate of the anti-calumenin-resin by both protein staining and immunoblot (Fig. 1B). Since BiP had previously been copurified with various microsomal proteins the presence of BiP in the eluate may be due to the known activity of BiP to interact with antibodies[15]. Thus calumenin appears to be a specific partner of Sec63p. This is supported by our previous observation that calumenin and reticulocalbin were not detected to any significant amount in the peptide eluates of anti-Sec62p- and anti-Sec61βp-resins[15], respectively but only in the peptide eluate of the anti-Sec63p-resin (Fig. 1A)

Here two EF-hand proteins of the mammalian ER lumen, calumenin and reticulocalbin, were identified as binding proteins of Sec63p, a subunit of the protein translocase that is present in the membrane of the endoplasmic reticulum. The two EF-hand proteins had previously been described as resident ER proteins with a low affinity for calcium ions in various mouse tissues (Table 1)[23,24] and with carboxy terminal ER retention motifs (Fig. 2, bold face). Our previous proteomic analysis of canine pancreatic rough microsomes had identified several additional calcium-binding proteins, such as the calcium-binding proteins/CaBPs 1 (also termed protein disulfide isomerase P5), 2 (protein disulfide isomerase Erp72), 3 (Erp60, calreticulin), and 4 (ERp99, Grp94) (Table 1)[25,26]. Furthermore, this proteomic analysis had allowed us to estimate and determine, respectively, the concentrations of these various proteins in both canine microsomes and the canine pancreatic endoplasmic reticulum (Table 1)[26]. Thus it is obvious that the pancreatic rough endoplasmic reticulum contains a whole variety of calcium-binding proteins and that the number of calcium-binding sites, provided by these proteins, by far exceeds the number of calcium ions that are present (Table 1)[25,27,28]. This contributes to the ability of the endoplasmic reticulum to act as a calcium-storage compartment. On the basis of the data that were presented here we propose that the pool of ER calcium-binding proteins is spatially inhomogeneous, i.e. organized in a way that the calcium-binding proteins with a low affinity are located at the membrane and near the protein translocases and where the calcium-binding proteins with a high affinity are distributed more or less equally. We propose that this asymmetric spatial organization of the calcium-binding proteins in the ER somehow accomplishes that there is no uncontrolled efflux of calcium ions from the ER under conditions of protein insertion and translocation, respectively. These conditions have been observed to allow the passage of small molecules through the Sec61p complex both in established *in vitro* systems[17-22] and in permeabilized cells[29,30]. Our proposal is consistent with immunoelectron microscopic analyses that showed human calumenin in association with the ER membrane[31]. Therefore, it had been proposed that calumenin interacts with a resident ER membrane protein.

However, additional considerations are necessary in this context. On the one hand it should be pointed out, that recent structural analysis of the archaean SecY complex that is functionally related to the mammalian Sec61p complex was interpreted as indication that the inner diameter of these complexes is very narrow and, therefore, should not allow the passage of ions to any

significant extent[32]. On the other hand it was suggested that the membrane of the endoplasmic reticulum is significantly more leaky than the membrane of other cellular membranes, in general[33] and therefore, that it should not provide a strict barrier for ions. At present, these apparent contradictions cannot easily be reconciled.

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