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Overexpression of CALNUC (Nucleobindin) Increases Agonist and Thapsigargin Releasable Ca^{2+} Storage in the Golgi

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Abstract. We previously demonstrated that CALNUC, a Ca^{2+}-binding protein with two EF-hands, is the major Ca^{2+}-binding protein in the Golgi by 45Ca^{2+} overlay (Lin, P., H. L. N. Niculescu, R. Hofmeister, J. M. McAffery, M. J. Jin, H. Henneman, T. McQuistan, L. De Vries, and M. Farquhar. 1998. J. Cell Biol. 141:1515–1527). In this study we investigated CALNUC’s properties and GolgiCa^{2+} storage pool in vivo. CALNUC was found to be a highly abundant Golgi protein (3.8 μg CALNUC/mg Golgi protein, 2.5 × 10^5 CALNUC molecules/NRK cell) and to have a single high affinity, low capacity Ca^{2+}-binding site (K_d = 6.6 μM, binding capacity = 1.1 μmol Ca^{2+}/μmol CALNUC). 45Ca^{2+} storage was increased by 2.5- and 3-fold, respectively, in HeLa cells transiently overexpressing CALNUC-GFP and in ErCr-CHO cells stably overexpressing CALNUC. Deletion of the first EF-hand α helix from CALNUC completely abolished its Ca^{2+}-binding capability. CALNUC was correctly targeted to the Golgi in transiently transfected cells as it colocalized and cosedimented with the Golgi marker, α-mannosidase II (Man II). A approximately 70% of the 45Ca^{2+} taken up by HeLa and CHO cells overexpressing CALNUC was released by treatment with thapsigargin, a sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) (Ca^{2+} pump) blocker. Stimulation of transfected cells with the agonist A TP or IP_3 alone (permeabilized cells) also resulted in a significant increase in Ca^{2+} release from Golgi stores. By immunofluorescence, the IP_3 receptor type 1 (IP_3R-1) was distributed over the endoplasmic reticulum and codistributed with CALNUC in the Golgi. These results provide direct evidence that CALNUC binds Ca^{2+} in vivo and together with SERCA and IP_3R is involved in establishment of the agonist-mobilizable Golgi Ca^{2+} store.

Key words: Golgi resident calcium-binding protein • EF-hand • IP_3 receptor • SERCA • nucleobindin

The Golgi complex is involved in posttranslational modification of newly synthesized proteins and serves as the main sorting station for protein and vesicular traffic (Farquhar and Hauri, 1997; Farquhar and Palade, 1998). Calcium is well known to be essential for cell signaling (Tsien and Tsien, 1990; Meldolesi and Pozzan, 1998) but also for cell processes such as protein processing and membrane traffic to and through the Golgi (Davidson et al., 1988; Ivessa et al., 1995; Duncan and Burgoine, 1996). Recently the Golgi has been identified as a Ca^{2+}-enriched compartment using ion microscopy and electron energy loss spectroscopy-electron spectroscopic imaging (EELS-ESI) (Chandra et al., 1991; Grohovaz et al., 1996; Pezzati et al., 1997). Ca^{2+} can be released from the Golgi by the Ca^{2+} ionophore A 23187 (Chandra et al., 1991), the Ca^{2+} channel blocker La^{3+} (Zha and Morrison, 1995), and histamine, an agonist known to be coupled to IP_3 generation (Pinton et al., 1998). How the high level of Ca^{2+} in the Golgi is maintained is unknown at present.

The ER Ca^{2+} pool (or Ca^{2+} store) has been studied more extensively and is known to be maintained by organelle-associated Ca^{2+} ATPase (Ca^{2+} pumps) and luminal Ca^{2+}-binding proteins of which there are many (Bastianutto et al., 1995; Meldolesi and Pozzan, 1998). There is also evidence for the existence of Ca^{2+} pumps on the Golgi based on ATP-dependent Ca^{2+} uptake into mamalian (Baumrucker and Keenan, 1975; Hodson, 1978; Neville et al., 1981; Vill and others, 1985) and yeast (Sorin et al., 1997) Golgi fractions. Both sarcoplasmic/ER calcium ATPase (SERCA) and plasma membrane calcium ATPase 1. Abbreviations used in this paper: CaM, calmodulin; CRT, calreticulin; GFP, green fluorescent protein; IP_3R, inositol 1, 4, 5 trisphosphate receptor; Man II, α-mannosidase II; PLC, phospholipase C; SERCA, sarcoplasmic/ER calcium ATPase; Tg, thapsigargin.
en route to the plasma membrane are essential for Ca\(^{2+}\) uptake into isolated Golgi fractions (Taylor et al., 1997). However, information on Golgi calcium-binding proteins is still limited and the detailed mechanisms of Ca\(^{2+}\) uptake, storage, and release from the Golgi apparatus remain to be elucidated. Previously, we demonstrated that CALNUC (nucleobindin) (M Iura et al., 1992; W Endel et al., 1995), a Golgi resident protein that faces the Golgi lumen, is the major Ca\(^{2+}\)-binding protein in the Golgi based on \(^{45}\)Ca\(^{2+}\) overlay (L in et al., 1998).

In this study we have investigated the role of CALNUC in establishing the Golgi Ca\(^{2+}\) pool in vivo by examining the effects of overexpression of CALNUC on Ca\(^{2+}\) uptake. We provide direct evidence that CALNUC possesses high affinity/low capacity Ca\(^{2+}\) binding properties and binds Ca\(^{2+}\) in the Golgi in vivo. The finding that the majority of the Ca\(^{2+}\) sequestered by overexpressed CALNUC is released by thapsigargin (Tg), A TP, and IP\(_3\) provides additional in vivo evidence for the existence of SERCA and inositol 1, 4, 5 trisphosphate receptor (IP\(_3\)R) on the Golgi. CALNUC together with SERCA and IP\(_3\)R on Golgi membranes constitute a cellular Ca\(^{2+}\) pool in the Golgi which may have distinct functions.

Materials and Methods

Materials

Polyclonal antibody (F-5059) against full-length, recombinant CALNUC was generated and affinity purified as previously described (Lin et al., 1998). Polyclonal anti-\(\alpha\)-mannosidase II (Man II) was prepared as described (Velasco et al., 1993). Monoclonal anti-Man II (53F-C3) and polyclonal antibody against denatured Man II were gifts from Drs. B. Burke (University of Alberta, Alberta, Canada) and K. Moremen (University of California, San Diego, CA), respectively. Monoclonal anti–mouse IP\(_3\)R-1 (18A10) was kindly provided by Drs. A. Miyawaki and K. Mikoshiba (University of Tokyo, Tokyo, Japan) (Furuechi et al., 1989). Polyclonal antibody against calnexin was a gift from Dr. J.J.M. Bergeron (McGill University, Montreal, Canada). Cross-absorbed Texas red–conjugated donkey anti–rabbit F(ab\(_2\)) was from Jackson Immunorsearch Laboratories, and affinity-purified goat anti–rabbit IgG (H + L) conjugated to HRP was from Bio-Rad. 

\(^{45}\)Ca\(^{2+}\) was obtained from NEN Life Science Products. Supersignal chemiluminescent reagent was purchased from Pierce. All chemical reagents were from Sigma Chemical Co. except as indicated.

Preparation and Purification of Hist6-CALNUC

Full-length CALNUC cDNA was amplified by PCR using 5'-CGCGGATCCATGCCATACCTCGTG-3' and 5'-CGGAAATTCGGAATCTTATGCAGTATGAAGAA-3' as primers. PCR was carried out using 100 pmol of each primer, 2 ng CALNUC cDNA, 200 \(\mu\)M dNTP, 2.5 U Pfu polymerase (Stratagene), and PCR reaction buffer in a total volume of 50 \(\mu\)L. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and subcloned into the pET-28a(+) vector (Novagen) at BamHI/HindIII restriction sites, followed by transformation into Escherichia coli BL21(DE3). Expression of CALNUC protein was induced with 1 mM isopropyl \(\beta\)-D-thiogalactoside (IPTG) (Pharmacia Biotech) at 18°C for 4 h at a bacterial density of OD\(_{600}\) \(\sim\) 1.0.

To purify His6-CALNUC protein, transformed E. coli were suspended in binding buffer containing 20 mM sodium phosphate and 500 mM NaCl (pH 7.3), and sonicated using a Misonix Ultrasonic Cell Disrupter (H.Eat Systems). Lysates were then treated with NiNTA agarose (Qiagen), washed with 20 mM sodium phosphate, 500 mM NaCl at decreasing pH (8.0, 6.0, and 5.3), and bound proteins were sequentially eluted with an imidazole gradient step gradient (10 mM to 1 M). Fractions containing a single band of purified CALNUC detected by silver staining were pooled and dialyzed against TBS containing 150 mM NaCl and 10 mM Tris-HCl (pH 7.4) at 4°C, and subsequently concentrated using an Ultrafree-15 (Biomax-50K) filter (Millipore). Highly purified His6-CALNUC [0.6 mg/ml of purified BL21(DE3)] was obtained.

Ca\(^{2+}\)-binding Analysis

Equilibrium dialysis was performed essentially as previously published (MacEnnan and Wong, 1971; Baksh and Michalak, 1991). Ca\(^{2+}\)-free solution was prepared by treatment of deionized water with a Unipure I Water Purification System (Solution Consultants) and Chelex 100 ion exchange resin (Bio-Rad) (Thielens et al., 1990). Equilibrium dialysis was performed using a Dialysis System (GIBCO BRL). 0.25 mg Ca\(^{2+}\)-depleted CALNUC (Thielens et al., 1990) was incubated with 0.35 \(\mu\)M Ca\(^{2+}\) and different concentrations of cold Ca\(^{2+}\) at 4°C for 16 h, followed by assay of radioactivity using a LS 6000IC Liquid Scintillation System (Beckman Instruments) in Ecolume liquid scintillation cocktail (ICN). Scatchard analysis was performed using CA Crickett Graph II software (Computer A Associates International).

Primary Structure Comparison

Amino acid sequences of CALNUC, Cab45 (Scherer et al., 1996), and calmodulin (Cam) were obtained through Entrez on the National Center for Biotechnology Information’s (NCBI) World Wide Web home page. A alignment of EF-hand motifs was performed using MacVector 6.0 software (Oxford Molecular Groups-IBI).

Cell Culture

HeLa cells were maintained in DME high glucose medium (Invirone Scientific) supplemented with 10% FCS (Life Technologies Inc.). Cells were used as 80% confluent monolayers for transfection. Transfected CHO cells were cultured in Ham’s F12 medium (CORC Cell Culture Faculty, University of California, San Diego, CA) with 10% FCS (Life Technologies), 250 \(\mu\)g/ml Zeocin (Invitrogen), and 750 \(\mu\)g/ml G418 sulfate (Calbiochem). All media contained 100 U/ml of penicillin G and 100 \(\mu\)g/ml of streptomycin sulfate. NRK cells were cultured as previously described (Lin et al., 1998).

Transient Overexpression of CALNUC-Green Fluorescent Protein (GFP) or Truncated CALNUC-GFP in HeLa Cells

CALNUC cDNA was amplified by PCR with the primers 5'-CGCGGATCCATGCCATACCTCGTG-3' and 5'-CCATGCCATGGCTAAATGCTGAGAATCC-3' as primers. PCR was also amplified by PCR with the primers 5'-TACGTCATGGTGGCAGGGAAGG-3' and 5'-ATGGTTA-GGCGGCCTGTTACCTTGCTACAGGC-3' to obtain 5'-ATGGTTAGGCACACCAATCTGGC-3' to obtain a CALNUC-GFP vector (Invitrogen) by threefragment ligation to obtain a CALNUC-GFP/pDNA3 construct with GFP ligated to the 3' (COOH terminus) of CALNUC.

CALNUC (JEF-1), in which the \(\alpha\) helix (A79–D109) of the first EF-hand (EF-1) domain (see Fig. 1) was deleted, was obtained by PCR with the primers 5'-CGCGGATCCATGCCATACCTCGTG-3' and 5'-CCCGCCAATGCATGCTAAATGCTGAGAATCC-3' as primers. PCR products were purified and digested, respectively, with BamHI, Ncol, and NotI (New England Biolabs). CALNUC and GFP cDNA were subcloned into the pcDNA3 vector (Invitrogen) by ligation using a fragment ligation to obtain a CALNUC-GFP/pDNA3 construct with GFP ligated to the 3' (COOH terminus) of CALNUC.
Establishment of a Stable HeLa Cell Line Overexpressing GFP Using Flow Cytometry

GFP cDNA amplified by PCR with the primers 5'-TGGTGAGCAAGGG-3' and 5'-TGAAGCTTTCGACCGGCGTTACTCTTGACGTC-3' was subcloned into the pcDNA 3 vector at BamHI/NotI restriction sites, followed by transfection into HeLa cells as described above and G418 selection (0.75 mg/ml) for 4 d. Cells expressing GFP were sorted by flow cytometry (Ex/Em: 488/530) more than 15 (FACStar Plus; Becton Dickinson) in the UCSD Flow Cytometry Core Facility. The top 0.12% of the positive cells was collected and maintained in media containing 0.75 mg/ml G418 until confluent. Selection by sorting was repeated three times until 100% of the cells (HeLa-GFP, GFP-1216) expressed GFP (data not shown).

Establishment of Stable Cell Lines Overexpressing CALNUC in the Ecdysone-inducible Mammalian Expression System

CALNUC cDNA was amplified by PCR and subcloned into the plND vector (Invitrogen) at BamHI/NotI restriction sites. Ecr-CHO cells (Invitrogen) stably expressing the ecdysone receptor (Rxr and VgECr) were transfected with CALNUC cDNA plasmid DNA using lipofectamine as described above followed by selection for G418 resistance (0.4 mg/ml) for 18 d. Cells were split into 96-well plates by serial dilution, 0.5 cells/well, and subsequently reseeded with G418 (0.75 mg/ml). Four clones overexpressing CALNUC after induction with muristerone A (Invitrogen) were obtained; one of these, Ecr-CHO-CALNUC-1 (CPC-22A), was used for these experiments.

Immunocytochemistry

CALNUC-GFP was directly visualized using a Zeiss Axiovert microscope and an FITC-filter (Ex/Em: 485/510). For immunofluorescence, cells on coverslips were fixed with 2% paraformaldehyde (50 min), permeabilized with 0.1% Triton X-100 (10 min), and incubated with affinity-purified anti-CALNUC IgG (6 μg/ml), anti-Mn II serum (1:1300), or anticalnexin serum (1:100) as previously described (Lin et al., 1998). Detection was with Texas red- or FITC-conjugated donkey anti-rabbit F(ab')2. In some cases cells were doubly stained for CALNUC and either a mouse mAb against Mn II (40 μg/ml) or the IP3-R-1 (1.25 μg/ml) and appropriate secondary antibodies. Specimens were examined with either a Zeiss Axiophot equipped for epifluorescence or a Bio-Rad confocal microscope (MRC 1024) equipped with Lasersharp 3.1 software (Bio-Rad) and a krypton-argon laser. Images were processed with Scion Image and Adobe Photoshop (Adobe Systems) software.

Subcellular Fractionation

Sucrose gradient flotation of Golgi fractions was carried out using a protocol similar to those previously published (Fries and Rothman, 1980; Brown and Farquhar, 1987) with minor modifications. In brief, microsomal membranes were resuspended in 1.5 ml 55% sucrose (wt/wt), loaded at the bottom of a sucrose step gradient consisting of 40, 35, 30, 25, and 20% (wt/wt in 1 ml Tris-HCl, pH 7.5), and centrifuged at 85,500 g for 16 h at 4°C using a SW-40T rotor (Beckman). 20 fractions were collected from the bottom, followed by SDS-PAGE and immunoblotting for calnexin (an ER marker), Man II (a Golgi marker), and CALNUC.

Rat liver Golgi fractions, membrane (100,000 g pellet) and cytosolic (100,000 g supernatant) fractions were prepared from postnuclear supernatants of transfected HE La or ECR-CHO-CALNUC cells as previously described (Saucan and Palade, 1994; J in et al., 1996; Lin et al., 1998).

Immunoblotting and SDS-PAGE

Proteins were separated by 5 or 10% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with affinity-purified anti-CALNUC IgG, anticalnexin, and anti–Mn II serum followed by HRP-conjugated anti–rabbit IgG and detection by ECL (Lin et al., 1998).

Results

Quantification of Endogenous CALNUC in Rat Liver Golgi Fractions and NRK Cells

To quantify endogenous CALNUC in rat liver Golgi fractions, a linear standard curve was obtained for purified His6-CALNUC (1.3–40 ng) by immunoblotting and densitometric analysis (data not shown). Endogenous CALNUC was found to be present in pooled Golgi light and heavy fractions from rat liver (Saucan and Palade, 1994; J in et al., 1996) at a concentration of 3.8 μg/ml Golgi protein, i.e., ~0.4% of the total Golgi protein (includes both Golgi resident proteins and cargo in transit through the Golgi). NRK cells were found to have 0.02 μg CALNUC/105 cells, or 2.5 × 105 CALNUC molecules/NRK cell.

CALNUC EF-1 Is an Ideal EF-Hand Ca2+-binding Motif and Constitutes a High Affinity, Low Capacity Ca2+-binding Site

An ideal EF-hand Ca2+-binding motif has an α-helix-loop-α helix structure in which oxygen ligands (O) provided by carboxy side chains of Asp (D)/Glu (E), carboxyl...
**A**

| Helix | Loop | Helix |
|-------|------|-------|
| CALNUC EF-1 | KTFILH | O     |
| CALNUC EF-2  | EHVYNK | O     |
| Cab45 EF-1  | MVIQSV | O     |
| Cab45 EF-2  | KLFRAV | O     |
| Cab45 EF-3  | DWYQAD | O     |
| Cab45 EF-4  | KEFRGL | O     |
| Cab45 EF-5  | KEFEEL | O     |
| Calm EF-1   | KEAFSL | O     |
| Calm EF-2   | QMINEV | O     |
| Calm EF-3   | KEAFRV | O     |
| Calm EF-4   | DEMIREA| O     |

**B**

| CALNUC EF-1 | KTFILH | O     |
| CALNUC EF-2  | EHVYNK | O     |
| Cab45 EF-5  | KFEELII | N     |
| Calm EF-4   | DEMIREA| O     |

**C**

![Figure 1](image)

*Figure 1.* CALNUC EF-1 is an ideal EF-hand Ca$^{2+}$-binding motif. (A) Alignment of the EF-hand loop domains constituting the key Ca$^{2+}$-binding sites in CALNUC, Cab45, and Calm. Consensus amino acids characteristic of ideal EF-hand motifs with oxygen atom–containing side chains (O), a hydrophobic amino acid (Φ), and Gly (G) are boxed. Amino acid numbering corresponding to each EF-hand is as follows: CALNUC (220–246 and 272–298), Z36277, Cab45 (103–129, 142–168, 201–227, 238–264, 283–309, 319–345, U45977), and Calm (14–40, 50–76, 87–113, 124–150, M26151). Numbers in parentheses refer to the amino acid positions and the GenBank accession numbers, respectively. (B) Primary structure of EF-hand sites in CALNUC, Cab45, and Calm. Sequence comparison shows that CALNUC EF-1 and EF-2 have the highest similarity to Cab45 EF-5 and Calm EF-4, except that CALNUC EF-2 has an Arg (R) instead of a Gly at residue 6 of the loop region. (C) Binding of Ca$^{2+}$ to CALNUC is high affinity and low capacity. CALNUC binding to Ca$^{2+}$ was examined by equilibrium dialysis. Scatchard analysis reveals the existence of one high affinity (K_d = 6.6 μM) and a low capacity, ~1.1 μmol Ca$^{2+}$/μmol protein, suggesting only one high affinity, low capacity Ca$^{2+}$-binding site on CALNUC.

Ca$^{2+}$-binding constants are not yet known, in order to predict and compare the Ca$^{2+}$-binding properties of these two proteins, we compared the EF-hand primary structures of CALNUC (two EF-hands), Cab45 (six EF-hands), and Calm (four EF-hands). Cab45's and Calm's EF-hand motifs are similar (Scherer et al., 1996), and Calm's Ca$^{2+}$-binding properties have been well characterized.

As shown in Fig. 1A, CALNUC EF-1, Cab45 EF-2 and -5, and all four Calm EF-hand structures constitute ideal EF-hand motifs. CALNUC EF-1 and EF-2 are strikingly similar to Cab45 EF-4 and Cab45 EF-5, but CALNUC EF-2 has an Arg (R) instead of a Gly at residue 6 (Fig. 1B). This suggests that CALNUC has only a single ideal EF-hand motif, EF-1.

To investigate the binding affinity of CALNUC for Ca$^{2+}$, we performed equilibrium dialysis. Purified recombinant His6-CALNUC was used based on a report that recombinant calreticulin (CRT) was comparable to native CRT in its Ca$^{2+}$-binding capability (Baksh and Michalak, 1991). Scatchard analysis of the binding curve (Fig. 1C) indicates that CALNUC binds Ca$^{2+}$ with a high affinity binding constant (K_d = 6.6 μM) and a low capacity, ~1.1 μmol Ca$^{2+}$/μmol protein, suggesting only one high affinity, low capacity Ca$^{2+}$-binding site on CALNUC.

**Overexpressed CALNUC Colocalizes with the Golgi Marker Man II**

To further investigate Ca$^{2+}$ binding to CALNUC in vivo, we expressed CALNUC-GFP by transient transfection in HeLa cells and generated an inducible cell line, EcR-CHO-CALNUC, stably expressing CALNUC. By immunoblotting, CALNUC-GFP (90 kD) was detected in transiently transfected HeLa cells but not in nontransfected cells (Fig. 2A). The majority of the CALNUC (~85%) was associated with membranes (100,000 g pellet) and the remainder (~15%) was present in the cytosolic fraction (100,000 g supernatant). Three additional bands (Fig. 2A), also visualized after in vitro translation (data not shown), were also seen. They could be products of protein degradation or mistranslated CALNUC retained in the cytosol. By immunofluorescence the distribution of CALNUC-GFP overlapped with that of the Golgi marker Man II (Fig. 3A), indicating that the majority of the CALNUC-GFP is correctly targeted to the Golgi.

In EcR-CHO-CALNUC cells induced with muristerone A or pravastatin A (0.1–10 μM) for 24 h, we found a linear increase in the expression of CALNUC with increasing amounts of added hormone (Fig. 2B). The ratio of CALNUC in membrane versus cytosolic fractions was similar to that of CALNUC-GFP in HeLa cells (data not shown). By immunofluorescence the distribution of CALNUC again overlapped with that of Man II in the Golgi region (Fig. 3C) in EcR-CHO-CALNUC cells induced with 10 μM muristerone A for 24 h, and was distinct from that of the ER marker, calnexin (Fig. 3B).

**Cosedimentation of Overexpressed CALNUC and Man II in Sucrose Gradients**

Next we analyzed the distribution of CALNUC in induced EcR-CHO-CALNUC cells using an established procedure for flotation of Golgi membranes and their separation.
purified anti-CALNUC IgG. were separated by SDS-PAGE and immunoblotted with affinity-librium in cultured cells (Mery et al., 1996). 

45Ca²⁺ shown previously to be long enough to reach 45Ca²⁺ uptake. The 45Ca²⁺ loading time was ~48 h, the time shown previously to be long enough to reach 45Ca²⁺ equilibrium in cultured cells (Mery et al., 1996). 45Ca²⁺ uptake by HeLa cells transiently overexpressing CALNUC-GFP was 2.5-fold that of nontransfected HeLa cells or those stably expressing GFP alone (Fig. 5 A). Similarly, there was a threefold increase in 45Ca²⁺ taken up by induced (5 μM muristerone A for 48 h) versus noninduced EcR-CHO-CALNUC cells (Fig. 5 B). These results demonstrate that Golgi-associated CALNUC binds Ca²⁺ in vivo and most likely is responsible for sequestering Ca²⁺ in the Golgi lumen.

To investigate whether EF-1 is indeed the sole Ca²⁺-binding motif in CALNUC, we examined Ca²⁺ binding in HeLa cells transiently transfected with truncated CALNUC-GFP mutants. When the α helix of EF-1 (Asp 227–Leu 239) or both EF-1 and EF-2 (Asp 227–Phe 293) were deleted from CALNUC, its Ca²⁺-binding capability was completely abolished (Fig. 5 A). Mistargeting could be ruled out since the majority of the mutant CALNUC-GFP was detected in the Golgi region by fluorescence. The results obtained from this in vivo Ca²⁺-binding analysis provide direct evidence that CALNUC binds Ca²⁺ in the Golgi, and EF-1 constitutes the sole Ca²⁺-binding site on CALNUC. The latter is in agreement with the data shown in Fig. 1.

**Release of Sequestered ⁴⁵Ca²⁺ by the SERCA Inhibitor, Tg**

To further investigate the characteristics of the Golgi Ca²⁺ pool, we performed experiments similar to those done previously to characterize the ER Ca²⁺ pool in cells overexpressing CRT (Bastianutto et al., 1995; Mery et al., 1996). When HeLa cells transiently overexpressing CALNUC-GFP or EcR-CHO-CALNUC cells stably expressing CALNUC were treated with the SERCA inhibitor Tg (Thastrup et al., 1990), ~73% and 70%, respectively, of the ⁴⁵Ca²⁺ was released (Fig. 6), suggesting the existence of SERCA on Golgi membranes. Since some Tg-insensitive organelles are capable of retaining Ca²⁺ after Tg treatment, we subsequently treated cells with the Ca²⁺ ionophore ionomycin to release the remaining stored ⁴⁵Ca²⁺. Nearly all the remaining ⁴⁵Ca²⁺ (~20–25%) was released by ionomycin (Fig. 6). In view of the fact that ionomycin is inactivated in acidic compartments such as secretory granules and endosomes, we further treated cells with monensin, a carboxylic sodium proton ionophore which releases Ca²⁺ from acidic compartments (Bastianutto et al., 1995; Mery et al., 1996) and found <5% of the ⁴⁵Ca²⁺ was released. Cells overexpressing CALNUC-GFP or induced EcR-CHO-CALNUC cells released twice as much ⁴⁵Ca²⁺ as nontransfected HeLa cells, HeLa cells stably expressing GFP alone, or noninduced EcR-CHO-CALNUC cells. The fact that the majority of the ⁴⁵Ca²⁺ taken up by CALNUC was released by Tg suggests that both CALNUC and SERCA play a key role in sequestering ⁴⁵Ca²⁺ in the Golgi, a conclusion in agreement with the recent description of SERCA associated with isolated Golgi fractions (Taylor et al., 1997).

**Release of Sequestered Ca²⁺ from the Golgi by Extracellular ATP**

We next examined whether or not Ca²⁺ sequestered in the Golgi is released after agonist challenge. Extracellular...
Figure 3. (A–C) Overexpressed CALNUC is correctly targeted to the Golgi. (A) HeLa cells transiently expressing CALNUC-GFP. CALNUC-GFP is concentrated in the Golgi region where it partially overlaps with the Golgi marker Man II. Cells were fixed in paraformaldehyde, permeabilized, and incubated with anti-Man II serum followed by Texas red-conjugated donkey anti-rabbit.
ATP is known to activate phospholipase C (PLC) (Brown et al., 1991) via binding to G protein–coupled nucleotide receptors on the cell surface (O’Connor, 1992). Activated PLC promotes production of IP3 which binds to IP3R on the ER and triggers Ca2+ mobilization. To investigate whether Ca2+ sequestered by overexpressed CALNUC in the Golgi could be released by agonist, we examined Ca2+ release in EcR-CHO-CALNUC cells by Ca2+ imaging after ATP challenge. The results (Fig. 7A) demonstrated that the ratio, 340:380, was doubled in cells induced with 2.5 μM ponasterone A for 24 h compared with noninduced cells, suggesting that more Ca2+ was released from induced cells. Similar results were also obtained when induced EcR-CHO-CALNUC cells were loaded with 45Ca2+ (Fig. 7B). These results obtained by two different methods suggest that the Golgi Ca2+ store is sensitive to IP3 generated after ATP binding.

Release of 45Ca2+ Sequestered in the Golgi by IP3

To obtain direct evidence that IP3 is able to release Ca2+ from the Golgi, 45Ca2+ uptake and release studies were performed on permeabilized EcR-CHO-CALNUC cells. Fig. 8A reveals that 45Ca2+ is rapidly taken up by both induced and noninduced permeabilized cells, but approximately twice the amount of 45Ca2+ was sequestered by cells overexpressing CALNUC. Steady state was achieved 45 min after loading, which was slower than reported for Swiss 3T3 cells (20 min) (Berridge et al., 1984). 45Ca2+ release was then stimulated by addition of IP3 (Fig. 8B). The ratio of 45Ca2+ released from induced versus noninduced cells was ~2:1. These results support the previous report of Pinton and colleagues (1998) suggesting that both Golgi membranes and ER membranes bear IP3R.

Localization of the IP3 Receptor on the Golgi and ER by Immunofluorescence

In view of the functional evidence for the existence of IP3R on the Golgi, we carried out immunofluorescence studies on NRK cells and induced EcR-CHO-CALNUC cells using a mAb that recognizes IP3R-1. IP3R-1 has an ER-type distribution and also overlaps with CALNUC in the Golgi region. Analysis was carried out on whole cells.

Figure 4. CALNUC cosediments with Man II in sucrose gradients. Total microsomes prepared from induced EcR-CHO-CALNUC cells were subjected to sucrose gradient flotation (85,500 g for 16 h) as described in Materials and Methods. 20 fractions were collected from the bottom, followed by 5 or 10% SDS-PAGE and immunoblotting analysis. CALNUC peaked in fractions 12-15 where it cosedimented with the Golgi marker Man II, whereas the ER marker calnexin peaked in denser fractions 7-11.
found throughout the cytoplasm and concentrated in the Golgi region (Fig. 3 D) which is compatible with both an ER and Golgi localization. Confocal analysis (Fig. 3 E) showed that the distribution of IP3R-1 overlaps with that of CALNUC in the juxtanuclear region, suggesting that IP3R-1 and CALNUC colocalize on Golgi membranes. As mentioned by Pinton and co-workers (1998), it was not possible to carry out reproducible immunogold localization by immunoelectron microscopy with the antibody available.

Discussion

The Golgi complex has been recently identified as a Ca$^{2+}$-enriched compartment whose total Ca$^{2+}$ concentration is $>0.1$ mM (Chandra et al., 1991; Pezzati et al., 1997; Pinton et al., 1998), but the question of how Ca$^{2+}$ is sequestered in the Golgi has remained unanswered. Previously we showed that CALNUC is the major Ca$^{2+}$-binding protein in Golgi fractions from rat liver detected by $^{45}$Ca$^{2+}$ overlay (Lin et al., 1998). In this study we provide evidence that CALNUC binds Ca$^{2+}$ in the Golgi in vivo, because overexpression of CALNUC in the Golgi led to a two- to threefold increase in Ca$^{2+}$ storage based on Ca$^{2+}$ equilibrium loading. This suggests that CALNUC is directly involved in maintenance of Ca$^{2+}$ storage and thereby in Ca$^{2+}$ homeostasis in the Golgi. Equilibrium dialysis demonstrated the existence of only a single high affinity ($K_d = 6.6$ $\mu$M)/low capacity (~$1$ mol Ca$^{2+}$/mol protein) binding site on recombinant CALNUC. CALNUC’s low Ca$^{2+}$-binding capacity in the Golgi might be compensated for by its abundance ($3.8$ $\mu$g/mg Golgi protein).

The demonstration of a single, high affinity Ca$^{2+}$-binding site in the Golgi is in keeping with the fact that CALNUC possesses two EF-hand motifs but only one, EF-1, has the structure expected for high affinity calcium binding. EF-2 has an Arg (R) instead of a Gly (G) at residue 6 of the EF-hand loop region. Arg is supposed to disrupt the EF-hand motif and abolish its Ca$^{2+}$-binding capacity (Branden and Tooze, 1991). CALNUC’s EF-1 has the highest homology to the COOH-terminal EF-4 of CaM which constitutes the high affinity Ca$^{2+}$-binding site of CaM (Crouch and Klee, 1980). Moreover, the Ca$^{2+}$-binding capability of CAL-
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NUC EF-1 was demonstrated previously by 45Ca\(^{2+}\) overlay on truncated CALNUC. When EF-2 was deleted, Ca\(^{2+}\)-binding was maintained, but when both EF-1 and EF-2 were deleted, Ca\(^{2+}\)-binding capability was lost (Miura et al., 1994). In this study, we further demonstrated that truncated CALNUC with either the EF-1 helix (Asp 227–Leu239) or both EF-1 and EF-2 domains (Asp 227–Phe291) deleted lost Ca\(^{2+}\)-binding capability completely. The majority of each of the CALNUC mutant proteins was still targeted to the Golgi region as monitored via the GFP tag. Collectively, these data suggest that EF-1 may constitute the sole high affinity Ca\(^{2+}\)-binding site on CALNUC.

Characterization of the Ca\(^{2+}\) pool in HeLa and CHO cells overexpressing CALNUC provides several important new pieces of information. 45Ca\(^{2+}\) sequestered in the Golgi in cells overexpressing CALNUC was largely released by Tg, an irreversible inhibitor of the SERCA Ca\(^{2+}\) pump, providing in vivo evidence for the existence of SERCAs on Golgi membranes. SERCAs were also assumed to be localized on Golgi membranes because it was shown previously that the p-type, Tg-sensitive SERCA Ca\(^{2+}\) pump was essential for Ca\(^{2+}\) uptake into isolated Golgi fractions in vitro (Taylor et al., 1997). Our results also suggest that the increase in 45Ca\(^{2+}\) uptake in cells overexpressing CALNUC is not likely to be due to the presence of CALNUC in the cytosol or another recently reported Tg- and IP\(_3\)-insensitive Ca\(^{2+}\) pool (Pizzo et al., 1997) since the majority of the Ca\(^{2+}\) was released only after SERCA was inhibited.

Our finding that only a small amount of the Ca\(^{2+}\) remaining after Tg treatment was released by subsequent

Figure 7. The Golgi Ca\(^{2+}\) pool is sensitive to stimulation with ATP. (A) Induced (2.5 μM ponasterone A for 24 h) or noninduced EcR-CHO-CALNUC cells were loaded with 1 μM Fura-2 AM for 1 h, followed by challenge with 100 μM ATP. Released Ca\(^{2+}\) was monitored by Ca\(^{2+}\) imaging. More Ca\(^{2+}\) was released after ATP stimulation from cells overexpressing CALNUC than from noninduced cells. Results represent the average of values from 14 induced cells and 15 noninduced cells. Reproducible results were obtained from experiments repeated twice. (B) Noninduced or induced EcR-CHO-CALNUC cells were loaded with 45Ca\(^{2+}\) (2 μCi/ml) for 48 h to reach 45Ca\(^{2+}\) equilibrium. Cells were washed and resuspended in KRH buffer supplemented with 3 mM EGTA, followed by stimulation with 100 μM ATP for 5 min at room temperature. Aliquots of 10⁶ cells were subsequently removed, the cells were sedimented, and 45Ca\(^{2+}\) in supernatants and pellets was assessed. Compared with noninduced cells, there was a twofold increase in the 45Ca\(^{2+}\) released in cells overexpressing CALNUC. Results (mean ± SD) represent the average of values obtained in three separate experiments performed in duplicate.

Figure 8. Ca\(^{2+}\) sequestered by CALNUC in the Golgi was released directly by IP\(_3\) treatment of permeabilized cells. (A) 45Ca\(^{2+}\) uptake. Noninduced or induced EcR-CHO-CALNUC cells (10⁶ cells/well) were permeabilized with 50 μg/ml saponin for 4 min, followed by loading with 40Ca\(^{2+}\) (10 μCi/ml) for the times indicated. Stored 45Ca\(^{2+}\) was extracted with 1 ml 0.1 N HCl for 30 min, and 0.5-ml aliquots were counted. Induced cells took up twice as much 45Ca\(^{2+}\) as noninduced cells. 45Ca\(^{2+}\) uptake reached equilibrium at ~45 min after loading. (B) 45Ca\(^{2+}\) release. Induced or noninduced EcR-CHO-CALNUC cells (10⁶ cells/well) were permeabilized and loaded with 10 μCi/ml of 45Ca\(^{2+}\) for 45 min to reach equilibrium and IP\(_3\) (10 μM) was added. Aliquots (10) were collected at 2-min intervals. Stored 45Ca\(^{2+}\) could be directly released by IP\(_3\). Twice as much 45Ca\(^{2+}\) was released from induced as noninduced cells. Results (mean ± SD) represent the average of values obtained in three separate experiments performed in duplicate.
ionomycin treatment might be due to incomplete depletion of Ca²⁺ from the Golgi by Tg, since the existence of a Tg-insensitive/ionomycin-sensitive-plasma membrane calcium ATPase Ca²⁺ pump on Golgi membranes has also been reported recently (Taylor et al., 1997). The fact that monensin treatment which depletes Ca²⁺ from acidic compartments (secretory vesicles, granules, trans-Golgi network) (Fasolato et al., 1991) did not release a significant amount of Ca²⁺ demonstrates that Ca²⁺ was not sequestered in an acidic compartment. Thus, our current results from in vivo studies suggest that the Ca²⁺-binding protein CALNUC together with SERCA Ca²⁺ pumps are responsible for the maintenance of the Golgi Ca²⁺ storage pool.

We also investigated the agonist sensitivity of the Golgi Ca²⁺ pool. It was shown recently that the Golgi Ca²⁺ store is sensitive to histamine, an agonist known to be coupled to IP₃ generation (Pinton et al., 1998), suggesting that there may be IP₃Rs on Golgi membranes. Here we used extracellular ATP, another agonist known to generate IP₃ after binding to plasma membrane nucleotide receptors (P₂Y₂-purinoceptors) (O’Connor, 1992), to investigate the sensitivity of the Golgi Ca²⁺ store to IP₃. A TAP challenge is coupled to IP₃ production via activation of PLC (Brown et al., 1991), and binding of IP₃ to IP₃Rs on the surface of Golgi pool releases intracellular Ca²⁺ (Irredale and Hill, 1993). When ADP was added to induced Ecr-CHO-CALNUC cells, there was a rapid release of sequestered Ca²⁺ revealed by both Ca²⁺ imaging and ⁴⁵Ca²⁺ which far exceeded that released from noninduced cells. Moreover, IP₃ directly triggered ⁴⁵Ca²⁺ mobilization from the Golgi in permeabilized Ecr-CHO-CALNUC cells. Thus, our biochemical results and those of Pinton et al. (1998) using histamine as agonist suggest that the Golgi apparatus bears IP₃Rs. The assumption that IP₃Rs on the Golgi are expressed on the Golgi is supported by our immunofluorescence observations suggesting a dual localization of IP3R-1 on both ER and Golgi membranes. CHO cells were found previously to express ample IP₃R-1 by immunoprecipitation (M onkawa et al., 1995) using mAb 18A10 (Furuichi et al., 1989) which specifically recognizes the COOH terminus of IP₃R-1.

A major controversy in the physiology of intracellular Ca²⁺ stores concerns the mechanism by which their depletion triggers influx of Ca²⁺ through the plasma membrane. In vertebrate cells, it has been assumed generally that the relevant store is the ER (Randriamampita and Tsien, 1993; Parekh and Penner, 1997). However, because both ER and Golgi accumulate Ca²⁺ via SERCA and release Ca²⁺ via IP₃ receptors, both should undergo depletion roughly in parallel, so one cannot yet exclude a role for the Golgi in controlling plasma membrane Ca²⁺ influx. In yeast, store-operated Ca²⁺ influx appears to be controlled mainly by the Golgi, because genetic deletion of the Golgi Ca²⁺ pump encoded by PMR1 increases the influx of extracellular Ca²⁺ (Halachmi and Eilam, 1996). Therefore, we tried to distinguish between ER and Golgi contributions by testing whether overexpression of CALNUC in Xenopus oocytes affected the store-operated Ca²⁺ current, Isoc (Y ao and Tsien, 1997). If the Golgi were important, increasing the quantity of Ca²⁺ buffer in its lumen should diminish or delay Isoc (M ery et al., 1996; Fasolato et al., 1998). Overexpression of CALNUC (via microinjection of its mRNA) increased the ⁴⁵Ca²⁺ content of oocytes analogously with Fig 5 and appeared by fluorescence microscopy to be colocalized with the Golgi marker galactosyltransferase fused to GFP (Llo pis et al., 1998). However, CALNUC overexpression did not significantly affect Isoc, either partially activated by the membrane-permeant Ca²⁺ buffer TPEN (A rslan et al., 1985; Hoffer et al., 1998) or maximally activated by the ionophore ionomycin. This negative result might seem to argue against a major role for the Golgi in controlling Ca²⁺ influx into oocytes, but a firm conclusion would require additional controls such as immuno electron microscopic localization of CALNUC and evidence that comparable increases in ER buffering do affect Isoc.

Previously, we demonstrated significant homology between CALNUC and CRT and two conserved motifs, AY(I/IA)EEX and QRLX(Q/E)E(I/E)E, located in the C-domain of CRT (aa337-341 and 365-372) (Lin et al., 1998). However, the homologous regions do not involve Ca²⁺-binding domains. CRT lacks EF-hand motifs but possesses a high affinity/low capacity and a low affinity (K_d = 2 mM)/high capacity (21 μmol Ca²⁺/μmol protein) Ca²⁺-binding site (Baksh and Michalak, 1991) constituted by clusters of ~35 Asp (D)/Glu (E) located in CRT’s C-domain. In the future it will be of interest to examine whether CALNUC can function like CRT, its ER-resident counterpart (Lin et al., 1998), to maintain a high Ca²⁺ concentration required for Golgi functions, e.g., sorting, lectin binding, budding, and concentration of cargo into regulated secretory granules.

In summary, this study demonstrates that CALNUC, an abundant Golgi resident protein and the major Golgi Ca²⁺-binding protein, together with SERCA Ca²⁺ pumps and IP₃Rs are involved in the maintenance of the Ca²⁺ storage pool in the Golgi. Further investigation of several remaining intriguing questions including whether the binding of Ca²⁺ to CALNUC regulates membrane traffic or posttranslational processing events in the Golgi, should shed light on the biological functions of CALNUC in the Golgi Ca²⁺ pool.

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