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 \(^{45}\)Ca\(^{2+}\) overlay (Lin, P., H. Le-Niculescu, R. Hofmeister, J.M. Mccaffery, M. J in, H. Henneman, T. M cQ uistan, L. D e V ries, and M. Farquhar. 1998. J. Cell Biol. 141:1515–1527). In this study we investigated CALNUC’s properties and capacity Ca\(^{2+}\) store. By immunofluorescence, the IP\(_3\) receptor type 1 (IP\(_3\)R-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\(_3\)R 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\(_3\)R 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 lumenal 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 mammalian (Baumrucker and Keenan, 1975; Hodson, 1978; Neville et al., 1981; Virk et al., 1985) and yeast (Sorin et al., 1997) Golgi fractions. Both sarcoplasmic/ER calcium ATPase (SERCA)\(^{1}\) and plasma membrane calcium ATPase

1 Abbreviations used in this paper: CaM, calmodulin; CRT, calreticulin; GFP, green fluorescent protein; IP\(_3\)R, inositol 1, 4, 5 trisphosphate receptor; M an II, α-mannosidase II; PLC, phospholipase C; SERCA, sarcoplasmic/ER calcium ATPase; T\(_9\), thapsigargin.
on 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 D\(^{45}\)Ca\(^{2+}\) overlay (Lin 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 thapsigargol (Tg), ATP, 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 A Iberita, A Iberita, Canada) and K. M oremen (University of Georgia, Athens, GA.), respectively. Monoclonal anti-mouse IP\(_3\)R-1 (1A8 10) was kindly provided by Drs. A. M yawaki and K. M ishiba (University of Tokyo, Tokyo, Japan) (Furuichi et al., 1989). Polyclonal antibody against calnexin was a gift from Dr. J. M. B ergeron (McGill University, Montreal, Canada). Cross-absorbed Texas red-conjugated donkey anti-rabbit F(ab\(^{\prime}\))2 was obtained from Jackson Immunoresearch Laboratories, and affinity-purified goat anti-rabbit IgG (H + L) conjugated to HRP was from Bio-Rad. 4\(^{Ca}\)C\(_{i}\) 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 His\(_6\)-CALNUC**

Full-length CALNUC cDNA was amplified by PCR using 5\(\prime\)-CGGCGG-GCAGGCATATGCTCACTCTCTTG-3\(\prime\) and 5\(\prime\)-CGGAAATTCGGATCTTATCA-3\(\prime\) as primers. PCR was carried out using 100 pmol of each primer, 2 ng CALNUC cDNA, 200 \(\mu\)M dNTP, 2.5 U PUFE polymerase (Stratagene), and PCR reaction buffer in a total volume as indicated.

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

Equilibrium dialysis was performed essentially as previously published (M. Alenann and W. Ong, 1971; Baksh and M ichalak, 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) (Thei lsens et al., 1990). Equilibrium dialysis was performed by using a Dialysis System (GIBCO BRL). 0.25 mg Ca\(^{2+}\)-depleted CALNUC (Thielsens et al., 1990) was incubated with 0.35 \(\mu\)C/mI 4\(^{Ca}\)C\(_{i}\) 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 Cricket Graph II software (Computer 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. Alignment of E. coli motifs was performed using MacVector 6.0 software (Oxford Molecular Groups-BI).
Establishment of a Stable HeLa Cell Line Overexpressing GFP Using Flow Cytometry

GFP cDNA amplified by PCR with the primers 5′-TGGCGGATCCCA-TGGTGGACGAAAGG-3′ and 5′-TATATGTTACCGGCGGCTTACTTTGTACAGCTC-3′ was subcloned into the pcDNA3 vector at BamHI/Not 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 nm; FACStar Plus®; Becton Dickinson) in the UCSD Flow Cytometry Core Facility.

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

CALNUC cDNA was amplified by PCR and subcloned into the pcDNA3 vector (Invitrogen) at BamHI/Not restriction sites. Er-Ca2+-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 reselected with G418 (0.75 mg/ml). Four clones overexpressing CALNUC after induction with muristerone A (Invitrogen) were obtained; one of these, Er-Ca2+-CHO-CALNUC-1 (CPC-22A), was used for these experiments.

Immunocytochemistry

CALNUC-GFP was directly visualized using a Zeiss Axiophot 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-Man II serum (1:300), 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 Man 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 lasersharpe 3.1 software (Bio-Rad) and a krypton-argon laser. Images were processed with Scion Image and Adobe Photoshop (A dobe 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/vol), loaded at the bottom of a sucrose step gradient consisting of 40, 35, 30, 25, and 20% (wt/vol in 1 mM 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 HeLa or Er-Ca2+-CHO-CALNUC cells as previously described (Saucan and Palade, 1994; Jin 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–Man II serum followed by HRP-conjugated anti–rabbit IgG and detection by ECL (Lin et al., 1998).

45Ca2+ Equilibrium Uptake and Release

The procedures followed were those reported previously (Bastianutto et al., 1995). Cells (2 × 106) transfected with CALNUC-GFP or GFP alone were incubated with 45Ca2+ (2 μCi/ml) for 48 h to reach 45Ca2+ equilibrium after which they were washed three times in Krebs-Ringer-Hepes (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 6 mM glucose, and 25 mM Hepes, pH 7.4) and five times with PBS. 45Ca2+ was extracted with 0.1 N HCl (30 min at room temperature) and radioactivity assessed as described above. To examine 45Ca2+ release after stimulation, washed cells were resuspended in KRH supplemented with 3 mM EGTA and stimulated at room temperature with 100 μM ATP or sequentially stimulated with 0.1 μM Tg, 2 μM ionomycin, and 2 μM monensin, 5 min each. Equal aliquots (106 cells) were collected after each stimulation, followed by centrifugation at 14,000 rpm (30 s) and quantification of 45Ca2+ in the supernatant.

Ca2+ Imaging

Noninduced or induced Er-Ca2+-CHO-CALNUC cells were loaded with 1 mM Fura-2 AM (Molecular Probes Inc.) in Ham’s F12 medium/0.5% FCS at 22°C for 1 h, washed with Ca2+-free HBSS (Irvine Scientific) followed by addition of 100 μM ATP. Ca2+ release was monitored by Ca2+ imaging performed on a Zeiss Axiosvert microscope equipped with a cooled charge-coupled CCD camera (Photometrics) and Metafluor software (Universal Imaging). Dual-excitation ratio imaging was obtained using two excitation filters (340 F20 and 380 F20) (Omega Optical and Chroma Technology) mounted on a filter wheel (Lambda 10-2, Sutter Instruments), a 420 RLP dichroic mirror, and a 510DF80 emission filter.

Assessment and Mobilization of Stored Ca2+ by IP3 in Permeabilized Cells

The procedures used were basically similar to those published (Berridge et al., 1984; De Smedt et al., 1997) with minor modifications. To examine equilibrium 45Ca2+ uptake, Er-Ca2+-CHO-CALNUC cells induced with 5 μM ponasterone A for 24 h in a 6-well culture plate (106 cells/well) were permeabilized at 20°C for 4 min with saponin (50 μg/ml) in loading buffer (140 mM KCl, 20 mM NaCl, 2 mM MgCl2, 2 mM ATP, 0.1 mM EGTA, 20 mM Pipes, pH 6.80), and 0.13 μM free Ca2+ calculated for conditions of 0.80, at 20°C (Tsien and Pozzan, 1989). Cells were washed four times with loading buffer and subsequently loaded with 45Ca2+ (10 μCi/ml) for various times (10–60 min). They were then rinsed five times with loading buffer (30 s), stored 45Ca2+ was extracted with 1 ml 0.1 N HCl for 30 min, and 0.5 ml aliquots were counted.

To investigate Ca2+ mobilization by IP3, induced and permeabilized Er-Ca2+-CHO-CALNUC cells were loaded with 10 μCi/ml of 45Ca2+ as above for 45 min. After washing (five times over 1–1.5 min), cells were challenged with 10 μM IP3 (o-myo-inositol 1,4,5-trisphosphate potassium salt) in loading buffer, 1 ml/well. Solutions were collected at 2-min intervals, replaced with loading buffer containing IP3, and counted.

Results

Quantification of Endogenous CALNUC in Rat Liver Golgi Fractons 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; Jin et al., 1996) at a concentration of 3.8 μg/mg 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/106 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...
Figure 1. CALNUC EF-1 is an ideal EF-hand Ca\(^2+\)-binding motif. (A) A alignment of the EF-hand loop domains constituting the key Ca\(^2+\)-binding sites in CALNUC, Cab45, and CaM. 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 272–304), Cab45 (309, 319–345), U45977), and CaM (14–40, 50–76, 87–113, 124–150, 298, Z36277), Cab45 (103–129, 142–168, 201–227, 238–264, 283–304, 319–345, U45977), and CaM (14–40, 50–76, 87–113, 124–150, 298, Z36277). Calnexin (Fig. 3 B). 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. 2 A), 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.

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 immunofluorescence, CALNUC-GFP (90 kD) was detected in transiently transfected HeLa cells but not in nontransfected cells (Fig. 2 A). The ratio 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. 2 A), 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.

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.
from ER membranes. As shown in Fig. 4, we found that CALNUC and Man II cosedimented and peaked in fractions 12–15 with sucrose densities similar to those previously reported (1.10–1.14 g/ml) (Dunphy and Rothman, 1983; Brown and Farquhar, 1987) for CHO cells. By contrast, the ER marker, calnexin, peaked in denser fractions 7–11 (1.16–1.19 g/ml). These results together with the immunofluorescence findings demonstrate that overexpressed CALNUC is found in the Golgi and is consistent with our previous conclusion (Lin et al., 1998) that overexpression does not lead to mistargeting of CALNUC.

Overexpression of CALNUC-GFP or CALNUC in the Golgi Increases 45Ca2+ Uptake

To assess whether overexpressed CALNUC-GFP binds Ca2+ in the Golgi, we carried out in vivo equilibrium Ca2+ 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 43Ca2+ 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 (A sp227–Leu239) or both EF-1 and EF-2 (A sp227–Phe293) 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). A 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 was detected in induced cells in a pattern distinct from that of the ER marker, calnexin. Virtually no signal is detected in noninduced cells. Induced (muristerone A, 10 μM for 24 h) or noninduced cells were labeled with affinity-purified anti-CALNUC or anticalnexin serum and processed as in A. (C) Double labeling of induced EcR-CHO-CALNUC cells with anti-CALNUC IgG and anti-Man II mAb. The distribution of overexpressed CALNUC overlaps with that of Man II in the Golgi. Detection was with Texas red–conjugated donkey anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG. (D) Codistribution of IP3R with both ER and Golgi. NRK cells immunostained as above with anti-CALNUC IgG and mAb anti-IP3R-1 (18A10). IP3R-1 has an ER-type distribution and also overlaps with CALNUC in the Golgi region. Analysis was carried out on whole cells. (E) Similar preparation of induced EcR-CHO-CALNUC cells analyzed by confocal microscopy, showing overlap of staining for IP3R and CALNUC. Bars, 10 μm.
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 \(\sim 0.1\) 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 (\(\sim 1\) 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).

Figure 5. Overexpression of CALNUC increases \(^{45}\)Ca\(^{2+}\) uptake. (A) Nontransfected HeLa cells (N.T.), or those stably transfected with GFP alone, or transiently expressing CALNUC-GFP or CALNUC-GFP deletion mutants were incubated with 2 \(\mu\) Ci/ml \(^{45}\)Ca\(^{2+}\) for 48 h to reach \(^{45}\)Ca\(^{2+}\) equilibrium. \(^{45}\)Ca\(^{2+}\) uptake by cells overexpressing CALNUC-GFP was 2.5-fold (2,500 vs. 1,000 cpm) greater than that found in nontransfected cells or those expressing GFP alone. Truncated CALNUC in which both EF-1 [CN\((\Delta EF-1)-GFP\)] or EF-1 and EF-2 [CN\((\Delta EF-1, 2)-GFP\)] were deleted completely lost Ca\(^{2+}\)-binding capability. (B) EcR-CHO cells stably transfected with CALNUC cDNA (EcR-CHO-CALNUC) were loaded with \(^{45}\)Ca\(^{2+}\) as in A. \(^{45}\)Ca\(^{2+}\) uptake in induced cells (10 \(\mu M\) muristerone A) was threefold that found in noninduced cells (7,400 vs. 2,500 cpm). Results (mean \pm SD) represent the average of values obtained in three separate experiments performed in duplicate.

The demonstration of a single, high affinity Ca\(^{2+}\)-binding site 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-

Figure 6. Most of the \(^{45}\)Ca\(^{2+}\) taken up by CALNUC in the Golgi is released by the SERCA inhibitor Tg. Cells were incubated with \(^{45}\)Ca\(^{2+}\) for 48 h as in Fig. 5 and sequentially treated with 0.1 \(\mu M\) Tg, 2 \(\mu M\) ionomycin, and 2 \(\mu M\) monensin. \(^{45}\)Ca\(^{2+}\) release into the medium was measured after each treatment. (A) \(\sim 73\%\), \(\sim 20\%\), and 7\% of the \(^{45}\)Ca\(^{2+}\) was released from HeLa cells transiently overexpressing CALNUC-GFP after Tg, ionomycin, and monensin treatments, respectively. Nontransfected HeLa cells (N.T.) or those stably expressing GFP alone did not release as much \(^{45}\)Ca\(^{2+}\) as HeLa cells overexpressing CALNUC-GFP. (B) Similar results (70\%, 25\%, and 5\%) for Ca\(^{2+}\) release were obtained for induced (5 \(\mu M\) ponasterone A for 24 h) EcR-CHO-CALNUC cells following sequential treatment as above. Induced cells release twice as much \(^{45}\)Ca\(^{2+}\) as noninduced cells. Results (mean \pm SD) represent the average of values obtained in three separate experiments performed in duplicate.
NUC EF-1 was demonstrated previously by 45Ca2+ overlay on truncated CALNUC. When EF-2 was deleted, Ca2+-binding capability was maintained, but when both EF-1 and EF-2 were deleted, Ca2+-binding capability was lost (Miura et al., 1994). In this study, we further demonstrated that truncated CALNUC with either the EF-1 helix (Asp227–Leu239) or both EF-1 and EF-2 domains (Asp227–Phe291) deleted lost Ca2+-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 Ca2+-binding site on CALNUC.

Characterization of the Ca2+ pool in HeLa and CHO cells overexpressing CALNUC provides several important new pieces of information. 45Ca2+ sequestered in the Golgi in cells overexpressing CALNUC was largely released by Tg, an irreversible inhibitor of the SERCA Ca2+ 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 Ca2+ pump was essential for Ca2+ uptake into isolated Golgi fractions in vitro (Taylor et al., 1997). Our results also suggest that the increase in 45Ca2+ 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 IP3-insensitive Ca2+ pool (Pizzo et al., 1997) since the majority of the Ca2+ was released only after SERCA was inhibited.

Our finding that only a small amount of the Ca2+ remaining after Tg treatment was released by subsequent

Figure 7. The Golgi Ca2+ 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 Ca2+ was monitored by Ca2+ imaging. More Ca2+ 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 45Ca2+ (2 μCi/ml) for 48 h to reach 45Ca2+ 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. A aliquots of 106 cells were subsequently removed, the cells were sedimented, and 45Ca2+ in supernatants and pellets was assessed. Compared with noninduced cells, there was a twofold increase in the 45Ca2+ released in cells overexpressing CALNUC. Results (mean ± SD) represent the average of values obtained in three separate experiments performed in duplicate.

Figure 8. Ca2+ sequestered by CALNUC in the Golgi was released directly by IP3 treatment of permeabilized cells. (A) 45Ca2+ 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 45Ca2+ (10 μCi/ml) for the times indicated. Stored 45Ca2+ 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 45Ca2+ as noninduced cells. 45Ca2+ uptake reached equilibrium at ~45 min after loading. (B) 45Ca2+ release. Induced or noninduced EcR-CHO-CALNUC cells (106 cells/well) were permeabilized and loaded with 10 μCi/ml of 45Ca2+ for 45 min to reach equilibrium and IP3 (10 μM) was added. Aliquots (10) were collected at 2-min intervals. Stored 45Ca2+ could be directly released by IP3. Twice as much 45Ca2+ 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 Ca\textsuperscript{2+} from the Golgi by Tg, since the existence of a Tg-insensitive/ionomycin-sensitive plasma membrane calcium ATPase Ca\textsuperscript{2+} pump on Golgi membranes has also been reported recently (Taylor et al., 1997). The fact that monensin treatment which depletes Ca\textsuperscript{2+} from acidic compartments (secretory vesicles, granules, trans-Golgi network) (Fasolato et al., 1991) did not release a significant amount of Ca\textsuperscript{2+} demonstrates that Ca\textsuperscript{2+} was not sequestered in an acidic compartment. Thus, our current results from in vivo studies suggest that the Ca\textsuperscript{2+-}binding protein CALNUC together with SERCA Ca\textsuperscript{2+} pumps are responsible for the maintenance of the Golgi Ca\textsuperscript{2+} storage pool.

We also investigated the agonist sensitivity of the Golgi Ca\textsuperscript{2+} pool. It was shown recently that the Golgi Ca\textsuperscript{2+} store is sensitive to histamine, an agonist known to be coupled to IP\textsubscript{3} generation (Pinton et al., 1998), suggesting that there may be IP\textsubscript{3}R on Golgi membranes. Here we used extracellular ATP, another agonist known to generate IP\textsubscript{3} after binding to plasma membrane nucleotide receptors (P\textsubscript{2y}-purinoreceptors) (O’Connor, 1992), to investigate the sensitivity of the Golgi Ca\textsuperscript{2+} store to IP\textsubscript{3}. ATP challenge is coupled to IP\textsubscript{3} production via activation of PLC (Brown et al., 1991), and binding of IP\textsubscript{3} to IP\textsubscript{3}R on the surface of Ca\textsuperscript{2+}-pool releases intracellular Ca\textsuperscript{2+} (Iredale and Hill, 1993). When ATP was added to induced Ecr-CHO-CALNUC cells, there was a rapid release of sequestered Ca\textsuperscript{2+} revealed by both Ca\textsuperscript{2+} imaging and 45Ca\textsuperscript{2+} which far exceeded that released from noninduced cells. Moreover, IP\textsubscript{3} directly triggered 45Ca\textsuperscript{2+} 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 IP\textsubscript{3}R. The assumption that IP\textsubscript{3}R are expressed on the Golgi is supported by our immunofluorescence observations suggesting a dual localization of IP\textsubscript{3}R-1 on both ER and Golgi membranes. CHO cells were found previously to express ample IP\textsubscript{3}R-1 by immunoprecipitation (Monkawa et al., 1995) using mAb 18A10 (Furuichi et al., 1989) which specifically recognizes the C-terminal tail of the IP\textsubscript{3}R.

A major controversy in the physiology of intracellular Ca\textsuperscript{2+} stores concerns the mechanism by which their depletion triggers influx of Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} via SERCA\textsubscript{s} and release Ca\textsuperscript{2+} via IP\textsubscript{3} receptors, both should undergo depletion roughly in parallel, so one cannot yet exclude a role for the Golgi in controlling plasma membrane Ca\textsuperscript{2+} influx. In yeast, store-operated Ca\textsuperscript{2+} influx appears to be controlled mainly at the Golgi, because genetic deletion of the Golgi Ca\textsuperscript{2+} pump encoded by PMR1 increases the influx of extracellular Ca\textsuperscript{2+} (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 Ca\textsuperscript{2+} current, Isoc (Yao and Tsien, 1997). If the Golgi were important, increasing the quantity of Ca\textsuperscript{2+} buffer in its lumen should diminish or delay Isoc (Mery et al., 1996; Fasolato et al., 1998). Overexpression of CALNUC (via microinjection of its mRNA) increased the 45Ca\textsuperscript{2+} content of oocytes analogously with Fig. 5 and appeared by fluorescence microscopy to be colocalized with the Golgi marker galactoside transferase fused to GFP (Llopis et al., 1998). However, CALNUC overexpression did not significantly affect Isoc, either partially activated by the membrane-permeant Ca\textsuperscript{2+} buffer TPEN (Arslan et al., 1985; Hofer 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 Ca\textsuperscript{2+} influx into oocytes, but a firm conclusion would require additional controls such as immunoelectron 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/A)EE and QRXL(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 Ca\textsuperscript{2+} binding domains. CRT lacks EF-hand motifs but possesses a high affinity/low capacity and a low affinity (K\textsubscript{d} = 2 mm) high capacity (21 \mu mol Ca\textsuperscript{2+}/\mu mol protein) Ca\textsuperscript{2+}-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 Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+}-binding protein, together with SERCA Ca\textsuperscript{2+} pumps and IP\textsubscript{3}R are involved in the maintenance of the Ca\textsuperscript{2+} storage pool in the Golgi. Further investigation of several remaining intriguing questions including whether the binding of Ca\textsuperscript{2+} to CALNUC regulates membrane traffic or posttranslational processing events in the Golgi, should shed light on the biological functions of CALNUC and on the Golgi Ca\textsuperscript{2+} pool.

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