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Critical Review

Calnuc: Emerging Roles in Calcium Signaling and Human Diseases

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Summary

Calnuc is a recently discovered multidomain protein with EF-hand calcium binding sites. Several studies have reported various interacting partners for calnuc and, therefore, also different sites of localization in the cell. It interacts with important molecules such as DNA, G protein, COX, and amyloid precursor protein among others in addition to being involved in stress response and trafficking. The immense possibilities (of various functions this protein might be involved in) implicate great future in medicine and physiology. Preliminary studies also implicate the possibility of calnuc being involved in some of the human diseases. These initial observations imply the functions that this protein might be involved in. This review emphasizes the importance of further research on this protein. © 2010 IUBMB

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Keywords Ca^{2+} signaling; protein function.

INTRODUCTION

Despite its omnipresence, calcium displays very distinct and extremely specific role in each part of the cell (*1*). With ever-increasing body of literature, it almost seems like there is no place in the cell that does not involve Ca^{2+} ions in some physiological function or other. Interestingly, cells actually exclude Ca^{2+} from their interior (intracellular concentration being in the range of hundreds of nanomolar compared with the extracellular concentration, which is in the range of millimolar) for physicochemical reasons (*2*). To avoid dangerous consequences of this divalent ion, cells have evolved ways to reduce its cytosolic concentrations and use its binding energy for signal transduction in a controlled manner. To achieve this goal, cells have not only compartmentalized the storage of Ca^{2+} but have also developed proteins that bind to it and transmit consequent structural changes as signals.

The favorable chemical nature of Ca^{2+} (*e.g.*, ionic structure, valence state, binding strength, its ionization potential and kinetic parameters in biological reactions) made it impossible for a total exclusion of this ion, and hence cells compartmentalized Ca^{2+} also into their organelles. Consequently, amounts of intracellular calcium ions became important determinants of several biological functions. The major organelles that store Ca^{2+} are the endoplasmic reticulum, mitochondria, nucleus and the Golgi apparatus. The ability of cells to adapt to variations in Ca^{2+} concentrations across the volume of a cell came to be mediated by various calcium binding proteins. Controlled release of Ca^{2+} from these organelles and the way in which it travels in the cell (as sparks, waves, *etc*.) transmit signals from one part of the cell to another and this is accomplished by several calcium binding proteins (CaBPs). Classic examples of such proteins are parvalbumin and calmodulin (*3*). Role of Ca^{2+} ions stored in the mitochondria and endoplasmic reticulum is well understood, whereas the importance of Golgi as a Ca^{2+} store is only beginning to be studied (*4–5*). The calcium binding proteins in the Golgi apparatus are calnuc (*6*), Cab45 (*7*), and P54/NEFA (*8*). This review will focus on the biochemistry and physiological functions of calnuc. (In this review calnuc has been used to represent nucleobindin1 (Nucb1) and calnuc2 refers to Nucb2/NEFA).

CALNUC—A NOVEL CALCIUM BINDING PROTEIN

Calnuc is a highly conserved protein found in species as varied as C*iona intestinalis* and *Homo sapiens* (Fig. 1). An alignment and analysis of its amino acid sequences reveals differences between lower organisms and higher ones (*9*). The lower organisms have a single polypeptide chain, whereas the higher organisms (mammals) probably have two isoforms of the

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Figure 1. Phylogenetic analysis of calnuc sequences. Phylogenetic analysis of calnuc among different species shows that it is a highly conserved protein that has matured and segregated evolutionarily. All the complete sequences obtained from NCBI were used to create a multiple sequence alignment (MSA). This was used as the input for constructing the phylogenetic tree using PHYLIP. A rooted tree was obtained, which means that calnuc protein in all these organisms evolved from a single common ancestor. MSA of calnuc sequences reveals the presence of common highly conserved domains, viz. EF-hand motifs, DNA-binding region and leucine zipper, which only exerts the important evolutionarily conserved functional, and hence, structural relationship this protein might be playing across such diverse animal kingdom. Phylogenetic tree also unfolds the evolutionary segregation of this protein. Invertebrates like *Schistosoma* and lower chordates like *Spodoptera*, *Drosophila*, and *Ciona* are all clustered farther away in the tree stressing the fact that these might be the oldest organisms which expressed calnuc. Although all higher organisms expressing calnuc clustered together as one group, even these were segregated into two groups based on isoform of calnuc. All higher organisms expressing isoform 2 of calnuc clustered separately from isoform 1 of calnuc in the phylogenetic tree. Gene duplication is probably responsible for the presence of these two different isoforms of calnuc in higher organisms. Organisms included in this study: *Schistosoma mansoni, Spodoptera frugiperda, Drosophila melanogaster, Caenorhabditis elegans, Ciona intestinalis, Homo sapiens, Bos Taurus, Mus musculus, Rattus norvegicus, Gallus gallus, Taeniopygia guttata, Xenopus tropicalis, Aristichthys nobilis, Danio rerio, Salmo salar, Xenopus laevis, Xenopus tropicalis, Ornithorhyncus anatinus, Monodelphis domestica, Pongo abelii, Pan troglodytes, Canis familiaris*, and *Equus caballus*. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

protein. The differences in expression levels and functions of these isoforms, if any, are still unknown. The amino acid sequences are conserved to a greater degree in the higher organisms compared with the ones from lower organisms. A theoretical analysis of the probable secondary structure of the protein shows it to be a largely helical protein with possible coiled–coiled regions, confirming the experimental observation from circular dichroism studies (*9, 10*).

The gene structure of calnuc in various species is fairly conserved, having 11 or more exons, with some organisms having untranslated regions, both at the 3′ and 5′ ends (*11*). The distribution of intron phase in most of these genes seems to be dependent on the size of the gene in the organism. As the size (and therefore the number of introns) increases from *Drosophila* to monkey, the number of "phase 2" introns seems to be going up (Fig. 2). The molecular basis and physiological consequence of this phenomenon are yet to be understood and might throw light on the structure, function, and evolution of this gene. It is an intriguing fact that, given the varied gene structure and the possibilities of exon shuffling, the corresponding primary structure of the protein is so highly conserved among all species, with large differences mostly in the C-terminal region.

Chromosomal location of the human calnuc gene has been assigned to 19q13.2-q13.4 (*12*). The entire polypeptide chain is encoded by 13 exons. The upstream region of the gene has characteristics common to promoters of house keeping genes (*e.g.*, Sp1-binding sites, CCAAT-box, or TATA-less promoter and multiple initiation sites). These observations suggest that calnuc may have a constitutive expression and plays a pivotal role in the maintenance of cellular machinery. Not surprisingly, therefore, the protein is expressed in almost all tissues and cell types (*6*).

Calnuc was first identified as a soluble factor secreted by KML1-7 cells cloned from a lupus-prone MRL/l mouse. This factor was found to promote the formation of antibodies against ss- and ds-DNA *in vitro* and *in vivo*. Calnuc enhances propensity of MRL1 mice toward development of autoimmune diseases. Additionally, it also positively affected growth activity; owing to this, it was proposed that it (a 55-KDa protein termed nucleobindin) might act as a growth factor (*13*).

LOCALIZATION, DISTRIBUTION, AND EXPRESSION

Northern blot analysis confirmed the ubiquitous expression of calnuc in all human tissues (*14*). Newly formed bone seems to be the tissue where calnuc is expressed maximally. The expression of calnuc has also been reported in and around rat metaphyseal cells and calvarial (skull) bone: *in situ* hybridization and Northern blot analysis of the bone tissues confirmed that the protein was a product of bone cells (*15*). Electron microscopy and immunolabeling experiments showed expression of calnuc in the osteoid of newly formed bone. In case of bone trabeculae, most of it was present in nonmineralized osteoid. Expression levels of calnuc were low during cell proliferation and mineralization, but high during differentiation and matrix maturation. Yet another similar report established the occurrence of calnuc in odontoblasts and dentin extracellular matrix in rats. It was also observed in the cells of the surrounding developing alveolar bone, specifically in the nuclei, endoplasmic reticulum, and mitochondria. Hence, calnuc probably plays a role in modulating the maturation of bone matrix during the process of mineralization (*16*). Using immunohistochemistry, calnuc has been clearly shown to possess both intra- and extracellular localizations within bone. An extrapolation that can be made from these studies is the probability that calnuc may be helping the accumulation and transportation of Ca^{2+} ions for mineralization before hydroxyapatite deposition.

One of the earliest reports supporting the presence of nucleobindin in other organisms came from Heinegard and group (*17*). While studying the protein composition of mineralized bone matrix in an attempt to understand the process of bone formation and bone remodeling, these researchers discovered a 63-kDa protein that forms a minor part of the mineralized matrix of the bovine bone. The cDNA analysis of this protein showed the presence of an N-terminal signal sequence, two consensus EF-hand motifs, a bipartite-nuclear localization signal and the heptad repeat-leucine zippers. Comparative sequence analysis of this 63-kDa protein showed close similarity between mouse and human calnuc.

In cells, expression levels of calnuc are particularly high in the Golgi apparatus and the cytoplasm (*18*). Calnuc is found in both cytosolic and membrane fractions. Immunofluorescence studies showed calnuc to be localized to both the cytosolic fractions and Golgi membranes. Within the Golgi, calnuc is concentrated along the *cis*-golgi network and *cis*-golgi cisternae facing the Golgi lumen. A pI of 4.9 for calnuc also means that it is tightly bound to the Golgi membranes and cannot be easily released by alkaline treatment (*6*).

Calnuc is also secreted constitutively into the extracellular medium. Farquhar and her colleagues have shown that it is synthesized in the endoplasmic reticulum and then transported to the Golgi. In Golgi, it undergoes post-translational modifications such as O-glycosylation, sialylation, and sulfation, after which it is released into the culture medium (*14, 17*). The divergence in the localization of Golgi and cytoplasmic pools of calnuc lies in its peptide sequence. Although the N-terminal signal peptide sequence targets the protein to the ER, its subsequent transport to Golgi is mediated by the ER export signal (Pro28 or the proline in second position after the signal peptide cleavage site). This was proved by mutational studies wherein mutation of this specific proline residue abolished localization of calnuc to the Golgi apparatus (*19*). Similar to calnuc, NEFA or Nucb2 is also localized to the Golgi as evidenced by N-terminal deletion mutants (*20*). This stresses the fact that the signal for sorting to Golgi apparatus lies in the N-terminal region. The N-terminal signal sequence of calnuc2 is similar to the signal sequences of the conserved multiple domains of the mitochondrial carrier family (MCF) of proteins, whereas calnuc has signal sequence

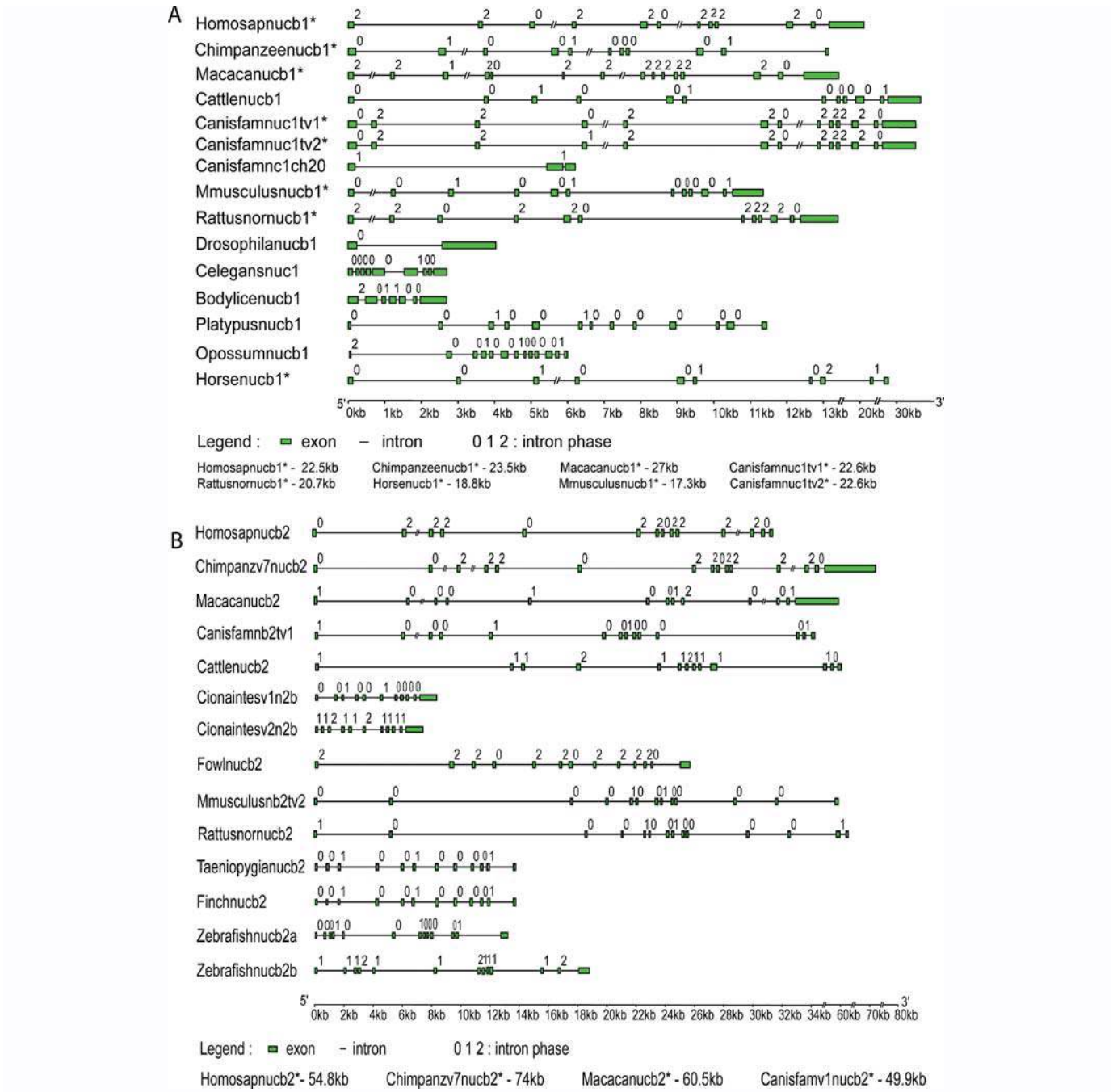

Figure 2. Gene strucutures of calnuc from various organisms. DNA sequences were retrieved form NCBI web site and used in fasta format. We were able to get 48 nonredundant sequences from the site. The sequences were uploaded on a gene structure drawing and displaying web server (www.gsds.cbi.pku.cn) that helps in marking introns, exons, and special regions (*11*). As is evident, the genes from various organisms are differently structured and exhibit multiple exons. The number of exons and the number of phase-2 exons increases with the increase in complexity of the organism, while few of the genes exhibit untranslated regions. An amazing feature is that despite such large variations in the gene structure the protein sequences and the various domains are very well conserved. Some of the longer intron regions have been cut short and their complete gene lengths indicated below. The levels of expression of calnuc and its correlation with the structure–function relationship of the genes should give great insight about the molecule. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

similar to those found in membrane and/or secretory proteins. Despite these differences in the signal sequence, the two proteins are trafficked to the Golgi apparatus largely due to the inherent hydrophobic nature of their signal sequences (*21*). Electron microscopy and immunohistochemical studies have revealed the localization of insect calnuc also to the *cis*-Golgi cisternae and *cis*-Golgi network, just as observed for human and mouse calnuc (*22*).

ISOFORMS OF CALNUC AND ITS MULTIPLE DOMAINS

Calnuc is one of the most abundant Golgi proteins, occurring in concentrations of ~3.8 μg calnuc/mg Golgi protein (*23*). Human calnuc is a 461 amino acid protein with a signal peptide at its N-terminus. It was first shown to exhibit almost 30% sequence homology to another Ca^{2+}-binding protein, calreticulin (*6*). It is a multidomain protein consisting of an N-terminal signal peptide sequence, a basic DNA-binding region, an acidic region between the two EF-hand motifs, and a leucine zipper domain toward the C-terminus.

A serendipitous observation from Hilschmann's group led to the discovery of a protein very similar in sequence to calnuc, a probable isoform (nucleobindin-2 or Nucb-2) (*24*). The protein was accidentally discovered while studying cDNA libraries of human acute lymphoblastic leukemia KM3 cell line in search of CALLA (common acute lymphoblastic leukemia antigen). In KM3 cells, calnuc2 is expressed in the plasma membrane, cytosol, and also secreted to the extracellular culture media, exhibiting a 55-KDa band on SDS gel electrophoresis. This protein was initially named NEFA (nucleic acid (DNA)-binding, EF-hand, and acidic amino acid region containing protein) because of its structural characteristics. In addition to these motifs/regions, NEFA has a probable nuclear localization signal, a basic amino acid region sandwiched between the two EF-hands, and a leucine zipper. Because of its extensive similarity to calnuc (61.56% sequence similarity), NEFA was alternately named as Nucb2 (*25*).

Phylogenetic analysis revealed that calnuc2 and calnuc have a common four-domain EF-hand ancestor, CTER (*25*). EF-hand motifs in proteins such as calmodulin and troponin C seem to have evolved from the CTER domains after undergoing a long process of genetic transformation, involving splicing, gene duplication, translocations, genetic fusions, and other similar methods of genetic evolution (*25*). Calnuc, with its various domains, seems to have also evolved in a similar fashion.

To understand the biochemical properties and functions of calnuc, Kanai et al., expressed the recombinant protein in *Escherichia coli* (*26*). The DNA-binding properties of calnuc were attributed to a combination of the "basic" DNA-binding region near the N-terminus and the leucine zipper region closer to the C-terminus. DNA-binding property of calnuc was lost when the leucine zipper or the basic amino-acid-rich region was deleted (*26*). Hence, it was concluded that the presence of the region rich in basic amino acids is required for specific binding to DNA, whereas the leucine zipper regions probably act as a dimerization motif, facilitating DNA-binding similar to transcription factors such as *fos* and *jun* (*27*). This observation generates the probability of any abnormalities or defects in calnuc folding and synthesis leading to induction of anti-DNA antibodies.

CA^{2+} BINDING STUDIES

The presence of two EF-hand-type calcium binding sites in calnuc was shown by Kanai's group based on the sequence comparison with other calcium binding proteins (*10*). To further investigate its Ca^{2+}-binding efficiency, two recombinant mutants, one with both the EF-hands deleted (Δa.a.157–221) and another with the region after second EF-hand deleted (Δa.a.294–455) were generated. These two mutants, along with human and mouse recombinant calnuc (rcalnuc), were used to determine and compare the Ca^{2+}-binding property of calnuc. As expected, experimental results showed that human and mouse rcalnuc and rcalnucΔ294–455 bound Ca^{2+} very efficiently, whereas the rcalnucΔ157–221 did not. Structural analysis by circular dichroism (CD) showed that Ca^{2+} ions increased the α-helical content of calnuc by almost 5%. In correspondence with Ca^{2+}-binding studies, the rcalnucΔ157–221 mutant did not exhibit any structural changes (*10*).

Further proof of Ca^{2+}-binding and a probable function of calnuc in mammalian cell lines came from $^{45}Ca^{2+}$ overlay studies in HeLa cell lines. HeLa cells overexpressing calnuc showed a 2.5-fold increase in Ca^{2+} storage when compared with normal cells that do not overexpress calnuc (*23*). The increased Ca^{2+} storage due to calnuc was further proved by treating the cells with thapsigargin (a SERCA pump inhibitor), causing a release of almost 70% of the stored Ca^{2+} from Golgi. Stimulation of these cells with the agonists, ATP or IP_3, also led to the release of Ca^{2+}. Immunofluorescence studies that show colocalization of IP_3 receptors with calnuc in the Golgi are reminiscent of a similar phenomenon on the ER, thus implying the participation of this complex in mobilizing the Golgi Ca^{2+} store (*23*).

Similar calcium-binding studies were done with rcalnuc2 also. Calnuc2 and its mutants with various deletions in the EF-hand region, expressed in *Pichia pastoris*, were used to determine the calcium-binding activity of this protein (*28*). Wild-type protein and mutant forms were secreted into the extracellular medium. Fluorescence titration experiments showed the presence of two different types of EF-hands in calnuc2, one with k_d of 0.08 μM and other with k_d of 0.2 μM. It was also observed that calnuc2 bound with Ca^{2+} at 2 mol Ca^{2+}/mol protein. CD studies showed an increase in α-helical content of calnuc2 by 13% upon addition of Ca^{2+}. Such conformational changes were also confirmed by differences in proteolytic digestion pattern of the Ca^{2+}-bound calnuc2 compared with the Ca^{2+}-free form (*28*).

Most recently, the NMR solution structure of the calcium-binding domain of human calnuc has been solved (*29*). Consistent with previous predictions, the NMR structure of both peptides shows the presence of EF-hand motifs. The orientation of the α-helices toward the N-terminus of the canonical EF-hand is in correspondence with EF-hand motifs as seen in most other calcium-binding proteins, although the orientation of the noncanonical EF-hand deviates from the average orientation. This calcium-binding EF-hand domain is unstructured in the absence of Ca^{2+} but folds properly upon addition of Ca^{2+}, and the helices have been observed to open up in presence of Ca^{2+} (*29*). The Ca^{2+} overlay experiments had predicted the first EF-hand of calnuc as a "high-affinity but low-capacity binding site" with an affinity constant of 6.6 μM. Our results have also shown that Ca^{2+} ions are able to compete off Stains-all from both the sites, whereas Mg^{2+}, on the other hand, is able to displace the dye only from one of the sites. Mg^{2+} ions, therefore, have a specific affinity to one of the sites, unlike Ca^{2+} ions, which did not differentiate between the two sites. Although ITC is unable to report Mg^{2+} binding, structural order, brought about by Mg^{2+}, is further enhanced by the binding of Ca^{2+} ions (*9*). The hypothesis that, in some proteins with EF-hand motifs, Mg^{2+} ions are involved in maintaining an intermediate structure that helps Ca^{2+} bind quickly to it is true in the case of calnuc also.

INTERACTING PARTNERS AND PROBABLE FUNCTIONS

Proteins with multiple domains have been shown to be central to several signal transduction pathways (*30*). Modular protein domains that mediate protein–protein interactions are critical elements of this process. With its several modular domains, it will not be surprising if calnuc is involved in new signal transduction pathways. Work on the molecular dissection and functional significance of the protein–protein interactions will eventually lead to an understanding of many of these pathways. These interacting partners will establish a specific functional and physiological role of calnuc (Fig. 3). The section below elaborates on many of the known interactions and possible physiological importance.

Signal Transduction

One of the first reports of an interacting partner for calnuc came from Paul Insel's group, who discovered that calnuc interacted with $G_{\alpha i}$ class of G-proteins. While trying to bait cellular proteins that interacted with $G_{\alpha i}$ using yeast two-hybrid system, the authors found that calnuc was one of the prey proteins (*31*). An *in vitro* binding assay also showed the interaction per se to be independent of the type of guanine nucleotide, while the colocalization and complex formation was confirmed by immunofluorescence (*18*). Transfection of COS cells with calnuc seemed to elevate levels of $G_{\alpha i}$ expression. Interaction of $G_{\alpha i}$ with calnuc also rendered mutual protection to these proteins from protease degradation. This opens up newer avenues to be explored with respect to the involvement of G-proteins in maintaining Ca^{2+} homeostasis in the Golgi (*6*).The possibility of calnuc interacting with other classes of G-proteins like, $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o1}$, $G_{\alpha z}$, and G_s was also observed, leading to the likelihood of cytosolic calnuc interacting with these different G-proteins (*6*).

Deletion mutants with both the EF-hands and the acidic region (Δ227–320), sandwiched between them, showed that this region is necessary for interaction with $G_{\alpha i3}$. This interaction is regulated by the divalent cations, Ca^{2+} and Mg^{2+}, being strongest when both the ions were present and was weakest when only Ca^{2+} was present (*31*). Inhibition of this interaction by the addition of 5-mM EDTA proved the importance of these two divalent ions in promoting the interaction between calnuc and $G_{\alpha i3}$. Mutational analysis involving truncation of $G_{\alpha i3}$ at the C-terminus, peptide competition strategy, and pull-down assays showed that α5 helix of the G-protein is involved in interaction with calnuc. We showed that the affinity of calnuc to $G_{\alpha i1}$ to be 13 nM by isothermal calorimetric experiments (*9*). Further biochemical evidence, using Stains-all and terbium chloride as probes, has shown that the Ca^{2+}-binding ability of calnuc is dependent on the activity-based conformational state of the G-protein. Interaction with GTP bound α-subunit of G protein leads to an increase in Ca^{2+} uptake by calnuc, thereby indicating its probable involvement in signaling phenomena (*9*).

Inflammation

Using yeast two-hybrid technique, cyclooxygenases have been shown as interaction partners of calnuc (*32*). Immunoprecipitation experiments showed that the interaction of COX-1 with calnuc was stronger than COX-2. It has been observed that the binding of calnuc to COX enzymes (anchored to luminal surface of ER) helps in its intracellular retention. Coexpression of calnuc with either COX-1 or COX-2 regulated the release of calnuc from cells (>80% being held back). This implies that COX and calnuc might be acting as regulators of each other's function, controlling the release of the partner. Immunofluorescence, confocal, and electron microscopy studies have colocalized calnuc and COX-2 to the Golgi and endoplasmic reticulum of human neutrophils (*33*). Addition of recombinant calnuc to COX-2 in a dose-dependant manner increased the synthesis of prostaglandin E2, thereby indicating a probable role for calnuc in inflammation.

Cancer and Apoptosis

One of the interesting interacting partners of calnuc2 is ARTS-1 (aminopeptidase regulator of TNFR-1 shedding type 1). Their interaction was found to be essential for releasing TNFR1 (tumor necrosis factor receptor type 1) to the extracellular matrix (*34*). Yeast two hybrid screening of human placental cDNA library and pull-down assays have shown that calnuc2 binds to the extracellular domain of ARTS-1 *via* its helix–loop–helix motif. Coimmunoprecipitation studies have shown that

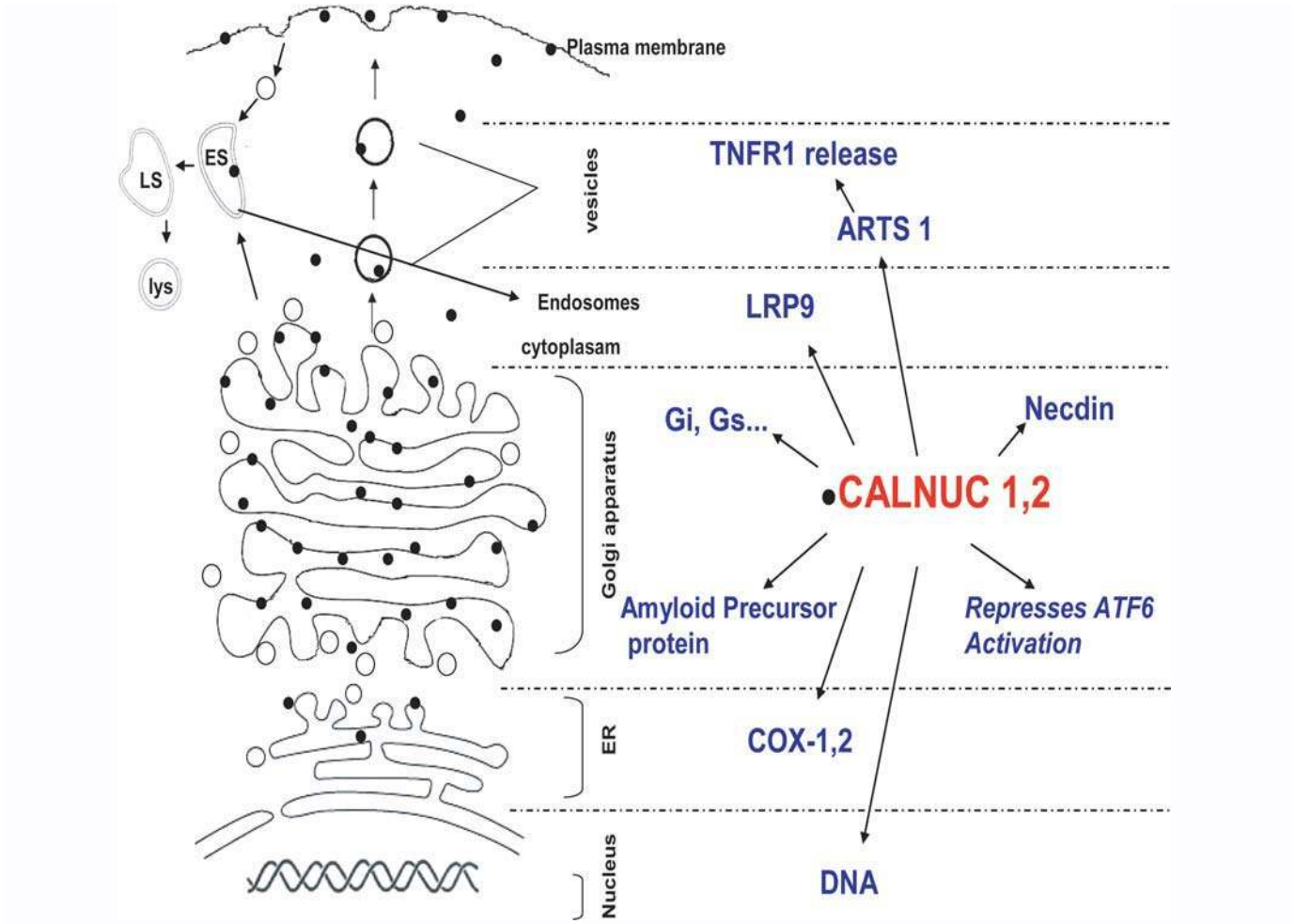

Figure 3. Distribution of calnuc and its interacting partners. Several studies have shown the localization of calnuc in different compartments of the cell. Calnuc has interacting partners in each of these compartments. The left pane of the figure shows the distribution of calnuc (represented by the symbol ●) in endoplasmic reticulum, Golgi apparatus, cytoplasm, vesicles, endosomes, and plasma membrane. The right pane of the figure shows the various interaction partners of calnuc in these different compartments of the cell. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

this interaction between endogenous ARTS-1 and calnuc2 is Ca^{2+}-dependent. Driven by the formation of this complex, calnuc2 associates with TNFR1 (before its commitment to other pathways) that result either in the inducible proteolytic cleavage of TNFR1 ectodomain or the consecutive release of TNFR1 exosome-like vesicles. RNAi knockout experiments for calnuc2 and ARTS-1 have proved the importance of these two molecules for releasing full-length TNFR-1.

While screening for cellular interaction partners of Necdin from cDNA libraries of neurally differentiated embryonal carcinoma P19 cells and adult rat brain, a postmitotic growth suppressor, calnuc2 gave a positive signal (*35*). The necdin binding domain in calnuc2 was determined by constructing various calnuc2 deletion mutants. Yeast two-hybrid analysis of these mutants showed that the two EF-hand motifs and the intervening acidic region (214–358) of calnuc2 are important for its interaction with necdin. Coexpression of necdin with calnuc2 blocks the secretion of calnuc2 in extra cellular space and enhances its retention in the lumen of the ER. As necdin binds to calnuc2 at a site homologous to the G protein binding site of calnuc, it could be playing an important role in the regulation of Ca^{2+} homeostasis under physiological and pathological conditions (*9*).

The search for putative downstream substrates for caspase activity has shown up Calnuc-1 and 2 as potential substrates (*36*). Calnuc is cleaved by caspases at one of the Ca^{2+}-binding EF-hand motifs. A putatively conserved caspase-cleavage site "DGLDP" has been found in both calnuc1 and calnuc2. Whereas calnuc-1 is a target for activity by caspases 6 and 8, calnuc2 acts as a substrate for caspases 3, 6, and 8. This caspase cleavage site is highly conserved among calnucs from different organisms such as bovine, rat, mouse, chicken, *Xenopus*,

zebrafish, and *Drosophila* (*36*). Regulation of Ca^{2+} homeostasis is probably regulated by caspase-mediated cleavage of calnuc in apoptotic conditions.

Alzheimers

Calnuc is an active interacting partner of amyloid precursor protein and binds to its C-terminal region in a Ca^{2+}-sensitive manner (*37*). Immunofluorescence studies have shown that both APP and calnuc are colocalized to the Golgi. Overexpression of calnuc in neuroblastoma cells led to a decrease in APP levels, whereas downregulation of calnuc by siRNA has shown to increase the levels of APP. Calnuc down regulates APP mRNA levels, which in turn inhibits APP biosynthesis, resulting in reduction of APP proteolytic metabolites. Also, levels of calnuc in the brains of patients with Alzheimer's disease are very low compared with age-matched non-Alzheimer's patients. This shows the extensive role calnuc might have in regulating the onset of Alzheimer's disease by modulating the levels of APP and plaque formation.

Stress Response

Under ER stress conditions, ATF6 (activating transcription factor 6), an ER membrane-bound transcription factor, is transported to the Golgi apparatus where it is subsequently cleaved by S1P (site-1-protease) to activate the unfolded protein response. Calnuc acts as a repressor of S1P-mediated ATF6 activation, and hence inhibits induction of the unfolded protein response. Further examination has shown that calnuc is an ER stress inducible gene. The promoter for this gene has functional *cis*-elements for transcriptional activation of ATF6. Overexpression of calnuc inhibits S1P-mediated cleavage of ATF6 whereas siRNA knockdown promotes ATF6 cleavage under ER stress conditions. Calnuc mutants, with their inability to localize to Golgi, were unable to inhibit ATF6 activation. Hence, calnuc acts as a negative feedback regulator of ATF6 activation by S1P in unfolded protein response (*38*).

Trafficking

Yeast two-hybrid and pull-down assays showed that the conserved N-terminal region of calnuc is essential for the interaction of arginine-rich region of the cytosolic tail of LRP9 (LDL receptor-related protein 9). Both calnuc and LRP9 colocalize at the surface of *trans*-Golgi network and early endosomes (*39*). Calnuc is essential for retrieving the LRP9 molecules from endosomes to the trans-Golgi network (TGN). siRNA mediated knock down of calnuc, missorted the LRP9 molecules in the late endosomal/lysosomal compartments, thus promoting their lysosomal degradation. This stresses the role played by calnuc in receptor trafficking.

Nucleic Acid Binding

Using gel-shift assay, Kanai's group was able to zero in on a 160-bp nucleosomal DNA fragment that appeared in the sera of mice injected with rcalnuc. Administration of rcalnuc and anti-CD3 monoclonal antibody seemed to accelerate the appearance of this nucleosomal DNA in the sera of mice. This was accompanied by DNA fragmentation in the thymus. However, it is important to note that the appearance of nucleosomal DNA is one of the characteristic features of apoptosis occurring in the thymus, and it might be interesting to study the cause/affect process in this case. It was found that clearance of this nucleosomal DNA from the sera was delayed considerably. This could explain the generation of autoantibodies against DNA in the presence of calnuc (*40, 41*).

Another study reports the possible interaction of calnuc2 as a tRNA-binding protein. This might prove to be an important step for screening proteins with Ca^{2+}-binding activities, which regulate protein translation levels (*42*).

CALNUC-RELATED DISEASES

With such diverse localizations and many interacting partners, we speculate calnuc to have a major role in maintaining the physiological balance of an organism. Thus, calnuc promises to be an important central molecule and any abnormality in the folding or functioning of this protein will expose us to a wide range of disease processes.

Calnuc was first identified as an autoimmune antigen in lupus prone mice. N-terminal amino acid sequencing confirmed the isolate as calnuc from the sera of lupus prone MRL/l mice that did not exist in the normal substrain MRL/n mice (*43*). Calnuc also efficiently induces other autoantibodies as well, like the anti-U1RNP (ribonuclease P) and rheumatoid factor of IgG class. Surprisingly, intraperitoneal injection of calnuc caused lupus-like symptoms even in normal mice. Detailed mechanistic studies and further analysis needs to be done to elucidate the role of calnuc in this disease and to show whether the secretion of calnuc (to the sera) alone is sufficient and capable of inducing lupus syndrome or that other factors are also involved in developing this condition (*44*).

Wang *et al*. studied the antigenic expression of calnuc in gastric adenocarcinomas with lymph-node metastasis by immunohistochemical reactivity studies in patients with and without this tumor (*45*). It was observed that calnuc was expressed in ~56% of patients with lymph-node metastatic tumor when compared with just 10% of patients with tumors not related to lymph node metastasis. Although further research is required to study the underlying biochemistry of calnuc in this disease, it might act as a probable marker for detecting tumors of the stomach associated with lymph-node metastasis. Exploring the possibilities of calnuc as a tumor-associated antigen that can induce autoantibodies in human cancers has shown that though autoantibodies to calnuc are found in only 4.7% of human cancers, an exorbitant 11.5% of colon cancers carried autoantibodies to calnuc, when compared with 1.2% in normal individuals (*46*). Immunohistochemical studies have shown that 59.4% of colon cancers overexpress calnuc when compared with normal

colon tissues. Strangely though, calnuc2 expression levels are decreased in gastric tumors, compared with the surrounding normal cells (*47*). This seems to present yet another avenue for evaluation of the prospects of calnuc as a potential marker in cancer detection.

Interestingly, expression levels of calnuc are upregulated in patients suffering from "non-Hodgkin's lymphoma" [other proteins associated with this disease are (i) interferon regulatory factor 4 and (ii) tissue inhibitor of metalloproteinase-1]. A comparative systems level study of the expression levels of these proteins would lead to interesting clues toward the pathophysiology of the disease (*48*). While mitogen stimulation increased the expression of calnuc mRNA in normal peripheral blood mononuclear cells, immunohistochemical staining of tumor specimens obtained from patients (for calnuc with anti-human calnuc monoclonal antibody) showed the presence of this protein in almost all the specimens. The degree of staining was directly proportional to the level of malignancy. Deeper insights into the biological role played by calnuc will shed more light into the biochemistry of non-Hodgkin's lymphoma and probably calnuc could work as a marker for the detection of the malignant stage of this disease.

Yet another role of calnuc has been linked to serotonin receptors. Calnuc2 has been shown to be a precursor for a novel satiety molecule, nesfatin. Indeed, nesfatin comprises of the first 82 residues of Calnuc2. It has also been linked to leptin and melanocortin signaling in the central nervous system in an independent manner (*49*). Administration of serotonin receptor agonist to mice leads to an increased expression of calnuc2 in the hypothalamus causing limited food intake. Consequently, hyperphagic and nonobese mice express lower levels of calnuc2. Also, treatment with an antisense probe of nesfatin resulted in weight gain of these animals. Hence, it can be effectively concluded that serotonin receptors upregulate expression of calnuc2 in the hypothalamus and initiate anorexia in mice *via* a leptin independent pathway (*49*). Calnuc2 and its N-terminal peptide, nesfatin, are expressed at normal levels in the hypothalamus and nuclei of the spinal cord. Nesfatin also crosses the blood–brain barrier, which means that a peripheral source of calnuc2 also exists (*50*). Studies to examine the distribution of nesfatin in the stomach have revealed their localization in the small endocrine cells of gastric mucosa although expression levels were much higher than in brain and heart. This exhibits the role played by calnuc2 in energy homeostasis since calnuc2 gene expression is regulated by the nutritional status of the organism (*50, 51*). Another study has shown that pronesfatin or calnuc2 colocalize with insulin in islets of pancreatic cells in mice and rats (*52*). Thus, pronesfatin and its derived peptides, such as nesfatin, probably play an important role in islet biology and glucose homeostasis. Calnuc2 is also phosphorylated in the gastric mucosa, as has been proved by Western blotting studies (*47*). Its expression is predominant in the cytoplasm and secretory granules of parietal cells of glands in the stomach. Hence, calnuc2 acts by establishing an agonist-releasable Ca^{2+} pool in the endoplasmic reticulum and the Golgi. It transfers its signals *via* G-proteins and mediates exocytosis of secretory granules.

WHAT LIES AHEAD?

Despite extensive, though sporadic, research efforts to study and characterize the functional and pathophysiological role of calnuc in such diverse conditions, many questions still loom large in the open. With every new interaction partner being added to the database of calnuc, there will be a multifold increase in functional range of calnuc. Interaction of calnuc with G-proteins opens up the area of signal transduction to be studied, including the concomitant search for molecules involved in the downstream signaling. Interaction of calnuc with many receptors, like the serotonin receptors, LDL receptors, and indirectly with TNF receptor is yet another area with immense scope to study signaling, including receptor sorting and trafficking. Its link with autoimmune disorders, such as systemic lupus erythematosus and Alzheimer's diseases is a subject of immense medical importance. Relating calnuc with cyclooxygenases and the subsequent prostanoid synthesis stresses the role played by calnuc in inflammation and pain. In the form of nesfatin precursor, calnuc plays an important role in regulating food intake and eating disorders. Being upregulated or downregulated in cancers, keeping in mind its interactions with caspases, creates outlets for a common domain of research, linking caspases, calnuc, cell death, and cancer. This emphasizes the need to look at calnuc as a marker in cancer biology and/or cell death.

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