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Ion-binding properties of Calnuc, Ca^{2+} versus Mg^{2+} – Calnuc adopts additional and unusual Ca^{2+}-binding sites upon interaction with G-protein

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Calnuc is a novel, highly modular, EF-hand containing, Ca^{2+}-binding, Golgi resident protein whose functions are not clear. Using amino acid sequences, we demonstrate that Calnuc is a highly conserved protein among various organisms, from *Ciona intestinalis* to humans. Maximum homology among all sequences is found in the region that binds to G-proteins. In humans, it is known to be expressed in a variety of tissues, and it interacts with several important protein partners. Among other proteins, Calnuc is known to interact with heterotrimeric G-proteins, specifically with the α-subunit. Herein, we report the structural implications of Ca^{2+} and Mg^{2+} binding, and illustrate that Calnuc functions as a downstream effector for G-protein α-subunit. Our results show that Ca^{2+} binds with an affinity of 7 μM and causes structural changes. Although Mg^{2+} binds to Calnuc with very weak affinity, the structural changes that it causes are further enhanced by Ca^{2+} binding. Furthermore, isothermal titration calorimetry results show that Calnuc and the G-protein bind with an affinity of 13 nM. We also predict a probable function for Calnuc, that of maintaining Ca^{2+} homeostasis in the cell. Using Stains-all and terbium as Ca^{2+} mimic probes, we demonstrate that the Ca^{2+}-binding ability of Calnuc is governed by the activity-based conformational state of the G-protein. We propose that Calnuc adopts structural sites similar to the ones seen in proteins such as annexins, c2 domains or chromogrannin A, and therefore binds more calcium ions upon binding to Giα. With the number of organelle-targeted G-protein-coupled receptors increasing, intracellular communication mediated by G-proteins could become a new paradigm. In this regard, we propose that Calnuc could be involved in the downstream signaling of G-proteins.

Calnuc is a novel Ca^{2+}-binding protein whose functions are not clearly known. It has multiple functional domains, including two EF-hand Ca^{2+}-binding sites, a DNA-binding site, a cyclooxygenase-binding site, and a leucine zipper region. Calnuc was originally discovered as a factor promoting the formation of antibodies associated with lupus [1,2]. Assigning a specific function to Calnuc has been difficult, because it is targeted to the Golgi apparatus, nucleus, cytoplasm, and extracellular region [3]. In humans, Calnuc is expressed in a wide variety of tissues, and has been shown to interact with DNA and proteins such as cyclooxygenase, necdin, and Alzheimer's β-amyloid precursor protein [4–9]. Lin *et al.* have demonstrated that Calnuc, one of

Abbreviations
ANS, 1-anilinonaphthalene-8-sulfonic acid; GPCR, G-protein-coupled receptor; ITC, isothermal titration calorimetry; MSA, multiple sequence alignment.

the two Golgi resident Ca^{2+}-binding proteins (the other being Cab45), is involved in the establishment and maintenance of the agonist-mobilizable Ca^{2+} storage pool in the Golgi apparatus [7]. Using a yeast two-hybrid system, it has also been recently demonstrated that Calnuc interacts with the α-subunit of heterotrimeric G-proteins. Further studies have shown that the interaction (monitored by using yellow and cyan fluorescent protein chimeras) is localized to the Golgi bodies. It has been shown that this interaction is specific to the α5 helical domain of Giα, and that the binding is Ca^{2+}/Mg^{2+}-dependent [10].

The Golgi apparatus is a genuine Ca^{2+} store, as has been reported in literature [11,12]. Ca^{2+} gradients across the Golgi membranes (the Ca^{2+} concentration in the Golgi apparatus is 0.3 mM) have also proven to be very important for its function, underlining the importance of the Ca^{2+}-binding proteins targeted to it [13–15]. The available literature indicates that, among all the organelles, the Golgi bodies seem to show an abundance of G-proteins, involved in their biogenesis, trafficking, membrane organization, and many other important functions [16–19]. G-proteins on the Golgi membranes also engage in a plethora of very specific protein–protein interactions, recognizing downstream effectors [20–22]. Understanding the origins of these specificities is central to elucidating the mechanism of new signal transduction pathways. The physiological implications of the presence of core signaling molecules on the Golgi membranes and the fact that it acts as a store for calcium ions is an emerging and interesting area for investigation. In view of these observations, interactions between the Ca^{2+}-binding protein Calnuc and signaling molecule G-proteins assume extreme importance.

The present study was aimed at elucidating the ion-binding properties of Calnuc and the physiological relevance of its interaction with G-proteins. Bioinformatic analysis has demonstrated that Calnuc is highly conserved in various organisms with high sequence homology, showing its potential functional importance. Furthermore, the region involved in G-protein interactions is more conserved in all the organisms than the other functional domains of the protein.

We compared the Ca^{2+}-binding and Mg^{2+}-binding properties of Calnuc, and elucidated the physiological relevance of its interaction with G-proteins, using a variety of spectroscopic techniques. Results from isothermal titration calorimetry (ITC) showed that Calnuc binds to Ca^{2+} with an affinity (K_d) of 7 μM, whereas binding of Mg^{2+} could not be detected. Both Ca^{2+} and Mg^{2+} caused structural changes in the protein, to varying extents. Studies on the protein–protein interaction between Calnuc and G-protein α-subunit using ITC showed the affinity constant (K_d) to be 13 nM. Our results further demonstrated that an interaction with GTP-bound G-protein mediates increased Ca^{2+} binding by Calnuc. We hypothesize that Calnuc adopts a structure similar to that of the unusual Ca^{2+}-binding sites seen in c2 domain/annexin-like domain/chromogrannin-like sites and/or that of a pseudo-EF-hand domain, resulting in the increased Ca^{2+} binding.

Results

Multiple sequence alignment of Calnuc

We were able to retrieve 46 Calnuc sequences from various organisms by querying the HOMOLOGENE database and ENSEMBL HUMAN GENE VIEW. Apart from these sequences, many incomplete sequences from other mammals, such as *Echinops*, *Erinaceous*, Feline, *Loxodonta*, *Monodelphis* and *Ornithorhynchus*, were also obtained but not included in the analysis. Multiple sequence alignment (MSA) of Calnuc from these organisms was performed to extrapolate the sequence similarity of the proteins to structural, functional and evolutionary similarity (Fig. 1). On the basis of the MSA of Calnuc in all organisms, a phylogram was constructed using CLUSTALW. A rooted tree was obtained, meaning that the operational taxonomic units (different organisms) have a common ancestor.

Thermodynamic analysis of Ca^{2+} binding to Calnuc by ITC

To measure energetic variables such as enthalpy change (ΔH) and entropy change (ΔS), along with the affinity of binding (association constant, K_a) of Ca^{2+} and Mg^{2+} to Calnuc, we performed ITC. From ITC experiments, it is evident that Ca^{2+} binding to Calnuc is exothermic, with significant heat change. Figure 2A shows the favorable enthalpy change upon binding for each injection as a function of the concentration of $CaCl_2$. Nonlinear least square fitting of the ITC data from the bottom trace of Fig. 2A fit best into a 'one set of sites' model. As shown in Table 1, the dissociation constant ($K_d = 1/K_a$) of the Ca^{2+} binding was 7 μM at 30 °C having one set of binding sites (1.04 ± 0.0118), suggesting that the macroscopic binding constants for both functional EF-hands are similar. Calnuc has two functional EF-hands, and previous equilibrium binding studies revealed that Calnuc binds Ca^{2+} in the micromolar range (6 μM) [10]. ITC can

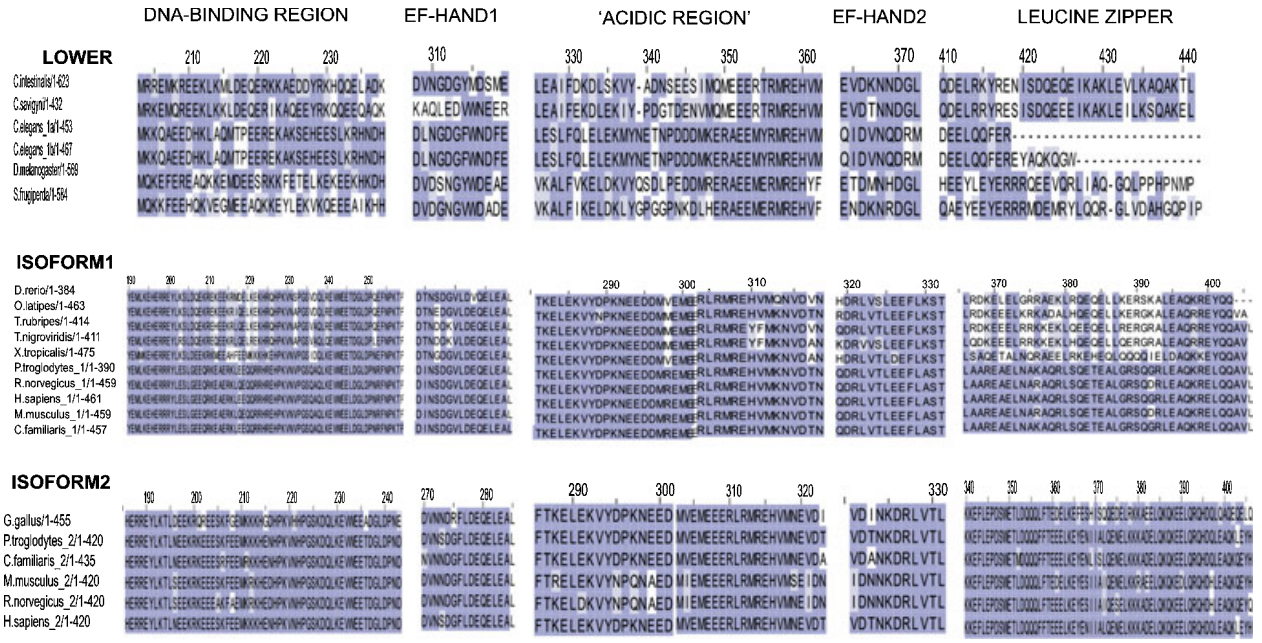

Fig. 1. On the basis of the MSA of Calnuc, the organisms can be conveniently grouped as nonmammals or lower organisms and mammals. Among mammals, two different isoforms of Calnuc are found in most, isoform 1 and isoform 2. Lower organisms include *Ciona*, *Caenorhabditis*, *Drosphila*, and *Spodoptera*. Isoform 1 includes Calnuc from *Macaca*, *Danio*, *Oryzias*, *Tigro*, and *Xenopus*, in addition to *Rattus*, *Mus*, *Pan*, *Canis*, and *Homo*. Isoform 2 includes Calnuc from *Rattus*, *Mus*, *Pan*, *Canis*, and *Homo*, in addition to Calnuc from *Gallus*. It is evident that the different domains in Calnuc are well conserved among the specific groups, which implies that Calnuc in these organisms has similar and conserved functions to perform. The phylogram showed the segregation and evolutionary pattern of Calnuc in different organisms. Although all of them seem to have a common ancestor, there seem to be different branching patterns based on the evolutionary status of the organism in the tree of life. The two isoforms in the higher organisms probably arose from a gene duplication process.

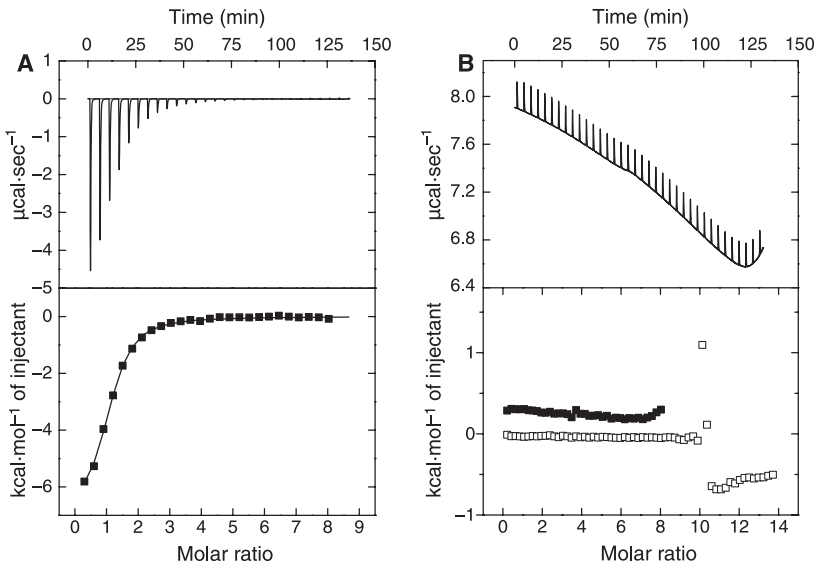

Fig. 2. ITC. Calorimetric titration of 3 µL aliquots of 7 mM $CaCl_2$ (A) or $MgCl_2$ (B) solution into 50 µM apo-Calnuc at 30 °C. All solutions were prepared in 20 mM Tris/HCl (pH 7.5) containing 50 mM NaCl. A plot of kcal·mol^{-1} of heat per injection of $CaCl_2$ or $MgCl_2$ at 30 °C as a function of molar ratio (metal/protein) is shown in the lower trace (heat differences obtained per injections). The best least-squares fit of the data to a one set of sites model is given by the solid line. An integrated curve with experimental points (■) and the best fit (—) are shown.

Table 1. Summary of macroscopic binding constants and thermodynamic parameters obtained from ITC for Ca^{2+} binding to Calnuc and Giα binding to Calnuc at 298 K. Data from the ITC thermograms were fitted using MICROCAL ORIGIN software. The data fit well for a one site model. *N* is the stoichiometry coefficient.

Interacting partners	*N*	K_a (M^{-1})	ΔH (kcal·mol^{-1})	ΔS (cal·mol^{-1})
Calnuc and calcium	1.04 ± 0.0118	$1.28 \times 10^5 \pm 6.96 \times 10^3$	−7027 ± 103.3	0.192
Calnuc and Giα	0.412 ± 0.00384	$7.45 \times 10^7 \pm 2.45 \times 10^7$	$2.058 \times 10^5 \pm 3367$	715

resolve dissociation constants of multiple sites on the basis of differences in their binding enthalpy (ΔH); however, these were similar, and data fitting in 'one set of sites' suggests that both EF-hands have similar binding affinities. We next examined Mg^{2+} binding to Calnuc by ITC; however, there was no heat change, as shown in Fig. 2B, suggesting that the binding of Mg^{2+} is weak and probably in the millimolar range, as such affinities cannot be detected by ITC.

Monitoring conformational (surface hydrophobicity) changes by 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence

ANS is a fluorescent probe that binds to hydrophobic sites on proteins and enables the monitoring of conformational changes in proteins upon their binding to Ca^{2+} and Mg^{2+} [23,24]. The spectrum of ANS alone in buffer exhibited a maximum at 530 nm. Upon binding to apo-Calnuc (metal ion-free), there was a shift in its peak maximum to 460 nm, accompanied by an increase in the emission intensity (Fig. 3). Mg^{2+} binding to a complex of ANS and apo-Calnuc caused a further 20% increase in ANS fluorescence intensity. Further addition of Ca^{2+} to a complex of Mg^{2+}-saturated Calnuc and ANS led to a marked increase ($\sim$ 30%) in fluorescence intensity, due to the binding of calcium ions to the protein. Hence, it is clear that Mg^{2+} binding led to the exposure of hydrophobic sites on Calnuc, and Ca^{2+} was able to further enhance the surface hydrophobicity of Mg^{2+}-bound protein. Structural changes in Calnuc that are a result of the binding of these metal ions could be extended to its function.

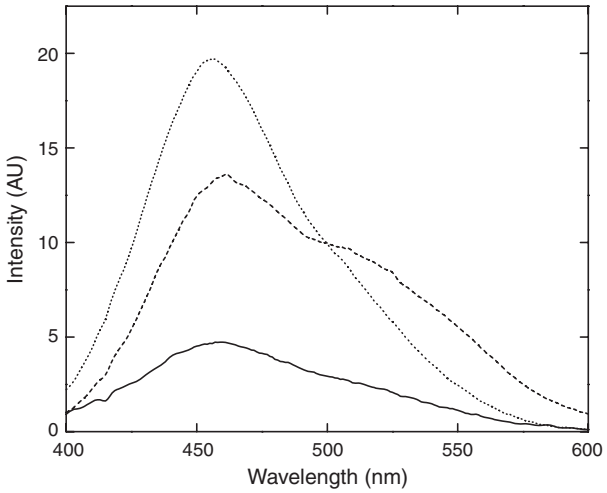

Fig. 3. Interaction of ANS with Calnuc and effects of metal ions. Ca^{2+} and Mg^{2+} change the exposed hydrophobicity pattern on the surface of Calnuc. In order to monitor the effect of different metal ions on the ANS–Calnuc complex, fluorescence spectra were recorded using an excitation wavelength of 365 nm. Solid line: complex of ANS (115 μM) and apo-Calnuc (99 nM). Dashed line: addition of Mg^{2+} to the dye–protein complex. Dotted line: addition of Ca^{2+} to the Mg^{2+}:dye:protein complex. The concentration of metal ions was 5 mM. All spectra were recorded in 20 mM Tris/HCl and 50 mM NaCl (pH 8.0). The spectra were recorded with 3 nm slits on the excitation and emission sides. The scan speed was maintained at 200 nm·min^{-1}. The results shown are representative spectra that were repeated several times. All experiments were performed at ambient room temperature (25 °C) in a final volume of 3 mL. Buffer blanks have been subtracted from these spectra.

Monitoring conformational changes by tryptophan fluorescence

The fluorescence signature from the intrinsic residues in Calnuc was monitored, in order to study the extent of conformational changes due to ion binding. Addition of Ca^{2+} or Mg^{2+} to apo-Calnuc led an increase in fluorescence intensity. Furthermore (as with the effect on ANS fluorescence), addition of Ca^{2+} to Calnuc saturated with Mg^{2+} led to an increase in its tryptophan fluorescence (from 25 to 45 U) (Fig. 4A). These observations were confirmed by monitoring changes in the tertiary structure of the protein upon ion binding, using CD spectroscopy. The near-UV CD spectrum of Calnuc has six peaks with maxima in the range 274–278 nm. The CD spectrum of Calnuc with Ca^{2+} and Mg^{2+} bound showed a similar signature to that of ion-free Calnuc, but with higher intensity maxima. Ca^{2+} and Mg^{2+} binding results in significant changes in aromatic side chain interactions in Calnuc (Fig. 4B). These data confirm that binding of calcium ions affects the structure adopted by the Mg^{2+}-bound protein.

Protein–protein interactions

We also studied the interaction of Calnuc with the α-subunit of GDP-bound G-protein in the presence of 2 mM Mg^{2+}, 2 mM Ca^{2+}, and 50 μM GDP (Fig. 5). Buffer–Calnuc titration was subtracted from G-protein–Calnuc titration isotherm data to take into account the heat of dilution. Binding isotherm data were used to calculate the lowest χ^2 value by calculating least squares, and it was taken as the best fit model, fitting was 'one set of sites'. The binding affinity (K_d) of Calnuc for G-protein was calculated to be 13 nM at 25 °C. Initial binding of Calnuc is endothermic, indicating that it is an entropically driven (ΔS is positive) binding event but not enthalpically favourable, as ΔH is positive.

Stains-all as a Ca^{2+} mimic probe

Next, we used Stains-all as a probe to study the local conformation of the EF-hand Ca^{2+}-binding sites of Calnuc. Free Stains-all, in 2 mM Mops (pH 7.2) and 30% ethylene glycol, gave an absorption spectrum showing the α-band at 575 nm and the β-band at 535 nm. Stains-all bound to Ca^{2+}-binding sites is known to generate the J-band (610–650 nm) and/or the γ-band (480–510 nm). Upon binding to Calnuc, the dye displayed prominent J-band and γ-band in both absorption and CD spectra (Fig. 6A,B). Furthermore, Ca^{2+} 'competes off' the dye (attenuating both the J-band and the γ-band), indicating that the dye binds in the EF-hand motif. Binding of Mg^{2+} caused a decrease only in the γ-band, without disturbing the J-band (Fig. 7A). Although the dye itself did not show any CD spectral signature, it showed a biphasic signature in both the J-band and the γ-band regions (Fig. 6B) on binding to the protein. CD results confirmed the absorbance data: Ca^{2+} binding is able to attenuate both the J-band and the γ-band, whereas Mg^{2+} binding affects only the γ-band (Fig. 7B).

Stains-all has been used previously to study protein–protein interactions between mellitin and calmodulin [25]. We used this assay to study the interaction between Calnuc and Giα, and report, for the first time, its physiological consequence. The concentrations of dye and the proteins used are as given in the figure legends. The data are representative, and the experiment was repeated several times. Addition of G-protein α-subunit to the Calnuc–Stains-all complex led to a change in intensity of the J-band (Fig. 8A). The signal intensity of the J-band signature is dependent on the nucleotide-bound state of the G-protein α-subunit. In the GDP-bound form, the G-protein α-subunit caused

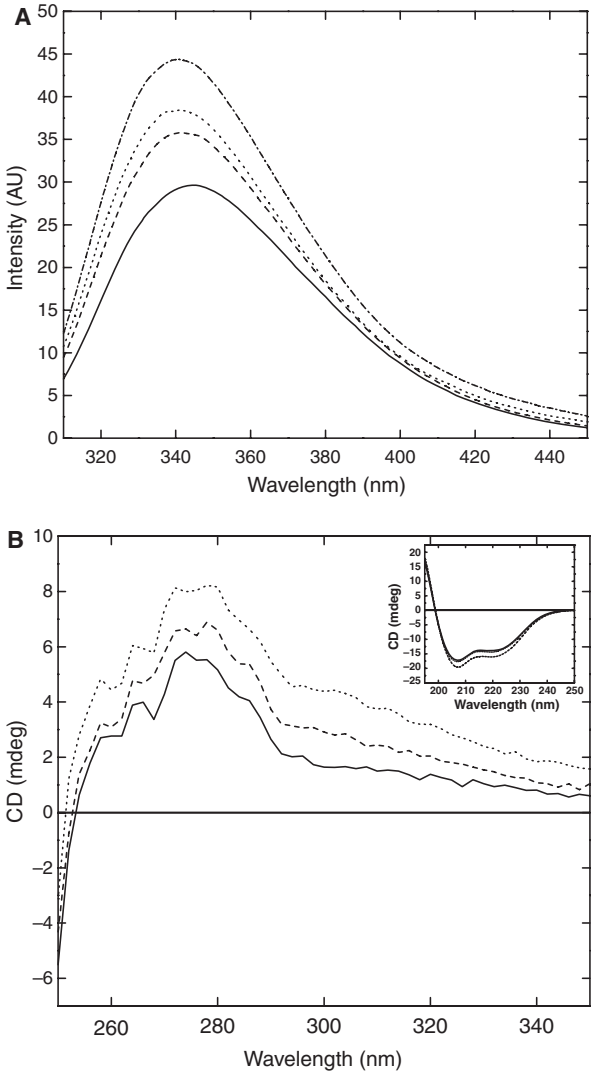

Fig. 4. Effect of different metal ions on the structure of Calnuc. Ca^{2+} and Mg^{2+} affect the structure of Calnuc differently. Tryptophan fluorescence spectra from Calnuc were recorded by exciting the protein with 295 nm light. (A) A fresh protein sample was used for addition of metal ion in order to study its effect on the structure of the protein. Solid line: apo-Calnuc (0.04 μM). Dashed line: Calnuc with 500 μM Mg^{2+}. Dotted line: Calnuc with 500 μM Mg^{2+} and 100 μM Ca^{2+}. Dash–dot–dash line: Calnuc with 500 μM Mg^{2+} and 500 μM Ca^{2+}. All spectra were recorded in 20 mM Tris buffer containing 50 mM NaCl (pH 7.5). Spectra are representative, and the experiments were repeated several times. The excitation light was stopped by a shutter in between spectra in order to minimize photobleaching. All spectra were recorded at 25 °C. (B) Changes in the tertiary structure of the protein upon ion binding as determined using CD. The near-UV CD spectrum of Calnuc has six peaks; the solid line shows the spectra corresponding to apo-Calnuc (39 μM), and the dotted and dashed lines represent the changes upon addition of Ca^{2+} (500 μM) and Mg^{2+} (2 mM), respectively. The inset shows changes in the secondary structure of Calnuc caused by the addition of Ca^{2+}, and that Mg^{2+} does not perturb the secondary structure of the protein.

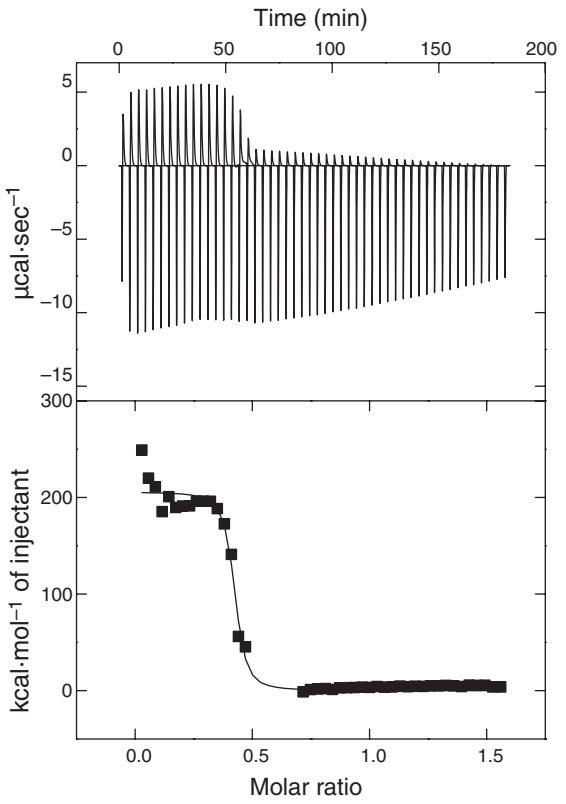

Fig. 5. Titration of Gi with Calnuc. Calorimetric titration of 3 μL aliquots of 10 μM Calnuc solution into 80 μM Giα at 30 °C. All solutions were prepared in 20 mM Tris/HCl (pH 7.5) containing 50 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 50 μM GDP. A plot of kcal·mol^{-1} of heat absorbed/released per injection of Calnuc as a function of the Calnuc/Gi ratio is also shown. The best least-squares fit of the data to a one site model is shown by the solid line.

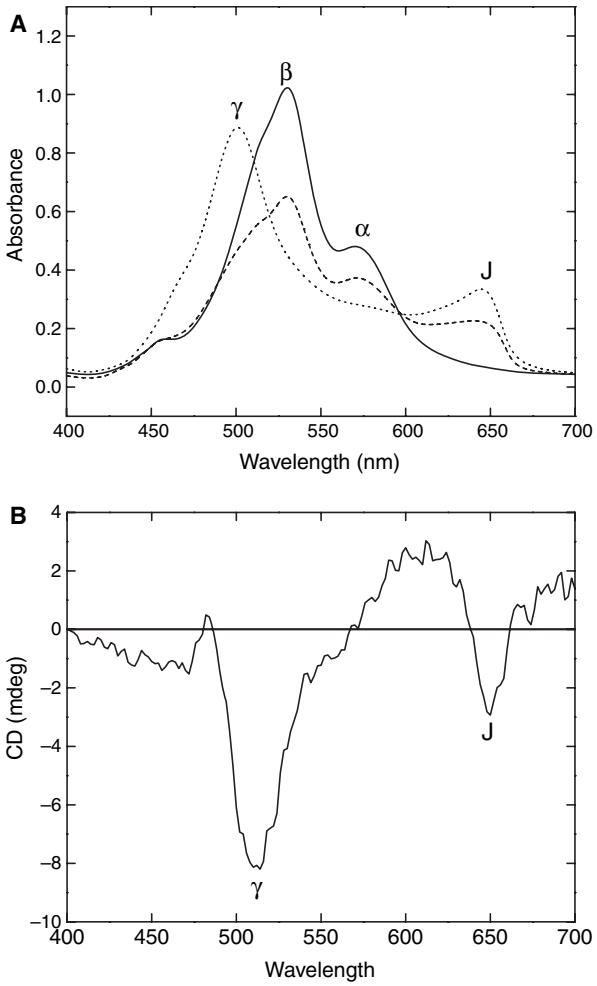

Fig. 6. Stains-all as a Ca^{2+} mimic probe. (A) Absorption spectra of free Stains-all and the dye complexed to Calnuc. The solid line represents the spectra of dye only (1.45×10^{-4} M) in 2 mM Mops buffer (pH 7.2) containing 30% ethylene glycol. The dotted line and dash–dot–dash line represent the dye–Calnuc complex (Calnuc concentration 280 μM and 844 μM, respectively). Increasing amounts of the protein elicit the J-band (at ~ 650 nm) and γ-band (~ 500 nm) as a result of the dye binding to the Ca^{2+}-binding sites. The spectra of dye alone and dye–protein complex were recorded at a scan speed of 1920 nm·min^{-1} with 2 nm slit widths. All spectra were recorded at 25 °C. The use of 30% ethylene glycol helps to prevent the time-dependent self-aggregation of Stains-all in aqueous solution, a complication that would have interfered with the interpretation of spectral changes. Also, any complications that might arise from photobleaching of the dye were avoided by working, as far as possible, in the dark or in very low levels of light. (B) CD spectra of the dye–Calnuc complex (Calnuc concentration is 1.76 μM). The J-band and γ-band can be seen.

a small drop in the J-band intensity, whereas the GTP-bound form enhanced the intensity of the J-band. Confirmation of this phenomenon was provided by the CD spectral data; no change of the CD signal was observed upon binding with GDP-bound G-protein, whereas an increase in the J-band intensity was elicited upon interaction of Calnuc and GTP-bound G-protein (Fig. 8B). Interestingly, why G-protein binding did not affect the γ-band in CD, is still not known.

Terbium binding is enhanced by GTP-bound α-subunit

We used the lanthanide ion, terbium, in order to study the interaction of G-protein with Calnuc. Terbium is generally used as a Ca^{2+} mimic, because of its size and the fact that it binds in the Ca^{2+}-binding EF-hand domain [26]. Fluorescence resonance energy transfer from a nearby tryptophan to the lanthanide ion leads to its showing fluorescence emission in the visible region (λ_{ex} = 295 nm; λ_{em} = 400–560 nm). Addition of Calnuc (4.7 mM protein to 9 μM Tb^{3+}) elicited fluorescence from the bound terbium ion in a dose-

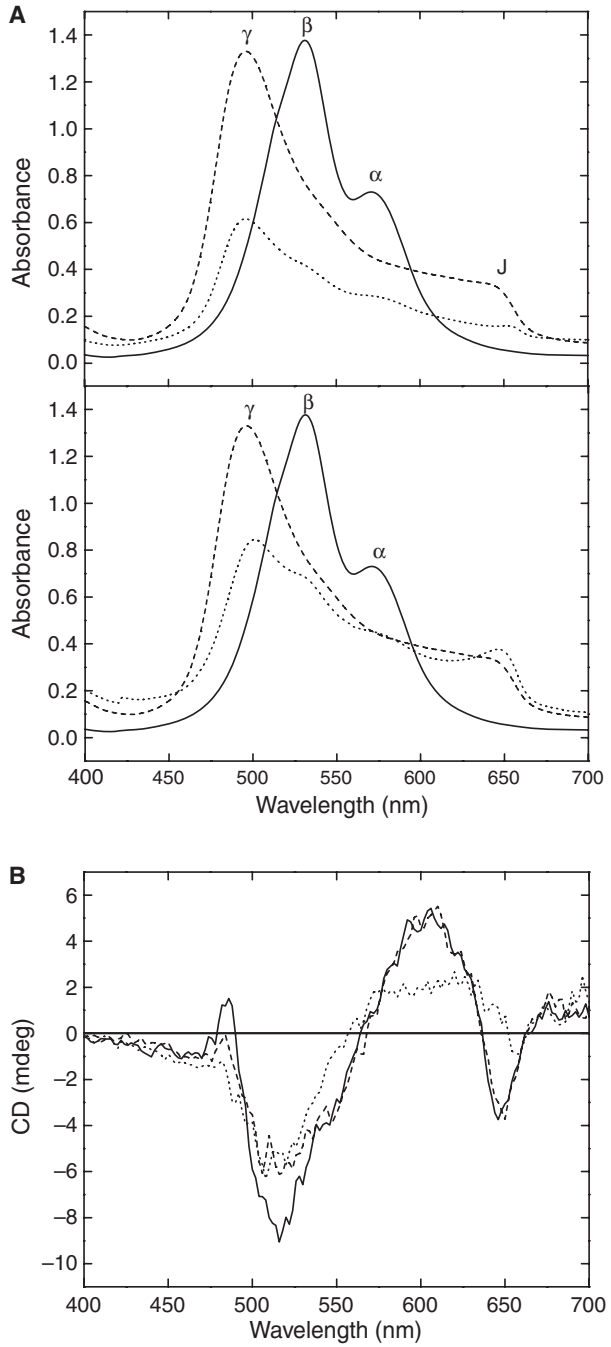

Fig. 7. Effect of metal ions on Stains-all binding to Calnuc. Absorption spectra of the dye–Calnuc complex upon ion binding. In both panels, the solid line represents the free dye (concentration 2.46×10^{-4} M) in 2 mM Mops buffer (pH 7.2) containing 30% ethylene glycol, the dashed line represents the dye–Calnuc complex (Calnuc concentration 110 μM), and the dotted line represents the dye–Calnuc complex in the presence of different ions. Whereas Ca^{2+} binds to both of the sites, Mg^{2+} seems to be able to bind to only one of the sites. (A) The top panel shows spectral changes induced by the addition of Ca^{2+} (100 μM); the bottom panel shows spectral changes induced by the addition of Mg^{2+} (50 μM). All experiments were performed at 25 °C. The data shown are representative of experiments performed several times. All complexes of stains with the protein or protein and metal ions were incubated for 45 min before recording of the spectra. (B) Stains-all CD spectra; the solid line represents the dye–Calnuc complex (Calnuc concentration 1.76 μM), and the dotted and dashed lines represent the dye–Calnuc complex with Ca^{2+} (1 mM) and Mg^{2+} (2 mM), respectively.

dependent manner ($\lambda_{em,max}$ at 490 and 545 nm), demonstrating its ability to bind to Calnuc (Fig. 9A). Further addition of G-protein (600 μM) to a Calnuc–terbium complex led to changes depending on whether the G-protein had a GDP or a GTP bound to it. GDP-bound α-subunit led to a drop in the emission intensities of the two resonance energy transfer peaks, whereas addition of GTP-bound α-subunit increased the emission intensities of these two peaks (Fig. 9B).

Discussion

A common factor in the etiology of several human diseases is the malfunctioning of the Golgi apparatus as a Ca^{2+} store [27,28]. In response to agonist stimulation, the Golgi apparatus increases the cytosolic Ca^{2+} levels, as does the endoplasmic reticulum [29]. Ca^{2+} released from the Golgi apparatus can also modulate the duration and pattern of cytosolic Ca^{2+} signals [30]. Normal intracellular Ca^{2+} signals are affected by disruption of the Golgi apparatus [30]. Several molecules with important functions, and normally associated with signal transduction pathways, have been shown to translocate to the Golgi apparatus in response to elevated cytosolic calcium levels, e.g. hippocalcin, neurocalcin, phospholipase C, phospholipase A2, GTPase KRas and Ras guanine nucleotide exchange factor, and RasGRP1 [31–35].

The Golgi apparatus has three Ca^{2+}-binding proteins – Calnuc [36], Cab45 [37], and P54/NEFA [38] – which play an important role in buffering the Ca^{2+}, and a Ca^{2+} pump (secretory pathway Ca^{2+}-ATPases). Investigations on the function of one of these proteins within the Golgi lumen, Calnuc, are underway. It was shown that overexpression of secretory pathway Ca^{2+}-ATPases in mammalian cell lines increased Calnuc levels [39]. On the other hand, it was also observed that overexpression of Calnuc led to enhancement of agonist-evoked Ca^{2+} release [36]. It is therefore very important that the physiological functions of these Ca^{2+}-binding proteins be understood. In this work, we elucidated the functional significance of the interaction between Calnuc and G-proteins.

Calnuc is one of the two (the other being the photoreceptor centrin) known Ca^{2+}-binding proteins that

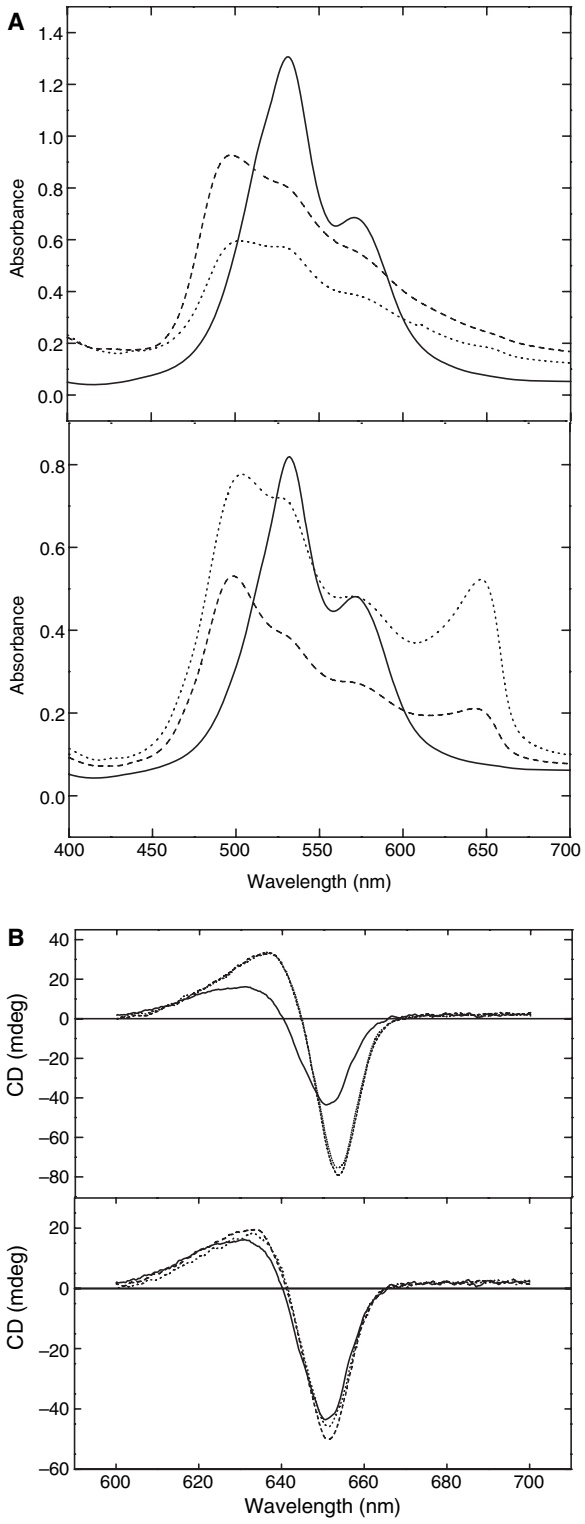

Fig. 8. G-protein modulates the Ca^{2+}-binding ability of Calnuc. The dye Stains-all was used as a Ca^{2+} mimic to monitor the function of the interaction between Calnuc and G-protein. (A) Top panel: absorption spectra of the dye–Calnuc complex when treated with GDP-bound Giα. The solid line represents dye only (2.46×10^{-4} M); the dashed line represents the dye–Calnuc (110 μM) complex; and the dotted line represents the addition of GDP-bound Giα (600 μM) to the dye–Calnuc complex. The bottom panel shows the absorption spectra of the dye–Calnuc complex when treated with GTP-bound Giα. The solid line represents dye only (concentration 2.46×10^{-4} M); the dashed line represents the dye–Calnuc (520 μM) complex; and the dotted line represents the addition of GTP-bound Giα (600 μM) to the dye–Calnuc complex. (B) Stains–all CD spectra. The solid line represents the dye–Calnuc (0.07 μM) complex, and, in both the top and bottom panels, the dotted lines represent the dye–Calnuc complex with GTP-bound Giα (0.74 μM) and GDP-bound Giα, respectively. The two dotted lines represent two different experiments performed under identical conditions. For the figures shown in both of the panels, the dye was dissolved in 30% ethylene glycol, in 2 mM Mops buffer. Absorption spectra were recorded at a scan speed of 1920 nm·min^{-1} with 2 nm slit widths. All spectra were recorded at 25 °C. Experiments were performed in dark or dim light conditions. All samples were incubated for 45 min before recording of the spectra.

have been reported to interact with the heterotrimeric G-proteins (specifically Giα). The binding site on Giα for Calnuc was mapped to the C-terminal region by yeast dihybrid analysis and by using a peptide competition assay [40]. The modular structure of Calnuc (with separate protein-binding and DNA-binding motifs) helps in its interaction with many other important biological molecules, i.e. cyclooxygenase, necdin, and Alzheimer's β-amyloid precursor protein [3–8]. Although the site of interaction on Giα is known, the physiological consequence of the interaction is not known. The functional and structural significance of metal ion binding to the two EF-hand sites on the protein is also not well understood.

In this work, we used various lines of argument to demonstrate that Calnuc acts as an effector molecule for G-proteins and plays an important role in Ca^{2+} homeostasis in the cell. We provide evidence to establish novel properties of Calnuc that include its structure–function relationship and its possible role in signal transduction pathways as a downstream effector of G-proteins. Sequence alignment of Calnuc from various organisms revealed Calnuc to be a highly conserved protein across species, from *Ciona intestinalis* to *Homo sapiens*, thus reflecting its conserved structure–function relationship. The conserved pattern of specific motifs implies that this protein probably has the same functions in all organisms, namely, Ca^{2+} binding and DNA binding, which are probably aided by the leucine zipper region involved in dimerization of the protein. Five different blocks were observed in these sequences, which revealed a high degree of conservation of specific amino acids in important domains such as the basic DNA-binding region, EF-hands,

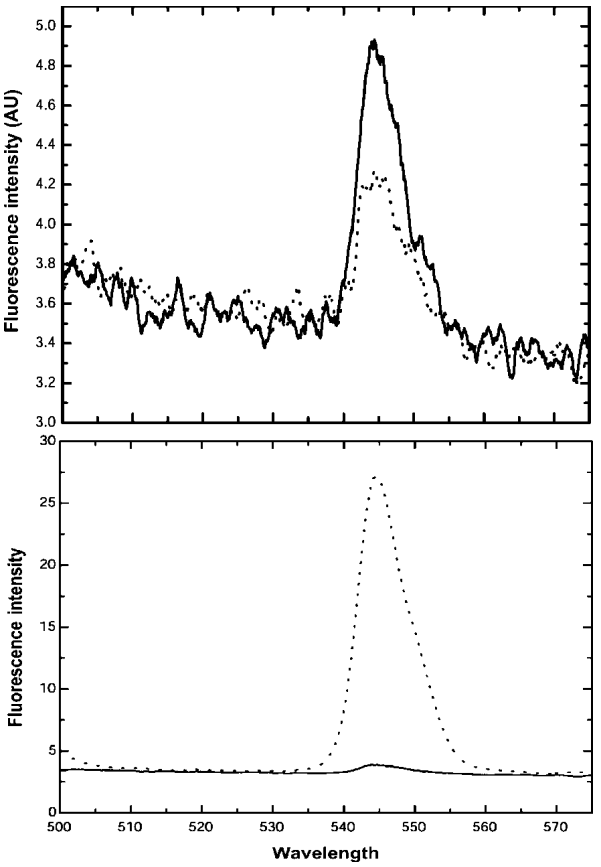

Fig. 9. Use of terbium as a Ca^{2+} mimic probe. The changes in the fluorescence intensity at 545 nm with increase in the concentration of terbium (1–9 μM) added to Calnuc. The top panel shows the fluorescence spectra of terbium-bound Calnuc (4.7 mM) when treated with GDP-bound Giα (600 μM). The solid line represents the fluorescence spectra of terbium-bound Calnuc, and the dotted line represents the fluorescence of terbium-bound Calnuc upon binding with GDP-bound Giα, showing a decrease in the intensity at 545 nm. The bottom panel shows the fluorescence spectra of terbium-bound Calnuc (4.7 mM) when treated with GTP-bound Giα (600 μM). The solid line represents the fluorescence spectra of terbium-bound Calnuc, and the dotted line represents the fluorescence of terbium-bound Calnuc upon binding with GTP-bound Giα, showing a five-fold increase in the intensity at 545 nm. The protein sample was in 20 mM Tris buffer excited at 295 nm, and the emission was recorded at 1 and 3 nm for excitation and emission slit widths, respectively. The data are representative of experiments performed several times. All recordings were made at 25 °C.

and the leucine zipper region. This high degree of conservation in these motifs across all species suggests the possibility of conserved functions for this protein in all these organisms, without any species differentiation.

The most conserved region among all the organisms is the G-protein interaction site, indicating the importance of this region and pointing to a role for the protein in signal transduction. Using MSA of Calnuc, a clear-cut differentiation can be drawn between lower organisms (*C. intestinalis*, *Ciona savignyi*, *Caenorhabditis elegans*, *Spodoptera frugiperda*, and *Drosophila melanogaster*) and higher organisms (mammals). Moreover, mammals themselves form two subgroups, based on the two isoforms of Calnuc present in them. A phylogram, obtained from all the sequences, clusters lower organisms and mammals as separate groups. The tree consists of two clades, representing clustering of the organisms containing this protein. *Ciona*, *Anopheles*, *Drosophila*, *Spodoptera* and *Caenorhabditis* seem to have evolved from a common ancestor, and the isoforms 1 of Calnuc in mammals, along with *Macaca*, *Oryzias*, *Danio*, *Tetraodon*, *Takifugu* and *Xenopus* share a common ancestor. Gene duplication processes in higher organisms probably led to the expression of two isoforms of Calnuc. Isoform 2 of Calnuc in mammals evolved as a separate group, along with Calnuc from *Gallus*. The phylogram thus shows a clear, distinct and early evolutionary pattern of isoform 1 of Calnuc in mammals. MSA of Calnuc from different organisms reveals that Calnuc is an important evolutionarily conserved protein.

Alignment of EF-hand motifs from all organisms revealed substitution of the conserved Gly and the hydrophobic residues that are necessary (in the loop region) to bind Ca^{2+} (Table S1). In EF-hand 1, the Gly at position six (inside the domain) has been replaced by Asp/Lys/Asn in *C. savignyi*, *Takifugu rubripes*, *Tetraodon nigroviridis*, and *Anopheles gambiae*, probably attenuating the Ca^{2+} affinity. The position of the hydrophobic residue is shared by the conserved Leu/Trp residues in all organisms (*C. intestinalis* being an exception, having a Met or Arg). In EF-hand 2, Gly is replaced by Arg, except in *Ciona*, *Aedes aegypti*, *D. melanogaster* and *S. frugiperda*, whereas the hydrophobic residue is Val/Ile in all organisms. Extrapolating the Ca^{2+}-binding efficiencies of EF-hands in human Calnuc from the literature to the EF-hands in other organisms, it can be said that the Ca^{2+}-binding efficiencies of the two EF-hands vary in Calnuc from organism to organism [40] (also see Table S1 for comparison of the Calnuc EF-hand sequence with the consensus sequence). The region between the two EF-hands, which is known to bind Giα, seems to be highly conserved in higher organisms (Fig. 1). Secondary structure analysis of this region (TKELEKVYDPKNEEDDMREMEERLRMREHVMKNDTN) has shown it to be largely unordered in nature. Upon interaction with Giα, this region probably adopts a more defined structure, and transmits structural changes through the entire protein backbone [41].

Although de Alba and Tjandra have reported the affinities (K_d of 47 and 40 μM) of Ca^{2+} for peptides comprising the EF-hands of Calnuc [42], there are no reports of the affinity of the metal ion for the protein as a whole. We show that Ca^{2+} binds to both sites with equal affinity. Ca^{2+} elucidates good isotherms (in ITC) and binds to Calnuc with an affinity of 7 μM (the data fit best to a single site model) (Table 1). Mg^{2+}, on the other hand, did not show any isotherms, making it impossible to detect affinities. Although Mg^{2+} does not show binding affinities in ITC it causes structural changes in Calnuc. To advance our understanding of this phenomenon, we have determined the effect of Ca^{2+} and Mg^{2+} on the structure of Calnuc with various techniques.

ANS is a hydrophobic fluorescence probe that binds to protein surfaces and indicates conformational changes. The extent of the structural changes that Mg^{2+} and Ca^{2+} induce in Calnuc is evident from observation of the changes in fluorescence of ANS bound to the protein. Although the qualitative changes brought about by the two ions are similar (a rise in intensity), Ca^{2+} causes changes in the surface hydrophobicity of Calnuc that are twice as great as those caused by Mg^{2+} (Fig. 3). These results indicate that, although Mg^{2+} binds Calnuc with very weak affinity, it causes changes in exposed hydrophobic surfaces, leading to structural changes as well.

Intrinsic tryptophan fluorescence is commonly exploited to study local structural changes occurring in proteins [43]. Calnuc has two tryptophans, one of them near EF-hand 1 (amino acid 200) and the other at position 300. We have used the intrinsic fluorescence properties of these two tryptophans to study conformational alterations occurring in Calnuc upon metal ion binding. Ca^{2+} and Mg^{2+} binding lead to an increase in the fluorescence intensity of the tryptophans in an ion-dependent fashion. Ca^{2+} binding leads to a two-fold increase in tryptophan fluorescence as compared to Mg^{2+}, without any significant shift in $\lambda_{max,em}$. Such changes in fluorescence emission spectral intensities have typically been attributed to conformational changes in the protein molecule. These changes not only confirm the ANS results, but are also supported by tertiary structural changes observed in the near-UV CD spectra. Whereas Ca^{2+} is known to cause an increase in total helical content in the protein as a whole [44], Mg^{2+} does not show the same effect. Mg^{2+} binding to EF-hands has been shown to be physiologically important, and several roles have been proposed, including preventing the overall protein structure from falling apart [45]. Mg^{2+}, being a potent competitor for the EF-hand ion-binding sites, also frequently plays a role in modulating the affinity of EF-hands for Ca^{2+} [46]. Changes observed in the tertiary structure upon binding to Mg^{2+} are half as great as those seen with Ca^{2+}. The data shown in Fig. 4B indicate that, upon ion binding the aromatic side chains show a high level of packing. These results reiterate the physiological function of both Ca^{2+} and Mg^{2+} binding to EF-hand protein, and emphasize that Ca^{2+} binding leads to further stabilization of the Mg^{2+}-bound structure. We propose that at least one of the ion-binding sites of Calnuc is of the mixed Ca^{2+}/Mg^{2+}-binding type.

Stains-all has been shown to be a very effective probe with which to differentiate between kinds of Ca^{2+}-binding proteins [25,47,48]. Caday and Steiner reported a change in the absorption spectral pattern of Stains-all upon binding to Ca^{2+}-binding proteins, and that it could be displaced by addition of Ca^{2+} [47]. The emergence of two peaks in the spectrum of Stains-all bound to Calnuc is an indication that, structurally, two distinct types of EF-hand conformations are present in Calnuc. One of the EF-hands may be present in the globular or compact region of the protein (J-band), whereas the other EF-hand may be in the exposed helical region (γ-band) [49]. Ca^{2+}, because of its higher affinity, displaces the dye from the EF-hands (as shown by the disappearance of both the γ-band and the J-band). Magnesium ions on the other hand, behave differently, and seem to have specific affinity for only one of the binding sites. Mg^{2+} binding reduces the intensities of only the γ-band, and does not cause any change to the J-band.

A comparison of the spectral band pattern properties of Stains-all upon binding to Calnuc with those when it is bound to other classic Ca^{2+}-binding proteins gives further insights into the functional properties of Calnuc. At high dye/protein molar ratios, calmodulin, troponin C and parvalbumin seem to complex with the dye similarly, and yield the J-band [25]. As the dye/protein ratio is decreased, the J-band is lost in the former two proteins, yielding the β-band and the γ-band, respectively; with parvalbumin, however, the J-band is retained at all stoichiometries. Also, whereas the J-band is replaced by the β-band and the γ-band upon the addition of Ca^{2+} to the dye complexes of calmodulin and troponin C, with parvalbumin, the J-band is simply lost and the bound dye is released. In the case of crystallins (eye lens proteins), it has been shown that, whereas β-crystallin generates only the J-band, δ-crystallin elicits only the γ-band. These single bands in both proteins can be titrated off by the addition of Ca^{2+} [49]. It is obvious that

β-crystallin and δ-crystallin behave similarly (show only one band), whereas calmodulin and troponin behave like each other. On the other hand, Calnuc and parvalbumin generate both the J-band and the γ-band, and show similar features. The functions of βγ-crystallin and δ-crystallin are largely to act as Ca^{2+} buffers in the eye lens [50,51]. Calmodulin and troponin have been assigned as Ca^{2+} sensors, and are involved in transducing signals upon binding to Ca^{2+}. On the other hand, parvalbumin has been designated as a Ca^{2+} buffer protein [52]. Calnuc seems to be able to generate signals from the dye that both the 'Ca^{2+} sensors' and the 'Ca^{2+} buffers' elicit. From the various features of the dye bound to Ca^{2+}-binding sites in different proteins, we propose that Calnuc acts both as a Ca^{2+} buffer and as a Ca^{2+} sensor, is involved in downstream signal transduction pathways, and hence interacts with G-proteins.

Results from ITC experiments are probably the ultimate proof of interactions between two proteins in solution, and provide a direct route to the complete thermodynamic characterization of protein interactions. Protein–protein interactions are distributed over a large surface area comprising many smaller local interactions. Advances in our understanding of these processes will generate insights into domain interactions. Calnuc and G-protein α-subunit interact with an affinity of 13 nM. The affinity between these two proteins is in tune with those shown between many other interacting protein partners [53].

Although the sites of interaction of Calnuc on $G_{i\alpha}$ are known, the lacuna in the present understanding is the physiological role of this interaction. Does Calnuc regulate the activity of G-proteins, or does the G-protein binding lead to release/uptake of Ca^{2+} by Calnuc? To help reach our goal of understanding the physiological role of the interaction between Calnuc and G-protein, we present here the use of Stains-all as a probe to study protein–protein interactions. It has already been established that Calnuc binds to G-protein α-subunit in both GDP-bound and GTP-bound forms [54]. Addition of G-protein α-subunit to a complex of dye and Calnuc elicits responses that are dependent on whether the G-protein is in the GDP-bound (off state) or GTP-bound (on state) form. Interaction with GDP-bound G-protein leads to a drop in the J-band, indicating that such an interaction leads to the release of Ca^{2+} by Calnuc. GTP-bound G-protein, on the other hand, causes a huge increase of the J-band, showing that interaction with an activated G-protein leads to an uptake of Ca^{2+} by Calnuc.

Terbium is also extensively used as a Ca^{2+} mimic [24]. Fluorescence resonance energy transfer from any tryptophan residue near the EF-hand Ca^{2+} site leads to the lanthanide ion showing fluorescence emission in the bound state. Calnuc shows binding to terbium, and the emergence of an emission peak at 545 nm can be observed that is due to fluorescence resonance energy transfer. Not surprisingly, however, the intensity of the 545 nm peak (after equilibrium saturation is achieved by Calnuc) is modulated by the interaction with G-protein. Upon binding to GDP-bound G-protein α-subunit, the terbium bound to Calnuc is released, as indicated by a drop in the peak intensity. Binding to the GTP-bound form of G-protein leads to huge uptake of the ion, as indicated by the rise in the intensity of emission.

Hypotheses for increased Ca^{2+} binding to Calnuc

In order to explain the increased binding of Ca^{2+} to G-protein bound Calnuc, we suggest the following four possible mechanisms.

The first is adoption of the c2 domain structure by Calnuc. Table 2 shows sequence comparisons between proteins classically known to adopt the c2 domain for binding to Ca^{2+} and Calnuc. In proteins containing c2 domains, the Ca^{2+}-binding sites are formed primarily by the side chains of Asp, which serve as bidentate ligands for two or three calcium ions [55]. Notably, solution and crystal structure data show the involvement of Asn, Ser and backbone carbonyl groups also. These essential amino acids could be widely separated in the primary sequences. A well-conserved distribution of Asp residues is observed in Calnuc, matching the distribution in other c2 domain-containing proteins. These amino acids probably coordinate with calcium ions. A scan of the amino acid sequence of Calnuc shows the presence of a phosphatidylserine-binding domain YHRYLQEVIDVLETDGHFREKLQAA(25–49) that provides further support for the possible presence of a c2 domain-like Ca^{2+}-binding site (MOTIFSCAN on http://www.expasy.org).

The second possible mechanism by which G-protein-bound Calnuc binds more calcium ions is by adopting an annexin-like structure. Table 3 shows the consensus sequence of the annexin domain that helps in the adoption of a structure that is able to bind Ca^{2+}. Replacement by other amino acids that retain the ability to adopt the required structure is also shown by the properties of their side chains. It can be observed that amino acids in Calnuc share 80% functional homology with the consensus sequence, and might play a role in increased Ca^{2+} binding.

The third possibility is the 'chromogranin and salivary acidic proline-rich protein' type of Ca^{2+}-binding

Table 2. c2-like domains of Calnuc. Bold letters indicate conserved amino acid residues.

	Strand 1							Strand 2											Strand 3								
Synaptotagmin	G	K	L	Q	Y	S	L	L	L	V	G	I	I	Q	A	A	E	L	**D**	**D**	P	Y	V	K	V	F	L
Protein kinase Cα	G	R	I	Y	L	K	A	L	H	V	T	V	R	D	A	K	N	L	**D**	**D**	P	Y	V	K	L	K	L
Phospholipase γ1								I	C	I	E	V	L	G	A	R	H	L	**N**	C	P	F	V	E	I	E	V
Calnuc	**G^{56}**	K	L	S	R	E								V^{205}	W	E	E	L	**D**	**D^{251}**	P	K	N	E	E	D	D

	Strand 4									Strand 5									Strand 6					
Synaptotagmin	P	V	F	F	T	F	K	V	L	L	V	M	A	V	Y	**D**	F	**D**	**D**	I	I	G	V	L
Protein Kinase Cα	P	Q	M	F	T	F	L	L	K	L	S	V	E	I	W	**D**	W	**D**	**D**	F	N	G	F	L
Phospholipase γ1	P	V	W	F	H	F	Q	I	F	L	R	F	V	V	Y	**E**	E	**D**	**N**	F	L	A	F	L
Calnuc	**P^{313}**	A	Y	**F^{395}**	H	P	D	T								**D^{410}**	Q	**KE**	**D^{415}**	T	S	E		**L^{421}**

Table 3. Consensus sequence of the annexin domain and that of the probable annexin-like domain in Calnuc. The grouping of amino acids into classes and class abbreviations (the key) used within consensus sequences are as follows: o, alcohol (S and T); l, aliphatic (I, L, V); (.), any (A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, Y); a, aromatic (F, H, W, Y); c, charged (D, E, K, R); h, hydrophobic (A, C, F, G, H, I, K, L, M, R, T, V, W, Y); –, negative (D, E); p, polar (C, D, E, H, K, N, Q, R, S, T); +, positive (H, K, R); s, small (A, C, D, G, N, P, S, T, V); t, turn-like (A, C, D, E, G, H, K, N, Q, R, S, T). As can be seen, there is very homology (~ 80%) among the properties of the amino acids in Calnuc (marked in bold) required for it to adopt an annexin-like structure. The consensus sequence table was taken from http://smart.embl-heidelberg.de.

Annexinconsensus	G **T** **N** E Q A I I D V L T K R S N T **Q** **R** Q Q I A K S F K A Q F G K D – L **T** E T L K S E L S G K F E – **R** **L** I V **A** L
Consensus/80%	G T s - t **h** **l** **h** **p** **l** h s p R **s** **t** **.** p h **.** **t** l **t** **t** **t** a **t** p **.** **h** t **p** s . L . **p** **t** l **t** t – **h** s G **p** h c **.** c h l l h l
Consensus/65%	G T – E **p** s l l c l l s o R o p h p h p p l p p t Y p p t a u + s . L . c s l p s – **h** S G **c** a c . c h l l s
Consensus/50%	G T D E s s L l c l L s o R S s s c l p p l + p s Y c c p a G K s . L c c s l c u – T S G – a c . + l L L u
Calnuc	D T N Q D R L **V** T L E E F L A S T Q R K E F D T G E G W E T V E M – Y T E E E L R R F E E E L A – R L E A Q

mechanism [56,57]. The presence of many acidic residues interspersed with Pro and Gly residues in the structure was shown to help in Ca^{2+} binding in both these proteins. Human and rat Calnuc have ~ 68 Glu residues (~ 15%), 29 Asp residues (~ 7%), 20 Pro residues (~ 6%), and 16 Gly residues (~ 4%), out of a total of 435 amino acids. Upon interaction with Giα, these residues could be brought close to each other, eventually adopting a structure that can bind Ca^{2+}.

The fourth possibility is formation of a pseudo-EF hand: Zhou *et al.* have used sequences from S100 and S100-like proteins to predict pseudo-EF-hand-like structures (with a positive predictive value of 99% and a sensitivity of 96%) that are generally N-terminal to the existing EF-hand Ca^{2+}-binding sites [58]. They have proposed that a consensus sequence of the pattern (LMVITNF)-(FY)-x(2)-(YHIVF)-(SAITV)-x(5,9)-(LIVM)-x(3)-(EDS)0-(LFM)-(KRQLE) will form a pseudo-EF-hand Ca^{2+}-binding site. In Calnuc, the sequence following residue 63 is D$\underline{F}^{2}$VS$\underline{H}^{5}$H$\underline{V}^{6}$RTKLDE$\underline{L}^{*}$ KRQ$\underline{E}^{\#}$VS$\underline{R}$ (amino acids that match the residues in the consensus sequence are underlined; superscript numbers correspond to the residue number in the consensus sequence; *residue following after a gap of five to nine amino acids; #residue following a three residue gap). His at position 6 and Val and Ser at positions 19 and 20 are amino acids that are extra in the Calnuc sequence. This sequence mostly has the required amino acids to be able to form a Ca^{2+}-binding site. The sequence is also positioned N-terminal to the first EF-hand domain. In this regard, it is notable that *D. melanogaster* Calnuc has been predicted to have an additional EF-hand domain at a similar position [59].

Association of two proteins and the establishment of a tight complex are fundamental to any signal transduction process. Thermodynamic analysis of binding complexes has shown much promise for providing a more detailed understanding of the binding process and the reasons underlying the architecture of binding sites. An interaction between two proteins involves large surfaces, with electrostatic forces, hydrophobic forces, hydrogen bonding etc. all playing important roles at the same time, making it extremely complicated to analyze. ITC was used to determine the affinity of the interaction between Calnuc and G-protein α-subunit. For the interaction, a K_d of 13 nM was determined. The affinity determined is in the same order of magnitude as observed in other protein–protein interactions [53].

These observations prompt us to suggest that Calnuc plays a dual role in the cell: that of a Ca^{2+} buffer and that of a Ca^{2+} sensor. This is probably also why it is targeted to both the Golgi membranes and the cytoplasm. On the Golgi membranes, where it is colocalized with G-proteins, it plays the role of a signaling molecule. Its physiological function is to act as a downstream effector of G-proteins, its function being governed by whether the G-protein is in an 'on state' [being activated by receptors, e.g. OA1 G-protein-coupled receptor (GPCR)], or in an 'off state'. In the cytoplasm, it acts as a Ca^{2+} buffer, controlling and maintaining the concentration at static/normal levels (Fig. 10).

Experimental procedures

Tris buffer, Sephadex G-75, 12 kDa cut-off dialysis membranes, ethylene glycol, ANS and Stains-all were purchased from Sigma Aldrich (St Louis, MO, USA). All other chemicals were purchased locally and were of the highest purity.

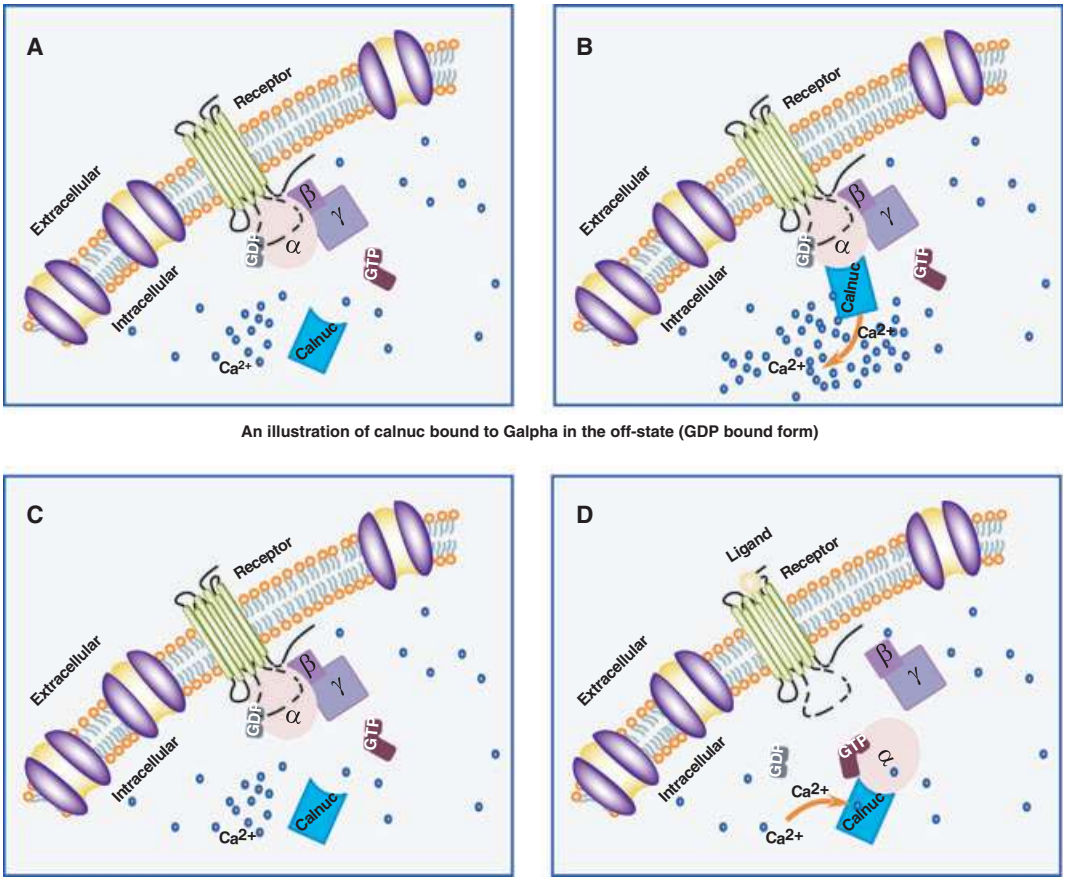

Fig. 10. A model for the physiological role of G-protein–Calnuc interactions. The model schematic diagram shows all of the possible interactions between Calnuc and G-proteins. (A) The initial state of the cell before the interaction. The heterotrimeric G-proteins are found coupled with the membranes, and are in the 'off' state. Some of the Calnuc expressed is present in the cytoplasm. (B) Calnuc might interact with the Giα that is in the 'off' state (GDP-bound state). This Calnuc–G-protein complex might be coupled with the receptor bound to a membrane of an organelle, resulting in the release of Ca^{2+}. (C) The same scene as in (A). Calnuc might interact with the Giα that is in the 'off' state (GDP-bound state), which is still coupled with the GPCR in the membrane, resulting in the release of Ca^{2+}. (D) Calnuc interacts with the Giα that is now in the 'on' state (GTP-bound state, activated by the ligand-bound GPCR). Ligand activation of the GPCR leads to receptor-mediated activation of the G-protein, in which GTP replaces the bound GDP, and therefore detachment of the α-subunit from the receptor/membrane. Activated Giα binds to Calnuc, leading to the uptake of Ca^{2+}. The illustration represents the data in the literature, that Calnuc is able to interact with both GDP-bound and GTP-bound G-protein, as well as our findings that its interaction with activated G-protein leads to an increased uptake of Ca^{2+}. Therefore, the physiological function of Calnuc is not only to maintain Ca^{2+} homeostasis in the cell, but also to act as a signaling molecule downstream of G-proteins.

Retrieval of sequences

The HOMOLOGENE database (http://www.ncbi.nlm.nih.gov/HomoloGene) of the National Center for Biotechnology Information (NCBI) was queried for 'nucleobindin'. Also, ENSEMBL HUMAN GENE VIEW (http://www.ensembl.org/Homo_sapiens/index.html) was 'mined' extensively to collect protein sequences orthologous to nucleobindin gene product from *H. sapiens*. MUSCLE was used to align the amino acid sequences of the proteins [60]. CLUSTALW aligned MSA was used to construct the phylogram, using the neighbor joining method and by ignoring gaps. The MOTIFSCAN database was used to search and predict different motifs present in the primary structure of human Calnuc. Checking for degree of conservation of motifs in Calnuc across all these species was performed using BLOCKS (http://blocks.fhcrc.org).

Overexpression and purification of Calnuc

cDNA of rat Calnuc cloned into pET28a was a kind gift from S. Menon (Rockefeller University, New York, NY, USA). The clone was further verified by performing PCR-based sequencing and matching with the cDNA sequence of Calnuc. This clone lacked the sequence comprising the first 31 amino acids at the N-terminus, most of which codes for a signal peptide. The protein has a His-tag at the N-terminus that is removable by precision protease. The cDNA clone for Giα in pET28b was a kind gift from T. P. Sakmar (Rockefeller University, New York, NY, USA). This clone was also sequenced, and its nucleotide sequence was confirmed. Calnuc and Giα were expressed in BL21(DE3) by inducing the cells with 100 μM isopropyl-thio-β-D-galactoside at 37 °C for 6 h at a bacterial density of $D_{600\ nm} \geq 0.6$. The cells were harvested by centrifugation at 18 500 ***g*** for 5 min at 4 °C.

To purify His6-Calnuc and G-protein α-subunit (GDP was added in all the buffers), the respective cell pellet was resuspended in 20 mM Tris/HCl, 300 mM NaCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 8.0) (resuspension buffer), and sonicated using an ultrasonicator (Vibracell Sonics and Materials, Inc. Newtown, CT, USA). The cell suspension was then centrifuged at 4 °C (18 500 ***g*** for 45 min). The supernatant was loaded on to a Ni^{2+}–nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) column equilibrated with 20 mM Tris/HCl, 300 mM NaCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 8.0) (equilibration buffer). The column was washed with wash buffer A (20 mM Tris/HCl, 300 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM imidazole, pH 8.0) and then with wash buffer B (20 mM Tris/HCl, 300 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 50 mM imidazole, pH 8.0). Protein was eluted with 20 mM Tris/HCl, 300 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 300 mM imidazole (pH 8.0) (elution buffer), and 1 mL fractions were collected. The fractions with the maximum protein content [as detected by SDS/PAGE (12%) gel] were pooled and dialyzed against sample buffer (20 mM Tris/HCl, 50 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 8.0) at 4 °C, and subsequently concentrated using an Amicon ultra 30K filter (Millipore Corp., Bedford, MA, USA). His-tag was removed with precision protease and the protein repurified (Fig. S1). The concentration and yield of protein obtained were estimated using Lowry's method [61].

Gel filtration chromatography of the concentrated sample was performed using Sephadex G-75 (Sigma Aldrich), packed to a 150 mL bed volume column and run under gravity flow. The column was washed thoroughly with four to five column volumes of resuspension buffer. Affinity-purified Calnuc (7 mg in 2 mL) was loaded, and three column volumes of 1 mL eluent fractions were collected. Full-length Calnuc, which elutes as the first peak, was pooled, concentrated, and analyzed by 12% SDS/PAGE. When Ca^{2+}-free Calnuc (apo form) was required, the protein sample obtained was dialyzed against several liters of 5 mM EGTA (Amresco, Solon, OH, USA), and then again against dialysis buffer, over a period of 24 h. Giα was also analyzed for its purity by 12% SDS/PAGE. Both proteins were purified to homogeneity, as shown by a single band on the gel.

Buffer preparation for Ca^{2+}-binding studies

Plastic labware was used in place of glass, with soaking and repeated rinsing of all the labware with dilute nitric acid and deionized water from a MilliQ system (Millipore Corp.). For Ca^{2+}-binding studies, extreme care was taken to remove calcium ions from all the buffers and other solutions. All the solutions were prepared using deionized water passed through a Chelex-100 (Bio-Rad, Richmond, CA, USA) column, to ensure the effective removal of contaminating metal ions.

ITC

Dissociation constants were determined from the binding isotherm of Ca^{2+} and proteins in a VP-ITC calorimeter (MicroCal Inc., Northampton, MA, USA). Ligand and protein solutions were prepared in 20 mM Tris/HCl (pH 7.5) containing 50 mM NaCl, and degassed before use. All titrations were carried out at 30 °C. Calnuc (50 μM) in the sample cell was titrated with 60 injections, 3 μL each, of 7 mM $CaCl_2$ solution loaded in the syringe. Similarly, Calnuc (50 μM) was titrated against 7 mM $MgCl_2$. Appropriate buffer titrations were carried out to determine the heat of dilution, and subtracted from the Ca^{2+}-binding and Mg^{2+}-binding thermograms before analysis of the data with MICROCAL ORIGIN 7.0.

Protein–protein interactions and dissociation constants were determined from the binding isotherm of the two proteins in a calorimeter. Calnuc and Giα protein solutions were prepared in 20 mM Tris/HCl (pH 7.5) containing 50 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and

50 μM GDP. All titrations were carried out at 30 °C. Calnuc (10 μM) in the sample cell was titrated with 60 injections, 5 μL each, of 80 μM Giα solution loaded in the syringe. Appropriate buffer titrations were carried out under similar conditions to determine the heat of dilution, and subtracted from the protein binding thermograms before analysis of the data.

Effect of different metal ions on ANS binding to Calnuc

All fluorescence data were recorded in a Jasco FP-6500 spectrofluorimeter. Changes in ANS fluorescence upon interaction with apo-Calnuc and Ca^{2+}-bound Calnuc were studied by exciting ANS (in 20 mM Tris buffer, pH 8, containing 50 mM NaCl) at 365 nm, and recording the emission spectrum from 400 to 600 nm. Emission and excitation slits were set at 3 nm. For studying the effects of various metal ions, a protein concentration of 99 nM was chosen, such that all of the ANS was in the Calnuc-bound state. In all of these experiments, 5 mM $CaCl_2$ or $MgCl_2$ was used. In the absence of the protein, the fluorescence of ANS was unaffected upon addition of the above-mentioned concentrations of ions (data not shown). All data are representative of experiments performed several times.

Effects of different metal ions on tryptophan fluorescence of Calnuc

The effects of Ca^{2+} and Mg^{2+} binding to apo-Calnuc were studied. Fluorescence spectra of Ca^{2+}-bound and apo-Calnuc were recorded in dialysis buffer at ambient temperature. Emission spectra were recorded from 310 to 450 nm, while the sample was excited with light of 295 nm wavelength. Emission and excitation slit widths were set to 3 nm each. Calnuc (0.04 μM in 3 mL), saturated with $MgCl_2$ (50 μM), was further titrated with $CaCl_2$ (up to 500 μM) in order to monitor conformational changes in the protein. All spectra were corrected for the contribution of the buffer by subtracting a 'blank' spectrum of only the buffer (recorded under similar conditions).

Stains-all assay

Absorption spectral changes of the cationic carbocyanine dye Stains-all can be used to monitor Ca^{2+} binding. The dye ($\sim$ 0.25 mM) was dissolved in 2 mM Mops buffer (pH 7.2) containing 30% ethylene glycol. The exact dye concentration was further established spectrophotometrically using the ε-value (M^{-1}), at 578 nm, of $\sim 1.1 \times 10^6$ in 100% ethylene glycol. Stains-all (6.1×10^{-5} M) was titrated with apo-Calnuc (46 μM), and the dye–protein complex was incubated at 4 °C for 45 min before recording of the spectra at room temperature (400–700 nm on a Perkin Elmer UV–visible spectrophotometer Lambda 35 model). The dye was titrated off from the protein by addition of 1 mM $CaCl_2$ or 2 mM $MgCl_2$. Ca^{2+}-free buffer was used as blank for all the scans, recorded with 2 nm slits. Interactions between Calnuc and G-protein α-subunit were also monitored using Stains-all as a probe. G-protein α-subunit (either GDP-bound or GTP-bound) was added to Calnuc in a final volume of 3 mL, and Stains-all spectra were recorded.

Terbium chloride experiments

In experiments using terbium chloride as a Ca^{2+} mimic, varying concentrations of terbium chloride (1–10 mM) were added to the protein solution in 20 mM Tris buffer. Samples were then excited at 295 nm, and emission was recorded from 450 to 550 nm using Jasco FP6500 fluorimeter. Slit widths of 1 and 3 nm for excitation and emission, respectively, were used, and spectra were recorded at a scan speed of 200 nm·min^{-1}. When saturation had been attained, Giα (600 μM) bound to either GDP or GTP was added, and its effect was monitored.

CD spectroscopy

Far-UV and near-UV CD spectra (for proteins) and visible region spectra (for Stains-all assay) were recorded at room temperature on a Jasco-815 spectropolarimeter with 0.1, 0.5 and 1 cm path length cuvettes, respectively. Appropriate buffer spectra were recorded and subtracted from the protein spectra.

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Supporting information

The following supplementary material is available:
Fig. S1. (A) Chromatogram and SDS/PAGE showing purification of Calnuc. The gel also shows the purity of G-protein α-subunit. (B) Fluorescence-based activation assay of the G-protein α-subunit.
Table S1. Comparison of EF-hand sequence of Calnuc with consensus amino acids at various positions.

This supplementary material can be found in the online version of this article.

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