



G α i3 binding to calnuc on Golgi membranes in living cells monitored by fluorescence resonance energy transfer of green fluorescent protein fusion proteins

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G α i3 is found both on the plasma membrane and on Golgi membranes. Calnuc, an EF hand protein, binds both G α i3 and Ca²⁺ and is found both in the Golgi lumen and in the cytoplasm. To investigate whether G α i3 binds calnuc in living cells and where this interaction takes place we performed fluorescence resonance energy transfer (FRET) analysis between G α i3 and calnuc in COS-7 cells expressing G α i3-yellow fluorescent protein (YFP) and calnuc-cyan fluorescent protein (CFP). The tagged proteins have the same localization as the endogenous, nontagged proteins. When G α i3-YFP and calnuc-CFP are coexpressed, a FRET signal is detected in the Golgi region, but no FRET signal is detected on the plasma membrane. FRET is also seen within the Golgi region when G α i3 is coexpressed with cytosolic calnuc(Δ N2–25)-CFP lacking its signal sequence. No FRET signal is detected when G α i3(Δ C12)-YFP lacking the calnuc-binding region is coexpressed with calnuc-CFP or when G α i3-YFP and calnuc(Δ EF-1,2)-CFP, which is unable to bind G α i3, are coexpressed. G α i3(G2AC3A)-YFP lacking its lipid anchors is localized in the cytoplasm, and no FRET signal is detected when it is coexpressed with wild-type calnuc-CFP. These results indicate that cytosolic calnuc binds to G α i3 on Golgi membranes in living cells and that G α i3 must be anchored to the cytosolic surface of Golgi membranes via lipid anchors for the interaction to occur. Calnuc has the properties of a Ca²⁺ sensor protein capable of binding to and potentially regulating interactions of G α i3 on Golgi membranes.

Many, if not most, important cell activities are regulated by either heterotrimeric G proteins or Ca²⁺. Calnuc (nucleobindin) is an unusual EF hand protein in that it is the only Ca²⁺-binding protein identified to date that is capable of binding both Ca²⁺ and heterotrimeric G proteins. We have shown that G α subunits bind calnuc in yeast two-hybrid (1) and coimmunoprecipitation assays (2, 3). Calnuc binds to the C-terminal α 5-helix region of G α i3 (3), and the site of G α i3 binding on calnuc is the region containing both EF hands (1).

G α subunits have been localized on both the plasma membrane (PM) and intracellular membranes. In particular, G α i3 has been localized on Golgi membranes as well as the PM (4, 5). The functions of G α i3 on Golgi membranes are not understood.

Calnuc is somewhat unusual in that it is both found in the cytosol and associated with the luminal surface of Golgi membranes with the ratio of the cytosolic to membrane pool varying (10–50%) among different cell types (1, 3). We have assumed, on the basis of topology, that it is the cytosolic pool of calnuc that interacts with G α i subunits, because G α i3 is anchored via lipid anchors to the cytoplasmic surface of the PM (6) and Golgi membranes (4, 5). We have characterized the Golgi luminal pool of calnuc and have shown that calnuc is the major Ca²⁺-binding protein in the Golgi apparatus and it is involved in establishing an agonist-mobilizable, Ca²⁺ store in the Golgi lumen (2). Several key questions were raised by our previous results. Do G α i3 and calnuc interact in living cells? If so, where in the cell do they interact? Which pool of calnuc interacts with G α i3?

In this paper, we set out to answer these questions by using fluorescence resonance energy transfer (FRET). FRET is a non-destructive spectroscopic method for measuring protein–protein interactions. It occurs when two fluorophores are in sufficient proximity (<100 Å) that an excited donor fluorophore can transfer its energy to a second, acceptor fluorophore producing light emission from the acceptor (7, 8). Thus, the intracellular site of protein–protein interaction can be visualized directly in living cells. Recently, mutants of the green fluorescent protein (GFP) have been developed and used to monitor protein–protein interaction via FRET with the preferred partners being CFP, a cyan variant of GFP, and YFP, a yellow variant of GFP (7, 9). By using this approach it has been possible to investigate a variety of problems including activation of heterotrimeric G proteins (10), oligomerization of G protein-coupled receptors (11), interaction between nuclear transport receptors and components of the nuclear pore complex (12), and activation of the small GTPase rac (13).

By using G α i3 fused to YFP and calnuc fused to CFP we have investigated the interaction between G α i3 and the Ca²⁺-binding protein, calnuc. We show here that G α i3 binds calnuc in living cells, that the interaction takes place on Golgi membranes, and that cytoplasmic calnuc binds to G α i3 anchored to the cytoplasmic surface of Golgi membranes. Our results indicate that G α i3 on Golgi membranes may have properties distinct from G α i3 on the PM.

Materials and Methods

Materials. Calnuc cDNA and G α i3 cDNA subcloned into a pcDNA3 vector were generated as described (2). cDNAs for ECFP, EYFP, α -mannosidase II-CFP, and galactosyltransferase-YFP were kindly provided by J. Llopis and R. Tsien (University of California at San Diego, La Jolla) and were prepared as described (14). Rabbit polyclonal antiserum against GFP (which recognizes CFP and YFP) was obtained from Charles Zuker (University of California at San Diego, La Jolla). Affinity-purified goat anti-rabbit IgG (H + L) conjugated to horseradish peroxidase was from Bio-Rad, and Supersignal chemiluminescent reagent was from Pierce.

Construction of Vectors for the Expression of GFP-Tagged Proteins.

pcDNA3/G α i3-YFP and pcDNA3/calnuc-CFP were obtained by using a Seamless Cloning kit (Stratagene). For construction of pcDNA3/calnuc-CFP, platinum pfx polymerase (Life Technologies, Grand Island, NY) and primers 5'-GACTCTTCAGGGC-

Abbreviations: CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; PM, plasma membrane; YFP, yellow fluorescent protein; wt, wild type.

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CATCTGTGTTT GCCCTC-3' and 5'-GACTCTTCATA-AATGCTGAGAATCCAGCTGTGG-3' and primers 5'-GACTCTTCATTAATGGTGAGCAAGGGCGAGGAG-3' and 5'-GACTCTTCA CCCTTACTGTACAGCTCGTCCATGCC-3' were used to amplify pcDNA3/calnuc and CFP cDNA, respectively. Primers 5'-GACTCTTCAGGCCATCTG TTGTTGCCCCCTC-3' and 5'-GACTCTTCATAAGTAAAGCCCA-CATTCTTTAAG-3' and primers 5'-GACTCTTCATTAATG-GTGAGCAAGGGCGAGGAG-3' and 5'-GACTCTTCACCC-TTACTTGTTCAGCTCGTCCATGCC-3' were used to amplify pcDNA3/*Gai3* and YFP cDNA, respectively. The resulting PCR products were digested with *Eam* 1104I and ligated. pcDNA3/calnuc(Δ EF-1,2), in which both EF-1 and EF-2 domains (Asp₂₅₂-Phe₃₁₆) were deleted, was obtained as described (2). Calnuc(Δ EF-1,2) cDNA was excised with *Bam*HI and *Nco*I. CFP cDNA was amplified with the primers introducing a *Nco*I site to 5' and *Not*I site to 3' end of CFP. Calnuc(Δ EF-1,2) and CFP cDNAs were subcloned into the pcDNA3 vector (Invitrogen) at the *Bam*HI and *Not*I sites to generate pcDNA3/calnuc(Δ EF-1,2)-CFP with CFP at the C terminus of calnuc(Δ EF-1,2).

To generate deletion mutants, we used an Exsite Mutagenesis kit (Stratagene). For the construction of deletion of the putative signal sequence, 5'-phosphorylated primers 5'-pGTGCCTGTGGACCGCGCAGC-3' and 5'-CATGGATCCGAGCTCGGTACCAA-3' were used to amplify pcDNA3/calnuc(Δ N2-25)-CFP with pfx polymerase and pcDNA3/calnuc-CFP as a template. The resulting PCR product was self-ligated. Similarly, for the construction of *Gai3*(Δ C12) the last 12 C-terminal aa of *Gai3* deleted, 5'-phosphorylated primers 5'-pTTAATGGTGAGCAAGGGCGAG-3' and 5'-GACATCTGTAACAGCATCAAAAAC-3' were used to amplify pcDNA3/*Gai3*(Δ C12)-YFP with pfx polymerase and pcDNA3/*Gai3*-YFP as a template. The resulting PCR product was self-ligated. To generate *Gai3*(G2AC3A) we replaced an N-terminal cDNA fragment of pcDNA3/*Gai3*-YFP with the corresponding N-terminal cDNA fragment of pAS2-1/*Gai3*(G2AC3A) (3) by double digestion with *Eco*RI/*Bsi*WI, obtaining pcDNA3/*Gai3*(G2AC3A)-YFP.

Fidelity of the constructs was verified by automated DNA sequencing (Molecular Pathology Shared Resource Facility, University of California at San Diego Cancer Center). cDNA constructs were transformed into *Escherichia coli* DH5 α , followed by extraction and purification by using Plasmid Midi Kits (Qiagen).

Transfection. COS-7 cells were grown either on coverslips (for live-cell imaging) or on 100-mm dishes (for cell fractionation) and maintained in DME high glucose supplemented with 10% (vol/vol) FCS (GIBCO/BRL). To transiently overexpress the fusion proteins, cells were transfected by using FUGENE6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. For double-transfection experiments, the DNA ratios of the two expression vectors were adjusted to obtain approximately equal amounts of the pair of interacting proteins.

Cell Fractionation. Cells (100-mm dish) were washed twice with ice-cold PBS, scraped from the dish, resuspended in homogenization buffer, and homogenized and fractionated as described (1). Proteins of the postnuclear supernatant, soluble (100,000 \times g supernatant), and membrane (100,000 \times g pellet) fractions were separated by SDS/PAGE and immunoblotted with anti-GFP antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. Detection was by enhanced chemiluminescence. The distribution of calnuc-CFP and *Gai3*-YFP in the supernatant and pellet was quantified by densitometry with SCANALYSIS software (Biosoft, Cambridge, U.K.).

Live-Cell Imaging and FRET. For live-cell imaging, COS-7 cells were plated on glass coverslips and transiently transfected 24 h before imaging. During microscopy, cells were kept in PBS plus 10%

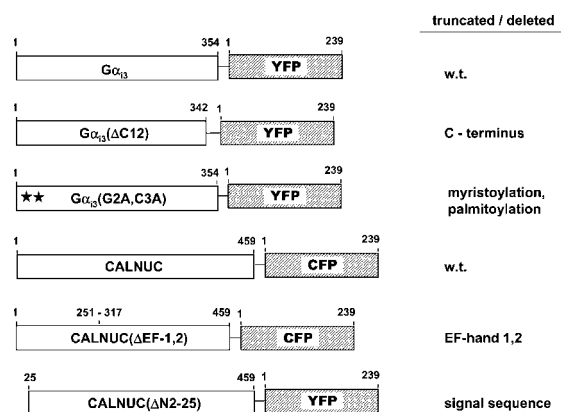


Fig. 1. *Gai3* and calnuc fusion proteins used for FRET. YFP was fused to the C terminus of wt *Gai3* and *Gai3* mutants, and CFP was fused to the C terminus of wt calnuc and its mutants. *Gai3*(Δ C12) with its last 12 C-terminal amino acids (amino acid 343–354) deleted, which represents the site of binding of calnuc to *Gai3*, does not bind calnuc. *Gai3*(G2AC3A), with the myristoylation and palmitoylation sites of *Gai3* replaced, represents a mutant in which anchoring of *Gai3* to membranes is expected to be abolished as shown for *Gai1* (16). Calnuc(Δ EF-1,2), lacking its two EF hands (amino acids 252–316), does not bind to *Gai3* (3). Calnuc(Δ N2–25) lacking its putative signal sequence (amino acids 2–25) is expected to be located in the cytoplasm.

(vol/vol) FCS and maintained at 37°C by using a temperature-controlled stage (20/20 Technologies, Wilmington, NC). Images were obtained as described (13, 15) with use of a cooled charge-coupled device camera with KAF 1400 chip (Photometrics, Tucson, AZ) and INOVISION software (ISEE, Raleigh, NC) for microscope automation and image analysis, and a Zeiss 40 \times 1.3 N.A. Fluor oil immersion objective. For detection of CFP, cells were viewed with an excitation filter of 436/20 nm, a dichroic beam splitter of 455 nm, and an emission filter of 480/40 nm. YFP was detected by using a filter set with an excitation filter of 500/25 nm, a dichroic beam splitter of 515 nm, and an emission filter of 535/30 nm. The filters for FRET were an excitation filter of 436/20 nm, a dichroic beam splitter of 455 nm, and an emission filter of 535/50 nm. Filters were obtained from the Chroma Technology (Brattleboro, VT). Each image was background subtracted, and all images were registered as described in detail (15). Emission appearing in the FRET image because of emission from CFP or direct excitation of YFP was removed by subtracting a fraction of the CFP and YFP images from the FRET image. This fraction depended on the filter set and exposure conditions used and was determined, as described (15), by taking images of cells containing only CFP or YFP alone and quantifying the relative intensity of emission in the FRET channel and that in the CFP or YFP channel. A broad range of intensities was examined, and a line was fit to these for accurate determinations. Controls were performed in which images were obtained in different orders. The order in which images were obtained had no effect. The exposure times were equal within each series of images and were chosen so that all pixel intensities were within the linear range of the camera.

Results

Construction of GFP Fusion Proteins. To examine protein–protein interactions of calnuc and *Gai3* *in vivo* by FRET analysis we fused YFP to the C terminus of *Gai3* and its mutants CFP to the C terminus of calnuc and calnuc mutants (Fig. 1). As controls for the FRET assay, we used *Gai3*(Δ C12) with the last 12 amino acids deleted, which does not bind calnuc (3), and calnuc(Δ EF-1,2) with the two EF hands deleted, which we have shown does not interact with *Gai3* (2). To explore whether *Gai3* must be anchored to membranes for interaction with calnuc to take place, we prepared *Gai3*(G2AC3A) with mutated myristoylation (glycine to alanine)

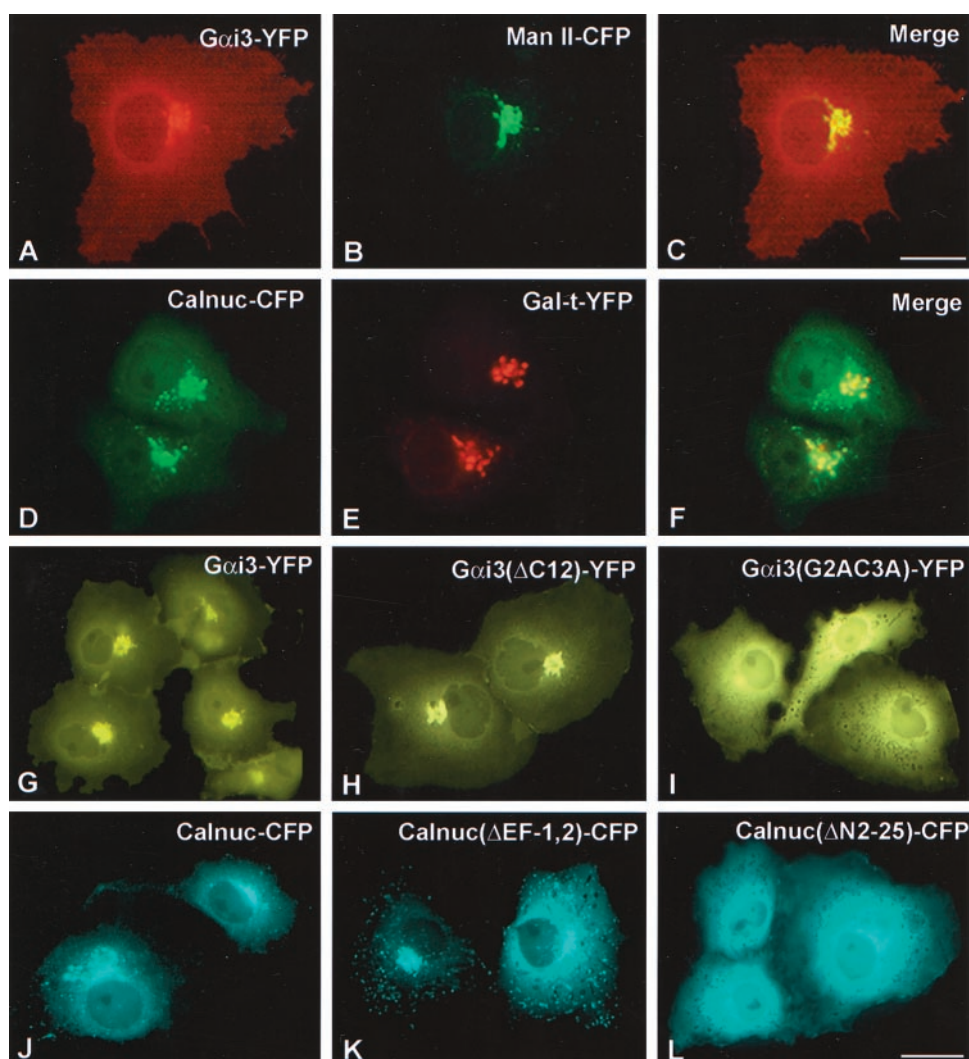


Fig. 2. Localization of $G\alpha i3$ and calnuc fusion proteins transiently expressed in COS-7 cells. COS-7 cells were cotransfected with the indicated cDNAs, and after 24 h the sites of expression of fusion proteins were monitored in live cells by using CFP and YFP fluorescence. (A and B) $G\alpha i3$ -YFP is detected on the PM and in the Golgi region where it overlaps with the Golgi marker α -mannosidase II-CFP (ManII-CFP). (C) Merged image showing areas of overlap (yellow). (D and E) Calnuc-CFP is correctly targeted to the Golgi region where it partially overlaps with the Golgi marker galactosyltransferase-YFP (Gal-t-YFP). It is also detected in the cytoplasm. (F) Merged image showing areas of overlap (yellow). (G–I) Distribution of $G\alpha i3$ -YFP and its mutants. $G\alpha i3$ -YFP (G) and $G\alpha i3(\Delta C12)$ -YFP (H) are concentrated on Golgi membranes and on the PM. $G\alpha i3(G2AC3A)$ -YFP (I) shows predominantly cytoplasmic staining. (J–L) Distribution of calnuc-CFP and calnuc-CFP mutants. Both calnuc-CFP (J) and calnuc(ΔEF -1,2)-CFP (K) are expressed in the juxtannuclear or Golgi region and in the cytoplasm. Calnuc($\Delta N2$ -25)-CFP (L), lacking its signal sequence, is located in the cytoplasm as expected. (Bar = 10 μm .)

and palmitoylation (cysteine to alanine) sites which, based on experiments on $G\alpha i1$ (16), would be expected to be located in the cytoplasm of transfected cells. To enhance the cytosolic pool of calnuc we prepared calnuc($\Delta N2$ -25) with its predicted N-terminal signal sequence (amino acid 2–25) deleted, which would be expected to be located exclusively in the cytoplasm of transfected cells.

Localization of Fusion Proteins in Transfected COS-7 Cells. To assess the targeting of the overexpressed wild-type (wt) and mutant fusion proteins we cotransfected COS-7 cells with $G\alpha i3$ -YFP and the Golgi marker α -mannosidase II-CFP and calnuc-CFP with the trans Golgi marker galactosyltransferase-YFP and determined the distribution of the fusion proteins by fluorescence microscopy after 24 h of expression. As shown in Fig. 2A, $G\alpha i3$ -YFP was detected both on the PM and on Golgi membranes where it overlapped with the Golgi marker α -mannosidase II-CFP (Fig. 2B). Calnuc-CFP (Fig. 2D) was expressed mainly in the Golgi region where it partially overlapped with the Golgi marker galactosyltransferase-YFP (Fig.

2E); it was also found to a lesser extent in the cytoplasm. Thus the subcellular localization of GFP fusion proteins of $G\alpha i3$ and calnuc was the same as that of the endogenous, untagged proteins described (1, 5).

$G\alpha i3(\Delta C12)$ -YFP (Fig. 2H), like wt $G\alpha i3$ -YFP (Fig. 2G), was localized predominantly in the Golgi region and was also found at the PM. By contrast, $G\alpha i3(G2AC3A)$ -YFP with myristoylation and palmitoylation sites eliminated was exclusively expressed in the cytoplasm of transfected cells (Fig. 2I).

Calnuc(ΔEF -1,2)-CFP (Fig. 2K) lacking its two EF hands had a distribution similar to that of wt calnuc-CFP (Fig. 2J) in that it was found both in the Golgi region and in the cytoplasm. Calnuc($\Delta N2$ -25)-CFP lacking its signal sequence was, as expected, expressed exclusively in the cytoplasm (Fig. 2L).

These results demonstrate that $G\alpha i3$ -YFP and calnuc-CFP are correctly targeted to their normal sites of residence in transfected COS cells and that elimination of lipid anchors from $G\alpha i3$ [$G\alpha i3(G2AC3A)$] or deletion of the signal sequence of calnuc

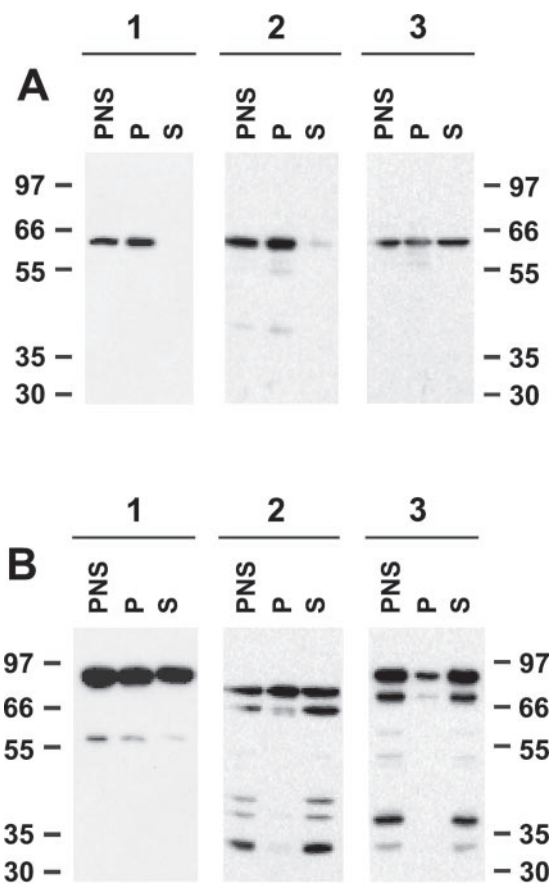


Fig. 3. Subcellular fractionation of *Gai3* and calnuc fusion proteins in transiently transfected COS-7 cells. Postnuclear supernatant (PNS) of COS-7 cells overexpressing CFP and YFP fusion proteins were fractionated by ultracentrifugation ($100,000 \times g$) into soluble (S) and particulate (P) fractions. Proteins were separated by SDS/10% polyacrylamide gel and immunoblotted with a polyclonal anti-GFP antibody as described in *Methods*. (A) *Gai3*-YFP (≈ 63 kDa) (1) as well as *Gai3*(Δ C12)-YFP (2) are exclusively associated with the pellet (P), whereas 70% of *Gai3*(G2AC3A)-YFP (3) is found in the soluble fraction (S). (B) Calnuc-CFP (≈ 90 kDa) (1) and calnuc(Δ EF-1,2)-CFP (≈ 75 kDa) (2) are about equally divided between the pellet (P) and the soluble (S) fractions. Calnuc(Δ N2-25)-CFP (3) with the signal sequence deleted is distributed mostly ($\approx 80\%$) in the soluble fraction. The additional bands detected by the polyclonal GFP antibody seen for calnuc fusion proteins may correspond to degradation products. No free CFP or YFP (≈ 30 kDa) is detected.

[calnuc(Δ N2-25)] resulted in the expected defects in targeting, and their accumulation in the cytoplasm.

Distribution of Overexpressed Fusion Proteins in Subcellular Fractions.

To confirm the subcellular targeting of the fusion proteins demonstrated by fluorescence, we also performed Western blotting of cytosolic ($100,000 \times g$ supernatant) and membrane ($100,000 \times g$ pellet) fractions prepared from transfected COS cells. *Gai3*-YFP and *Gai3*(Δ C12)-YFP were associated exclusively with the membrane fraction (Fig. 3A 1 and 2). *Gai3*(G2AC3A)-YFP, without its lipid anchors, was located largely ($\approx 70\%$) in the soluble fraction (Fig. 3A 3). Calnuc-CFP was equally distributed in the pellet and soluble fractions (Fig. 3B 1) as was calnuc(Δ EF-1,2)-CFP (Fig. 3B 2). The endogenous calnuc is also present in both the pellet and soluble fractions (1, 3). Calnuc(Δ N2-25)-CFP without its signal sequence was expressed mainly ($\approx 80\%$) in the soluble fraction and was strikingly reduced in the membrane pellet (Fig. 3B 3). Free CFP or YFP was not detected. The fractionation data are consistent with the fluorescence results in that mutating the lipid-anchoring sites of

Gai3 or deleting the signal sequence from calnuc led to a major increase in the percentage of the expressed protein associated with the soluble fraction. However, appreciable *Gai3*(G2AC3A)-YFP and calnuc(Δ N2-25)-CFP were also found in the pellet, which could be explained by binding of the cytosolic proteins to Golgi membrane proteins, e.g., soluble calnuc to membrane anchored *Gai3* and soluble *Gai3* to unknown Golgi membrane proteins (6).

***Gai3* Interacts with Calnuc at Golgi Membranes.** To investigate where in the cell *Gai3* and calnuc interact we performed FRET analysis on COS-7 cells transfected with calnuc-CFP and *Gai3*-YFP. As demonstrated in Fig. 4A and B, *Gai3*-YFP was found at both the PM and the Golgi apparatus, and calnuc-CFP was localized in the Golgi region plus the cytosol as described for cells transfected with a single protein alone. Both fusion proteins were expressed at similar levels on the basis of their specific fluorescence intensities. With appropriate filters the resulting image showed an intense FRET signal in the juxtannuclear region corresponding to the location of the Golgi apparatus (Fig. 4C and D), whereas no FRET signal was detected at the PM. FRET was observed in 9 of 11 cells tested. As a control we used a calnuc mutant, calnuc(Δ EF-1,2) without EF hands, that is not capable of binding *Gai3* *in vivo* (2) or *in vitro* (3). Eight of ten cells overexpressing *Gai3*-YFP and this mutant at about equal levels did not show a significant FRET signal in the juxtannuclear region or anywhere else in the cell (Fig. 4E).

From these data, we conclude that interaction between calnuc and *Gai3* takes place on Golgi membranes. Moreover, the findings confirm *in vivo* our previous *in vitro* findings (1) that the region of calnuc containing the EF hands is responsible for its interaction with *Gai3*. The FRET signal was not an artifact caused by bleedthrough of signal from one GFP mutant into the image of another as demonstrated both by the calnuc(Δ EF-1,2) used as a negative control (Fig. 4E) and analysis of bleedthrough intensities with cells expressing each of the fusion proteins alone.

Gai3 Must Be Membrane-Anchored to Interact with Calnuc.

By mutation of palmitoylation and myristoylation sites on *Gai3*-YFP we generated a mutant, *Gai3*(G2AC3A)-YFP, lacking its lipid anchors, that is highly enriched in the cytoplasm (see Figs. 2I and 3A). This mutant binds calnuc as strongly as wt *Gai3* in yeast two-hybrid assays (3). To explore if soluble *Gai3*(G2AC3A)-YFP binds to calnuc-CFP, cells overexpressing both fusion proteins were examined by FRET analysis (Fig. 4E). No fluorescence signal was detected either in the cytoplasm or in the juxtannuclear region in four of five cells overexpressing these proteins. We conclude that binding of *Gai3* to calnuc requires that *Gai3* be anchored to Golgi membranes. When *Gai3* is not anchored to membranes, it presumably is too dilute or does not have the proper conformation to produce a detectable FRET signal.

The Cytosolic Pool of Calnuc Interacts with *Gai3* Anchored to Golgi Membranes.

To address the question of which pool of calnuc interacts with the *Gai3*-YFP anchored to Golgi membranes facing the cytosol, we conducted FRET analysis on COS-7 cells doubly transfected with *Gai3*-YFP and calnuc(Δ N2-25)-CFP lacking its signal sequence that is expressed in the cytoplasm (see Figs. 2L and 3B 3). Overexpression of these fusion proteins resulted in a FRET signal similar to that of cells transfected with both wt proteins, with fluorescence in the Golgi region and no detectable signal at the PM in five of six cells examined (Fig. 4G). The only difference between the findings for calnuc-GFP and the cytosolic mutant was that the FRET signal was somewhat broader in the mutant. These results demonstrate that the cytosolic pool of calnuc binds to *Gai3*.

When *Gai3*(Δ C12)-YFP, which cannot bind calnuc (3), was cotransfected with cytosolic calnuc(Δ N2-25)-CFP, no FRET was seen in nine of the ten cells examined (Fig. 4H). This control

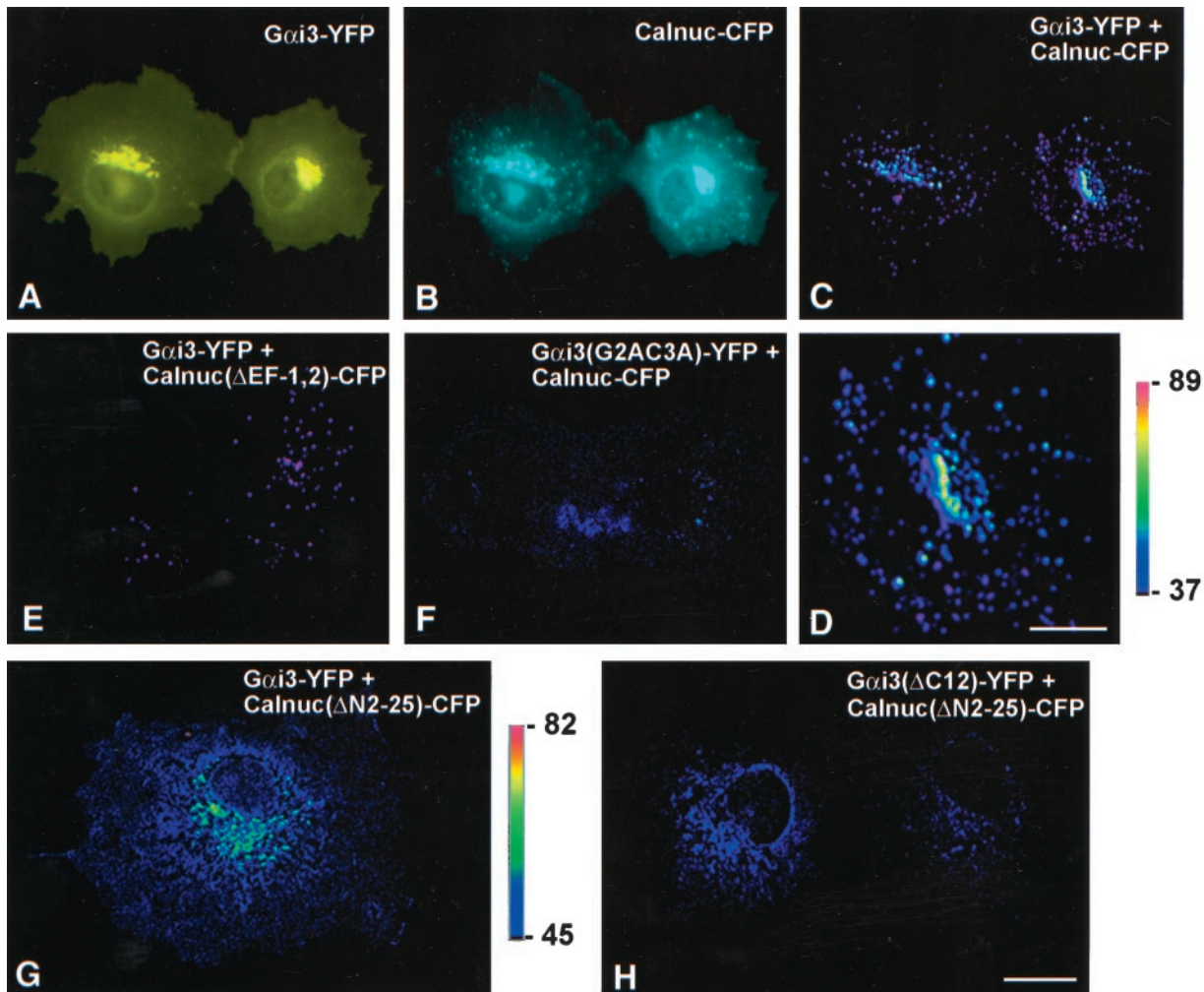


Fig. 4. Interaction of $G\alpha i3$ and calnuc fusion proteins monitored by live-cell FRET. COS-7 cells were transfected with various fusion proteins, and after 24 h of expression images were taken of live cells at 37°C. (A–C) Cells expressing $G\alpha i3$ -YFP and calnuc-CFP were imaged with the YFP filter set (A), the CFP filter set (B), and the FRET filters (C). The FRET image (C) shows an intense signal in the juxtannuclear (Golgi) region indicated by the yellow and red signal, but no significant FRET signal is detected on the PM. (D) Enlargement of the cell to the right in C showing FRET in the Golgi region. (E) In cells cotransfected with $G\alpha i3$ -YFP and calnuc(Δ EF-1,2)-CFP lacking the $G\alpha i3$ -binding site (2), no FRET signal can be detected. (F) Similarly, no FRET is seen in COS-7 cells cotransfected with calnuc-CFP and soluble $G\alpha i3$ (G2AC3A)-YFP with mutated myristoylation (G2A) and palmitoylation (C3A) sites. (G) In COS-7 cells transfected with $G\alpha i3$ -YFP and soluble calnuc(Δ N2–25)-CFP an intense FRET signal in the juxtannuclear region indicates that cytosolic calnuc interacts with $G\alpha i3$ anchored to the cytoplasmic surface of Golgi membranes. (H) Cells expressing $G\alpha i3$ (Δ C12)-YFP lacking the calnuc-binding site and soluble calnuc(Δ N2–25)-CFP do not show a significant FRET signal in the Golgi region. For C, D, and G, FRET intensities are encoded by using the color scales shown. FRET intensity within each image is represented by a range of colors. The lowest and highest values within each image are indicated next to the color bars. Colors range between blue (lowest FRET) and red and yellow (highest FRET). [Bar = 10 μ m (A–H) or 5 μ m (D).]

excludes nonspecific binding of calnuc to $G\alpha i3$ on Golgi membranes.

Discussion

The role of G proteins in signaling at the PM is well established, but very little is known about their function on intracellular membranes (17, 18). The only direct evidence for the role of G proteins on intracellular membranes comes from the work of Stow and coworkers (4), who showed that overexpression of $G\alpha i3$ slowed transport of a secretory protein, heparin sulfate proteoglycan, through the Golgi. This effect was reversed by pertussis toxin, which ADP ribosylates the C terminus of $G\alpha$ subunits (4). The C terminus of $G\alpha i3$ is a binding site for multiple receptors (19, 20) and effectors (21), but no such receptors or effectors have been identified on Golgi membranes to date. To gain insight into the functions of $G\alpha i3$ on Golgi membranes we undertook a quest for interacting

partners of $G\alpha i3$. We identified several new binding partners including calnuc (1), GATP (22) a member of the regulators of G protein-signaling family (RGS), and AGS3 (23), a GDI or guanine dissociation inhibitor for $G\alpha i3$. Calnuc corresponds to nucleobindin and is an EF hand containing Ca^{2+} -binding protein (24, 25). We have validated that calnuc is distributed in two pools (one cytosolic and one tightly associated with the luminal side of cis Golgi membranes) and have shown that the Golgi pool of calnuc, together with SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase) and IP_3 receptor type 1, is involved in the establishment of the agonist-mobilizable Golgi Ca^{2+} store (2). We have also demonstrated by multiple approaches [i.e., by immunoblotting of soluble (100,000 \times g supernatant) and membrane (100,000 \times g pellet) fractions (1), digitonin permeabilization (3), and pulse-chase experiments (3)] the existence of a cytosolic pool of calnuc (2, 3). Calnuc has a hydrophobic signal sequence and would be expected to

be translocated across endoplasmic reticulum membranes and to follow the secretory pathway. However, dual localization of proteins in both the cytoplasm and organelles of the secretory pathway is not uncommon and can be explained by either alternative splicing or regulation of the signal sequence (26, 27). The latter is most likely for calnuc, which undergoes posttranslational modifications in the cytoplasm (3). Because *Gai3* subunits are found on the cytoplasmic side of membranes, we speculated that the cytoplasmic pool of calnuc binds to *Gai3*.

In this paper, we performed FRET analysis in living cells by using fusion proteins with mutants of GFP and demonstrated that, although *Gai3* is expressed on both Golgi membranes and the PM, interaction between calnuc and *Gai3* is seen only on Golgi membranes and is not detectable on the PM. We further demonstrated that it is indeed the cytosolic pool of calnuc that interacts with *Gai3*, because expression of calnuc in the cytoplasm accomplished by deleting its signal sequence did not change the FRET signal. Furthermore, a *Gai3* mutant expressed in the cytoplasm was not able to produce FRET, indicating that *Gai3* has to be membrane anchored to interact with calnuc.

What is the function of calnuc? EF-hand proteins can serve as Ca^{2+} buffers and/or Ca^{2+} sensors that bind or modulate other proteins after Ca^{2+} binding (28). The intra-Golgi pool of calnuc was shown to be important in maintaining the Golgi Ca^{2+} stores through Ca^{2+} binding to its EF hands (2). The cytoplasmic pool is more likely to regulate other proteins through direct interaction as is the case with other Ca^{2+} -binding proteins, such as calmodulin, troponin C, and recoverin, located in the cytoplasm (28). Calnuc binds to *Gai3* in a Ca^{2+} - and Mg^{2+} -dependent manner (3) and undergoes a conformational change after binding Ca^{2+} (29) suggesting, in analogy to calmodulin and other calcium sensors, a putative role for calnuc in regulation of $G\alpha$ subunits. Calnuc binds to the C-terminal $\alpha 5$ -helix of *Gai3* (3) and therefore might interfere with binding of putative receptors to $G\alpha$ subunits.

The ability to investigate the site of protein interactions *in vivo* at specific subcellular locations has been greatly enhanced with the development of GFP mutants and their application in

FRET experiments (7). After important studies validating application of GFP mutants for analysis of FRET in living cells (30–32), it has recently become possible to use the technique with confidence to address unknown questions. Use of this approach has made it possible to study a variety of signaling and trafficking problems including receptor-mediated activation of heterotrimeric G proteins (10), oligomerization of G protein receptors (11), interaction between nuclear transport receptors and components of the nuclear pore complex (12), and activation of the small GTPase rac (13). We have taken advantage of FRET's unique capabilities to determine the site of interaction between *Gai3* and calnuc within living cells. Our experiments and controls demonstrate conclusively that *Gai3* and calnuc expressed in COS-7 cells interact on the cytoplasmic surface of Golgi membranes. Although we observed no detectable FRET at the PM, the limits of detectability are difficult to define, and negative results must be interpreted with caution. However, the strong signals obtained at the Golgi indicate that the number of protein complexes per unit area is greater in the Golgi than at the PM. Thus the results suggest that *Gai3* on Golgi has distinct functions and interactions from *Gai3* on the PM.

Because we found that the interaction between *Gai3* and calnuc is $\text{Ca}^{2+}/\text{Mg}^{2+}$ dependent and calnuc binds Ca^{2+} , it will be important in the future to ask if this interaction is regulated by the cytosolic Ca^{2+} concentration, i.e., if it is influenced by changes in the intracellular Ca^{2+} pool. Furthermore, it will be important to determine the functional consequences of the interaction. The fact that the interaction was observed on Golgi membranes but not on the PM might be caused by differences in the properties of *Gai3* in the two locations or by the involvement of additional unidentified proteins.

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