G Protein Binding Sites on Calnuc (Nucleobindin 1) and NUCB2 (Nucleobindin 2) Define a New Class of Gαi-regulatory Motifs

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Heterotrimeric G proteins are molecular switches modulated by families of structurally and functionally related regulators. GIV (Gα-interacting vesicle-associated protein) is the first non-receptor guanine nucleotide exchange factor (GEF) that activates Gαi subunits via a defined, evolutionarily conserved motif. Here we found that Calnuc and NUCB2, two highly homologous calcium-binding proteins, share a common motif with GIV for Gαi binding and activation. Bioinformatics searches and structural analysis revealed that Calnuc and NUCB2 possess an evolutionarily conserved motif with sequence and structural similarity to the GEF sequence of GIV. Using in vitro pulldown and competition assays, we demonstrate that this motif binds preferentially to the inactive conformation of Gα13 and Gα12 over other Gα subunits and, like GIV, docks onto the α3 switch II cleft. Calnuc binding was impaired when Lys-248 in the α3 helix of Gα13 was replaced with M, the corresponding residue in Gαi5, which does not bind to Calnuc. Moreover, mutation of hydrophobic residues in the conserved motif predicted to dock on the α3 switch II cleft of Gα13 impaired the ability of Calnuc and NUCB2 to bind and activate Gαi3 in vitro. We also provide evidence that calcium binding to Calnuc and NUCB2 abolishes their interaction with Gαi3 in vitro and in cells, probably by inducing a conformational change that renders the Gαi3-binding residues inaccessible. Taken together, our results identify a new type of Gα-regulatory motif named the GBA motif (for Gαi-binding and -activating motif), which is conserved across different proteins throughout evolution. These findings provide the structural basis for the properties of Calnuc and NUCB2 binding to Gα subunits and its regulation by calcium ions.

Recently it has become clear that in addition to G protein-coupled receptors and Gβγ subunits, the function of the Gα subunits of heterotrimeric G proteins is controlled by accessory proteins that regulate their activity and/or localization (1–3). The first group of such regulators to be described was the regulators of G protein signaling (RGS) protein family, which serve as GTPase-activating proteins for Gαi, Gαq, and Gα12 subunits via a 120-aa conserved domain, the “RGS box” (4, 5). Subsequent studies revealed another group of regulatory proteins with Gαi activity for Gαi subunits, which have a common signature motif, i.e. the GoLoco or G protein-regulatory (GPR) motif (1, 6, 7). Both the RGS box (8, 9) and the GoLoco/GPR motif (10) have been structurally resolved by X-ray crystallography, and their critical roles in metabolism, cell division, and cardiovascular function, among others, have made them emerging pharmacological targets (11, 12). We recently described another Gα-interacting protein, GIV (13), and showed that it is a GEF that activates Gαi subunits and mediates its biological functions via a defined motif (14) with structural similarity to the synthetic GEF peptide KB-752 (15). GIV is a metastasis-related protein (16) that enhances PI3K-Akt signaling and promotes macrophage, endothelial, epithelial, and tumor cell migration (17–19).

We identified Calnuc (nucleobindin 1 or NUCB1) as a Gα-binding protein in a yeast two-hybrid screen using Gα14 as bait (21). Calnuc, the most abundant protein in the Golgi (24) and the major calcium-binding protein within the Golgi lumen (21–23), regulates intracellular calcium stores via its two EF-hands (22). In addition, we have shown previously that there is a significant soluble pool of Calnuc in the cytosol (21, 25), which interacts with Gα13 in vivo on the surface of Golgi membranes as demonstrated by FRET and live cell imaging (26). The role of cytosolic Calnuc as a G protein regulator was further substantiated by the finding that it controls the intracellular localization of Gαi subunits in neuroendocrine cells (27). However, the mechanism by which Calnuc binds or regulates Gαi subunits remains unknown. Here we identified a conserved motif in Calnuc and the highly homologous protein NUCB2 (nucleobindin 2 or NEFA) (20) with similarity to the GEF motif of GIV and characterized how this motif binds and regulates Gαi subunits. These findings help define a new class of structurally defined G

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The abbreviations used are: RGS, regulator of G protein signaling; GIV, Gα-interacting vesicle-associated protein; GEF, guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor; GPR, G protein-regulatory; aa, amino acid(s); GBA, Gαi binding and activating; GTPγS, guanosine 5′-O-(thio)triphosphate; Sw1L, switch II; CFP, cyan fluorescent protein; GSP, Galphas(s)-binding peptide.
protein regulatory motifs and provide insights into how the interaction between Go13 and Calnuc is regulated.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—The peptide corresponding to the Calnuc Go13-binding motif, i.e., 309RLVTLEASTQRKE324 (Calnuc-(309–324) peptide, >95% purity), was synthesized and purified as described previously (14, 17, 29). The control peptide (EVVTLLQALEESEKLT, >95% purity) used in our experiments, corresponding to the GEF sequence of GIV with a remainder of the reagents and antibodies used were described previously (14, 17, 29).

cDNA was obtained from Open Biosystems. The sources of the cDNAs for GEFs were the pGEX-4T-1 vector to generate GST-Calnuc and GST-NUCB2. Briefly, His-tagged Calnuc-(1–459) and Calnuc-(171–459) (CalnucΔN) and full-length rat NUCB2 (NUCB2, respectively. His-tagged full-length Calnuc-(1–459) was cloned using the ligation-independent cloning vector pMCSG7 exactly as described previously (30). Cloning of rat GIVi3 and GIV is regulated. Calnuc-(309–324) peptide, 95% purity), was synthesized from rat brain membranes or 2–6 μg purified His-tagged proteins were added to each tube, and binding reactions were carried out overnight at 4 °C with constant tumbling. Beads were washed four times with 1 ml of wash buffer (4 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 10 mM MgCl2, 5 mM EDTA, and 2 mM DTT supplemented with GDP, GDP plus AlCl3, and NaF or GTPγS as during binding) and boiled in sample buffer for SDS-PAGE.

Immunoblotting—Proteins samples were separated on 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). In experiments using His-Calnuc, His-CalnucΔN, or His-NUCB2 all electrophoretic steps were performed in the presence of 4 mM urea, which increased the sensitivity of the immunodetection. Membranes were blocked with PBS supplemented with 5% nonfat milk before sequential incubation with primary and secondary antibodies. Infrared imaging was performed using an Odyssey imaging system (LI-COR Biosciences, Lincoln, NE). Primary antibodies were diluted as follows: anti-His, 1:2000; anti-Go13, 1:300, anti-Go13, 1:500; anti-GO13, 1:250.

**Steady-state GTPase Assay**—This assay was performed as described previously (14, 29). Briefly, His-GO13 (100 nm) was preincubated with different concentrations of His-CalnucΔN-(171–459) or GST-NUCB2 (173–333) for 15–30 min at 30 °C in assay buffer (20 mM Na-HEPES, pH 8, 100 mM NaCl, 1 mM EDTA, 2 mM MgCl2, 1 mM DTT, and 0.05% (w/v) C12E10). His-CalnucΔN and GST-NUCB2-(173–333) were used instead of full-length His-Calnuc or GST-NUCB2 because the protein concentrations used in these experiments were achievable for only the truncated proteins, which express at higher yields in bacteria. GTPase reactions were initiated at 30 °C by adding an equal volume of assay buffer containing 1 μM [γ-32P]GTP (50 cpm/fmol). Duplicate aliquots (50 μl) were removed at different time points, and reactions were stopped with 950 μl of ice-cold 5% (w/v) activated charcoal in 20 mM H3PO4 pH 3. Samples were then centrifuged for 10 min at 10,000 × g, and 500 μl of the resultant supernatant was scintillation-counted to quantify released [32P]Pi. To determine the specific P, the background [32P]Pi, detected at 10 min in the absence of G protein was subtracted from each reaction.

**GTPγS Binding Assay**—GTPγS binding was measured using a filter binding method. His-GO13 (100 nm) was preincubated in the presence or absence of 45 μM His-CalnucΔN for 30 min at 30 °C in assay buffer (20 mM Na-HEPES, pH 8, 100 mM NaCl, 1 mM EDTA, 25 mM MgCl2, 1 mM DTT, and 0.05% (w/v) C12E10). Reactions were initiated at 30 °C by adding an equal volume of assay buffer containing 1 μM [35S]GTPγS (50 cpm/fmol). Duplicate aliquots (25 μl) were removed at different time points, and binding of radioactive nucleotide was stopped by the addition of 3 ml of ice-cold wash buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 25 mM MgCl2). The quenched reactions were rapidly passed through BA-85 nitrocellulose filters.
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(GE Healthcare) and washed with 4 ml of wash buffer. Filters were dried and subjected to liquid scintillation counting. Experiments designed to study the effect of Ca²⁺ were performed as described above except that no EDTA was used and different concentrations of CaCl₂ were added.

Cell Culture, Transfection, and Immunoprecipitation—

COS-7 cells were grown at 37 °C in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 1% l-glutamine, and 5% CO₂, and transfected with plasmids encoding for ΔSS-Calnuc-CFP (lacking the signal sequence; aa 2–25, 0.5 µg) and Gα₅-FLAG (6 µg) or vector control (6 µg) using Genejuice as described previously (29). 36 h after transfection the cells were maintained overnight in DMEM alone (~1.8 mM calcium) and then stimulated or not with 1 µM thapsigargin or 100 µM ATP for 90 s, rinsed quickly twice with ice-cold PBS, scraped into lysis buffer (20 mM HEPES, pH 7.4, 5 mM Mg(CH₃COO)₂, 125 mM K(CH₃COO), 0.4% Triton X-100, and 1 mM EDTA) supplemented with phosphatase (Sigma) and protease (Roche Applied Science) inhibitor mixtures, passed through a 28-gauge needle at 4 °C, and cleared (14,000 × g for 10 min). COS-7 cell lysates (~1–2 mg) were incubated for 2.5 h at 4 °C with 2 µg anti-FLAG mAb (Sigma) followed by incubation with protein G-agarose beads (GE Healthcare) at 4 °C for an additional 45 min. Beads were washed four times with 1 ml of wash buffer (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 10 mM MgCl₂, 5 mM EDTA, and 2 mM DTT), and the bound immune complexes were eluted by boiling in SDS sample buffer.

Preparation of Detergent-soluble Extracts from Rat Brain Membranes—

Isolation of rat brain membranes was adapted from a fractionation procedure described previously for liver (32). Briefly, rat brains were homogenized in 10 mM HEPES-KOH, pH 7.4, 5 mM EDTA, and 0.5 mM sucrose with a Teflon-glass homogenizer and spun down at 1,000 × g for 10 min to sediment unbroken tissue and nuclei, and the resulting supernatant (postnuclear supernatant) was collected. Crude membranes were sedimented from the postnuclear supernatant by centrifugation at 100,000 × g at 4 °C. Lysates were cleared (14,000 × g for 10 min) and sediment unbroken tissue and nuclei, and the resulting supernatant (postnuclear supernatant) was collected. Crude membranes were sedimented from the postnuclear supernatant by centrifugation at 100,000 × g, aliquoted, and stored at −80 °C. Rat brain membrane lysates were freshly prepared prior to the pulldown experiments presented in Fig. 4 by solubilizing ~750 µg of protein/condition in binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% (v/v) Nonidet P-40, 10 mM MgCl₂, 5 mM EDTA, 2 mM DTT, and protease inhibitor mixture) for ~2 h at 4 °C. Lysates were cleared (14,000 × g for 10 min) before use in subsequent experiments.

Other Methods—Protein structure analysis and visualization was performed using ICM-Browser-Pro software (Molsoft Inc., San Diego). Data presented in Figs. 2C, 7C, and 8D and supplemental Fig. S4 were curve-fitted by nonlinear regression using Prism 4.0. (San Diego) to determine the Kᵦₛ, EC₅₀, and IC₅₀ values.

RESULTS

Identification of a Putative Gα₅-interacting Motif in Calnuc and NUCB2—By manual examination of the Calnuc sequence, we noticed a region with significant similarity to the GEF motif of GIV and two synthetic peptides with GEF activity, KB-752 (15) and GSP (33) (Fig. 1A). This motif (aa 309–324) overlaps with the C-terminal part of the second EF-hand of Calnuc and is also found in NUCB2, a protein highly homologous to Calnuc (62% aa sequence identity). Phylogenetic analysis revealed that this motif is evolutionarily conserved in both Calnuc and NUCB2 proteins from invertebrates to humans (supplemental Fig. S1). In addition, the structure of this motif extracted from the NMR coordinates of the calcium-binding domain of Calnuc (34) is very similar to the established crystal structure of the α3 helix.
KB-752 peptide bound to Goi1 (15) and the homology-based structural prediction for the GEF motif of GIV bound to Goi3 (14) (Fig. 1B). These observations indicate that Calnuc and NUCB2 possess an evolutionarily conserved motif with structural similarity to the GEF motif of GIV and GIV-related peptides that display GEF activity.

Calnuc and NUCB2 Bind Preferentially to Inactive Goi3—Based on the sequence and structural similarities described above, we reasoned that Calnuc and NUCB2 might have similar Go binding properties to those of the GEF motif of GIV and related peptides that bind specifically to inactive GDP-bound Go subunits. We found that this was indeed the case for both Calnuc (Fig. 2A) and NUCB2 (Fig. 2B). GST-Calnuc bound robustly to inactive, GDP-loaded His-Goi3, but not to the G protein when it was activated by either GDP-AlF4− or GTPγS loading (Fig. 2A). Similar results (data not shown) were obtained in pulldown assays using GST-Goi3 and His-tagged Calnuc or CalnucΔN-(171–459), an N-terminally truncated construct containing the putative Go-binding motif. Similarly, His-NUCB2 showed robust binding to GST-Goi3 in the presence of GDP but not in the presence of GDP-AlF4− (Fig. 2B). The interaction of GST-Calnuc and GST-NUCB2-(173–333) containing the putative Go-binding motif, with GDP-loaded His-Goi3, has a dissociation constant (Kd) of 3.7 ± 1.2 μM and 1.0 ± 0.3 μM, respectively (Fig. 2C). These results indicate that much like GIV and the GIV-related peptides, binding of Calnuc and NUCB2 to Goi3 is state-dependent with a marked preference for the inactive conformation.

Calnuc and NUCB2 Bind to the α3/Switch II Cleft on Goi3—Next we performed competition assays to determine whether Calnuc and NUCB2 shared a common binding site on Goi3 with the synthetic KB-752 peptide and GIV, which bind to the hydrophobic cleft circumscribed by the α3 helix and “switch II” (SwII) (14, 15). We found that increasing concentrations of KB-752, but not a control peptide (see "Experimental Procedures"), decreased His-Goi3 binding to GST-Calnuc (Fig. 3A). Similarly, increasing concentrations of a peptide (aa 309–324) corresponding to the putative Goi3 binding sequence of Calnuc, but not a control peptide, decreased the amount of His-GIV-CTs (aa 1660–1870, containing the GEF motif of GIV) that bound to GST-Go13 (Fig. 3B). We also performed similar competition assays with GST-NUCB2-(173–333) and found that it also competed with the KB-752 peptide (Fig. 3C) or His-GIV-CTs (Fig. 3D) but not with their respective controls for binding to Goi3. Taken together these results suggest that Calnuc and NUCB2 bind specifically to the α3/SwII cleft of Goi3-GDP via the newly identified motif.

Characterization of Calnuc Specificity for Go Subunits—We have previously shown that Calnuc can interact with Goi3, Goi5, and Goi6 but not Goi12/13 or Goi4 in yeast two-hybrid assays (21). Next we investigated the relative strength of the interaction of Calnuc with Goi3, Goi5, and Goi6 using in vitro protein-protein binding assays. We found that GST-Calnuc bound GDP-loaded Goi3 from rat brain membrane lysates, whereas binding of Goi5 was very weak and binding of Goi6 was undetectable (Fig. 4A). We further performed pulldown assays using purified GST-Go13, GST-Go12, and GST-Go13 and His-CalnucΔN to investigate whether Calnuc binds to other Goi subunits. We used CalnucΔN instead of full-length Calnuc because initial experiments indicated that they shared virtually identical Goi binding properties (data not shown). We found that His-CalnucΔN binds strongly to GDP-loaded GST-Go13 and “GST-Go13 but showed significantly less binding to Goi12 (Fig. 4B). These results indicate that the binding preference of Calnuc for Goi subunits is Goi13 ≈ Goi3 > Goi12 ≫ Goi5 ≈ Goi4.
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Lys-248 in Gα<sub>f3</sub> Determines the Preferential Binding of Calnuc to Gα<sub>f3</sub> versus Gα<sub>o</sub>.—Recently we found that a single residue differing between the Gα<sub>o</sub> and Gα<sub>f</sub> subunits, i.e. Trp-258 in Gα<sub>f3</sub> and Phe-259 in Gα<sub>o</sub> (Fig. 5A), accounts for the preferential binding of GIV to Gα<sub>f3</sub> versus Gα<sub>o</sub> (29). To determine whether this is the case for Calnuc, we investigated the effect of mutating Trp-258 in Gα<sub>f3</sub> and Phe-259 in Gα<sub>o</sub> on the binding of these G proteins to Calnuc. GST-Calnuc bound robustly to purified wild-type His-Gα<sub>f3</sub> but not to purified wild-type His-Gα<sub>o</sub> (Fig. 5B); this striking preference remained unchanged when Trp-258 in Gα<sub>f3</sub> was mutated to Phe or when Phe-259 in Gα<sub>o</sub> was mutated to Trp (Fig. 5B). This indicates that Trp-258 in Gα<sub>f3</sub> is not responsible for the preferential binding of Calnuc to Gα<sub>f3</sub> versus Gα<sub>o</sub>.

We reasoned that Lys-248 in Gα<sub>f3</sub> could be responsible for the preferential binding to Gα<sub>f3</sub> versus Gα<sub>o</sub> because it is the only amino acid that is not conserved between the two Gα<sub>f</sub> subunits in the Calnuc binding site (Fig. 5A), i.e. the α3/SwII cleft (Fig. 3). Mutation of Lys-248 in GST-Gα<sub>f3</sub> to Met, the corresponding residue in Gα<sub>o</sub>, dramatically decreased His-Calnuc binding (Fig. 5C). Importantly, GST-Gα<sub>f3</sub> K248M did bind to GIV, AGS3 (activator of G protein signaling 3), and Gβγ (Ref. 29 and data not shown), indicating that this mutation specifically affects Calnuc binding. Conversely, when Met-249 in His-Gα<sub>o</sub> was mutated to Lys, it enhanced its binding to GST-Calnuc compared with wild-type His-Gα<sub>o</sub> (Fig. 5D). Furthermore, structural analysis (supplemental Fig. S2A) revealed that the positively charged Lys-248 of Gα<sub>f3</sub> might establish an electrostatic interaction with negatively charged Glu-314 of Calnuc. We hypothesized that inverting the charge of the Gα<sub>f3</sub> Lys-248 alone would impair the Gα<sub>f3</sub>–Calnuc interaction by electrostatic repulsion of the Calnuc Glu-314 but that inverting the
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FIGURE 4. Calnuc binds to different Gαi subunits but not to Gαo or Gαz.
A, Upper panel, GST-Calnuc binds Gαo (lane 3) but not Gαo (lane 7) or Gαz (lane 11) from rat brain membrane lysates in the presence of GDP but not GDP-AlF4 (lanes 4, 8, and 12). Solubilized proteins from 750 μg of rat brain membranes were incubated with ~20 μg of purified GST (lanes 2, 6, and 10) or GST-Calnuc (lanes 3, 4, 7, 8, 11, 12, and 13) immobilized on glutathione beads in the presence of GDP (30 μM; lanes 2, 3, 6, 7, 10, and 11) or GDP and AlF4− (AlCl3, 30 μM; NaF, 10 mM; lanes 4, 8, and 12). An additional control without rat brain membrane lysate was performed to validate Gαo antibody specificity (lane 13). Input (lanes 1, 5, and 9), 10% of the membrane lysate. No binding of Gαo, Gαo, or Gαz to the negative control GST was detected (lanes 2, 6, and 10). The arrows (lanes 1, 3, 5, and 9) denote the specific bands corresponding to the different Gα subunits (including the long and short splice forms of Gα, lane 9), and the star (lanes 11, 12, and 13) denotes a nonspecific band recognized by the Gαo antibody. IB, immunoblot. Lower panel, equal loading of GST proteins was confirmed by Ponceau S staining. B, His-CalnucΔN binds to GST-Gαo-GDP (lane 3) and GST-Gαo-GDP (lane 5) to a greater extent (~20-fold) than to GST-Gαo-GDP (lane 4) and binds only marginally to either of the Gαo subunits preloaded with GDP-AlF4− (lanes 6, 7, and 8) or GST (lane 2). 10 μg of His-CalnucΔN was incubated with 15 μg of GST (lane 2), GST-Gαo, (lanes 3 and 6), GST-Gαo (lanes 4 and 7), or GST-Gαo (lanes 5 and 8) preloaded with GDP (lanes 2–5) or GDP-AlF4− (lanes 6–8), immobilized on glutathione beads, and analyzed as described for Fig. 2A. Input (lane 1), 1 μg of His-CalnucΔN.

stabilizes Gαi3-Calnuc binding. These results demonstrate that although GIV and Calnuc have an overlapping binding site on Ga subunits, i.e. the α3/SwII cleft, and display preference for Gαi versus Gαo, the critical residues in the Ga subunit that determine binding specificity are different.

Identification of Residues in Calnuc Required for Binding and Regulating Gαi—Calnuc, NUCB2, GIV and GIV-related peptides share hydrophobic residues in positions 3, 6 and 7 of the consensus sequence depicted in Fig. 1A. In the structure of the KB-752-Gαi3 complex these residues are packed against the hydrophobic cleft formed by the α3 helix and switch II to stabilize the interaction (Ref. 15 and Fig. 1B). We reasoned that residues in the same position might also be required for Calnuc and NUCB2 to bind Gαi3. We found that mutation of each of the corresponding residues in Calnuc, i.e. Leu-313, Phe-316, and Leu-317 to Ala dramatically reduced His-Gαi3 binding to GST-Calnuc (Fig. 6A). The double mutant L313A/L317A decreased the interaction even further than the single mutations (Fig. 6A, see high exposure blot). Similar findings were obtained for NUCB2 (Fig. 6B), indicating that these hydrophobic residues are required for both Calnuc and NUCB2 to interact with Gαi3. In addition, mutation of Gαi3 Trp-211 or Phe-215 in the predicted binding site for Calnuc (supplemental Fig. S3A) also disrupted the interaction (supplemental Fig. S3B), suggesting that they mediate a hydrophobic interaction with the Calnuc-Leu-313, Phe-316, and Leu-317. These results indicate that the interaction of Calnuc and NUCB2 with Gαi3 requires the hydrophobic residues found in their conserved motif shared with GIV and GIV-related peptides.

We next investigated the effect of Calnuc and NUCB2 on G protein activation. For this we measured the steady-state GTPase activity of His-Gαi3 (which depends directly on the rate of nucleotide exchange (29, 35)) in the presence of wild-type His-CalnucΔN or His-CalnucΔN L313A/L317A, which has dramatically impaired binding to Gαi3 (Fig. 6A) (negative control). His-CalnucΔN was used instead of full-length His-Calnuc because the protein concentrations used in these experiments were achievable only for the truncated protein, which expresses at higher yields in bacteria (see “Experimental Procedures”). Wild-type CalnucΔN but not CalnucΔN L313A/L317A increased the rate of steady-state GTP hydrolysis (Fig. 7A). Similarly, wild-type GST-NUCB2 but not its binding-deficient mutant, L315A/L319A, also increased the steady-state GTPase activity of Gαi3 (Fig. 7B). Other mutants of the Gαi-binding motif such as Calnuc F316A and NUCB2 F318A also impaired Gαi3 activation when compared with their respective wild-type controls (supplemental Fig. S4), suggesting that Calnuc and NUCB2 are GEFs for Gαi3 and that they work via their conserved Gα-binding motif. Wild-type CalnucΔN and NUCB2 increased the GTPase activity of His-Gαi3 1.74- ± 0.18-fold (n = 6) and 1.76- ± 0.13-fold (n = 3), respectively, at the maximal concentration tested (Fig. 7C). The EC50 values were 7.3 ± 1.0 μM and 4.0 ± 1.0 μM (Fig. 7C), which are in good agreement with their respective Kc values for Gαi3 binding (Fig. 2C). To further validate the role of Calnuc as a GEF, we performed GTPγS binding experiments, which are a direct measure of GDP for GTP exchange activity. Incubation of His-Gαi3 in the presence of CalnucΔN increased the initial rate of GTPγS bind-
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FIGURE 5. Lys-248 but not Trp-258 is responsible for preferential binding of Calnuc to Gαi3 versus Gαo. A, sequence alignment of Gαo, Gαi1, Gαi2, and Gαi3 indicating the Gαi3 and Gαo mutants studied. Rat Gαo, Gαi1, Gαi2, and Gαi3 sequences corresponding to the SwII and the α3 helix were obtained from the NCBI database and aligned using ClustalW. Conserved identical residues are shaded in black and similar residues in gray. The secondary structure elements (α = α-helix, β = β-sheet) indicated below the alignment are named according to their crystal structures. Residues within this region that are conserved among Gαi1, Gαi2, and Gαi3 but are different in Gαo were mutated in Gαi3 to the corresponding residues in Gαo (indicated below with arrows) or in Gαo, to the corresponding residues in Gαi3 (indicated above with arrows). B, wild-type His-Gαo-GDP (lane 1) and His-Gαo-GDP W258F (lane 5) but not wild-type His-Gαo-GDP (lane 3) or His-Gαo-GDP F259W (lane 7) bind to GST-Calnuc. No binding of any of the Gαi subunits loaded with GDP-AlF₄ to GST-Calnuc was detected (lanes 2, 4, 6, and 8). C, His-Gαi3 mutants study. Rat Gαi3 mutants (indicated below with arrows) were subjected to GST pull-down assay. No binding of His-Gαo (lanes 1 and 2), His-Gαi1 (lanes 3 and 4), His-Gαi2 (lanes 5 and 6), or His-Gαi3 W258F (lanes 7 and 8) with His-Gαi3 (lanes 3) or GDP-AlF₄ (lanes 2, 4, 6, and 8) was detected. D, pull-down assay of His-Gαi3 (lanes 3–6) and wild-type His-Gαi3 (lanes 1–2) in the presence of MgCl₂ or LiCl (data not shown). The two Ga subunits loaded with GDP, wild-type His-Gαi3 (lanes 1–2) or GDP-AlF₄ (lanes 3–6) were immobilized on glutathione beads, and analyzed as described in the legend for Fig. 2A. Effect of CaCl₂ on the interaction of Calnuc and NUCB2 has GEF activity toward Gαi3. E, COS-7 cells were co-transfected with Gαi3-FLAG and a truncated Calnuc (ΔSS-Calnuc-CFP), lacking the signal sequence which is present pre-
dominantly in the cytosol (26). Cells were stimulated with thapsigargin (which elevates the cytosolic levels of Ca\textsuperscript{2+} by blocking the endoplasmic reticulum Ca\textsuperscript{2+} pump) or ATP (which activates purinergic receptors at the cell surface) (22), and immunoprecipitation was carried out using anti-FLAG IgG followed by immunoblotting for Calnuc. We found that noprecipitation was carried out using anti-FLAG IgG followed by immunoprecipitation of G\textsubscript{i3} with either thapsigargin or ATP (Fig. 8). Co-immunoprecipitation of GFP with G\textsubscript{i3}/FLAG was not affected by thapsigargin or ATP, indicating that elevation of the intracellular levels of Ca\textsuperscript{2+} specifically affects the interaction of G\textsubscript{i3} with Calnuc but not other G\textsubscript{i}-binding proteins. These results suggest that elevation of intracellular Ca\textsuperscript{2+} levels can efficiently disrupt the interaction between Calnuc and G\textsubscript{i3} in living cells, corroborating our observations in vitro. Taken together, these data indicate that calcium binding can promote conformational changes in Calnuc (and presumably also in NUCB2) that block its interaction with G\textsubscript{i} subunits in vitro and in living cells (Fig. 8F), subsequently inhibiting its GEF activity.

**DISCUSSION**

In this work we identify a new class of G protein-binding motif with defined structural features. This motif is found in two closely related proteins, Calnuc and NUCB2, and was previously found in another unrelated protein, GIV, and in the synthetic peptides KB-752 and GSP, shown previously to have GEF activity for G\textsubscript{i} (15, 33). It consists of a relatively disordered N-terminal region followed by an \( \alpha \)-helix that docks onto the \( \alpha \text{3}\)/SwII cleft of the G\textsubscript{i} subunits only in the inactive conformation to enable GEF activity in vitro. We named this signature sequence the GBA motif (for G\textsubscript{i}-binding and -activating motif). We propose that the conserved GBA motif found in native proteins is a signature structure that defines a new family of G protein regulators with GEF activity, in the same fashion as the GoLoco/GPR motif or the RGS box define families of proteins with GDI or GTPase-activating protein activity, respectively. An important observation is that the GBA motif in Calnuc is evolutionarily conserved across species from sponges to man (supplemental Fig. S1), and the *Caenorhabditis elegans* orthologues of Calnuc and the G\textsubscript{i} subunits have been shown to interact (36), suggesting that its function as a G\textsubscript{i}-binding motif is also evolutionarily conserved. This evolutionary conservation suggests a selection imposed by a crucial biological function associated with the interaction with G\textsubscript{i}. It is interesting that a similar consensus motif was found in two synthetic peptides, KB-752 and GSP, which were identified by two different in vitro approaches, phage display of random sequence peptides (15) and iterative optimization of in vitro mRNA-translated peptides (33). In both cases the selection is determined solely by the chemical properties of the peptides and not their biological function. These observations suggest that the sequences found in vivo in the GBA motif of Calnuc, NUCB2, and GIV have highly optimized chemical properties for G\textsubscript{i} binding.

Based on the sequences of Calnuc, NUCB2, and GIV in different species (supplemental Fig. S1 and Ref. 14) and related synthetic peptides (15, 33), we propose that the GBA motif can be defined by a conserved core sequence of seven amino acids (Fig. 1A). \( \Psi^-[S/T]^-[\Phi/\Psi]^-[X^-][D/E]^-F^-\Psi \), in which \( \Psi \) are aliphatic residues and \( \Phi \) are aromatic residues. Residues in positions 3, 6, and 7 in this consensus motif, \( i.e., \text{Leu-313, Phe-316, and Leu-317 in Calnuc, correspond to hydrophobic residues aligned on one side of the } \alpha \text{-helical part of the motif, which are used to stabilize the interaction with G}\textsubscript{i} \text{by packing against the } \alpha\text{3/SwII hydrophobic cleft. Residues in positions 2 and 5 form a hydrogen bond in the structures of KB-752 and Calnuc, which is required for the motif to adopt its helical conformation. This design for molecular coupling resembles that observed for other signaling interfaces. For example, A-kinase-anchoring proteins (AKAPs) are characterized by a signature motif that} \)
forms an aliphatic helix that docks onto a hydrophobic pocket on the regulatory subunit of cAMP-dependent protein kinase (PKA) (37), and the N-terminal region of the GoLoco/GPR motif, also binds to the α3/SwII hydrophobic cleft of Gαi subunits via an aliphatic helix (10).

Our results also provide the structural basis for the regulation of Calnuc and NUCB2 binding to and activation of Gαi subunits by calcium. Calnuc is the major calcium-binding protein in the lumen of Golgi cisternae, where it regulates the intracellular calcium stores (21, 22). On the other hand, there is a cytosolic pool of Calnuc that interacts directly with Gαi3 (26). NUCB2 has been described as sharing a similar subcellular distribution (20). The regulation of the Calnuc/NUCB2-Gαi interaction by Ca2+ described in this work is not surprising considering that the Gαi-binding motif on Calnuc overlaps with the EF-hands responsible for calcium binding. Our finding that Gαi binding to Calnuc and NUCB2 is impaired by calcium binding is compatible with previous observations by NMR indicating that when calcium-bound, the Calnuc EF-hands fold into a globular domain that hides the Gαi-binding residues, whereas in the absence of calcium this domain is disordered and probably exposes the Gαi-binding motif (Fig. 8F). Thus, we propose that the conformational changes in Calnuc and NUCB2 that occur upon Ca2+ binding regulate their interaction with the G protein and its subsequent activation. This mode of regulation by Ca2+ is consistent with our results presented here indicating that Calnuc and Gαi3 interact in the cytosol of cells under resting conditions (in accord with our own previous observation using FRET and live cell microscopy (26)) but not upon stimulation with thapsigargin or ATP (Fig. 8E). This is probably because in resting conditions the cytosolic concentration of free Ca2+ (50–100 nM) is significantly lower than the $K_d$ value of Calnuc for Ca2+ binding (~7 μM (22)), thereby allowing the interaction of calcium-free Calnuc with Gαi3, whereas upon stimulation with thapsigargin or ATP the intracellular levels of Ca2+ are increased and calcium-bound Calnuc cannot interact with Gαi3. It will be important in the future to ask whether the regulation of the Calnuc-Gα3 interaction by Ca2+ might influence the interplay between G protein- and calcium-dependent signaling, two major signaling events that regulate a multitude of cellular functions.

Our data also unveiled the structural basis for the state-dependent binding of Calnuc and NUCB2 to Gαi subunits. Based on the structures of Gα1i and other Gα subunits bound to GTPγS and GDP-AlF4 (38, 39), the hydrophobic cleft circumscribed by the α3 helix and the switch II is occluded when the G protein adopts the active conformation, thereby hindering its interaction with Calnuc and NUCB2 by steric clashes. From this we concluded that conformational changes of Gα3 upon activation determine its interaction with Calnuc and NUCB2. We previously reported that Calnuc binds to a site different from the α3/SwII cleft (i.e. the α5 helix) of Gα3i by using C-terminal truncations of the G protein (25). Although we cannot rule out the presence of two binding sites for Calnuc on Gαi subunits, one likely explanation for the previous results is that truncation of the Gαi C terminus promotes constitutive activation of the G protein (40) which in light of the data presented here would abolish its interaction with Calnuc and NUCB2.

The studies presented here also provide insights into the specific features of the Calnuc-Gαi subunit interface and its differences from another Gαi motif-containing protein, i.e. GIV. Both Calnuc and GIV bind preferentially to Gαi subunits over Gαi or Gαo, but Calnuc shows preference for Gαi1 and Gαi3 over Gαi2, whereas GIV binds equally to Gαi1, Gαi2, and Gαi3 in vitro (14). The basis for the preference of Calnuc for Gαi1 and Gαi3 over Gαi2 is still unclear, because all Gαi subunits have identical residues in the switch II and the α3 helix (Fig. 5A), which based on our results presented here is a major binding site for Calnuc on the G protein. It is possible that other residues of the G protein outside of this major binding surface, i.e. the α3/SwII cleft, may also determine the specificity of binding by making additional contacts, as reported for other G protein regulators with preference for Gαi1 and Gαi3 over Gαi2 such as...
GAIP/RGS19 (41), RGS14 (42, 43), and RGS12 (44). As in the case of GIV (29), binding of Calnuc to Gαi is marginal compared with binding to Gαs. We provide evidence here that the preferential binding of Calnuc to Gαi over Gαs is determined by Lys-248, located in the middle of the α helix of the G protein, but not by Trp-258, located in the α3/β5 loop, which we have previously shown to be responsible for the preferential binding of GIV to Gαi versus Gαs (29). Based on the results presented here on Calnuc (Figs. 5 and 6 and supplemental Figs. S2 and S3) and our own published (14, 29) and unpublished data on GIV, we propose that conserved hydrophobic residues in Calnuc and the GIV GBA motif are required to dock onto a common spot of Gαi, i.e. the α3/β5 hydrophobic cleft, whereas nonconserved residues establish a set of contacts with the G protein that is different for Calnuc or GIV (see details in supplemental Fig. S5). These variations in the common theme highlight the uniqueness of the interfaces formed between different GBA motif-containing proteins and the G protein, which may be relevant for achieving spec-
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ificity in the pharmacological targeting of these interfaces for therapeutic purposes, as proposed for GIV (14, 29).

Our results suggest a role for Calnuc and NUCB2 as regulators of G protein activity. The affinity of Calnuc and NUCB2 for Goα3 (Kd ~ 4 and ~1 μM, respectively) is lower than that of GIV (Kd ~ 300 nm, data not shown) but similar to the GEF peptide KB-752 (Kd ~ 4 μM (15)) and other G protein regulators such as the GDI proteins LGN (Kd ~ 6 μM (45)) and G18/AGS4 (Kd ~ 2.5 μM (46)). Like GIV and related synthetic peptides, Calnuc and NUCB2 possess GEF activity in vitro, indicating that this is a common feature associated with the conserved GBA motif. While this manuscript was in preparation, Kapoor et al. (47) reported that Calnuc possesses GDI activity toward Goα1. Although the reason for the discrepancy between their work and ours is not clear, one possible explanation is the different physiological setting. Further investigations will be required to elucidate whether Gα3 binds to each other in living cells as determined by FRET and live cell imaging (26) and that Calnuc regulates the subcellular localization of Goα3 (27), suggesting a functional role for the Calnuc-Gα coupling in the physiological setting. Further investigations will be required to elucidate whether Goα3 activation by Calnuc occurs in vivo and to determine the functional consequences of the interaction.

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REFERENCES

1. Sato, M., Blumer, J. B., Simon, V., and Lanier, S. M. (2006) Annu. Rev. Pharmacol. Toxicol. 46, 151–187
2. Blumer, J. B., Smrcka, A. V., and Lanier, S. M. (2007) Pharmacol. Ther. 113, 488–506
3. Siderovski, D. P., and Willard, F. S. (2005) Int. J. Biol. Sci. 1, 51–66
4. De Vries, L., Zheng, B., Fischer, T., Elenko, E., and Farquhar, M. G. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 235–271
5. Ross, E. M., and Wilkie, T. M. (2000) Annu. Rev. Biochem. 69, 795–827
6. De Vries, L., Fischer, T., Tronchère, H., Brothers, G. M., Strockbine, B., Siderovski, D. P., and Farquhar, M. G. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 14364–14369
7. Peterson, Y. K., Bernard, M. L., Ma, H., Hazard, S., 3rd, Graber, S. G., and Lanier, S. M. (2000) J. Biol. Chem. 275, 33193–33196
8. Tesmer, J. I., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) Cell 89, 251–261
9. Soundararajan, M., Willard, F. S., Kimple, A. J., Turnbull, A. P., Ball, L. I., Schoch, G. A., Gileadi, C., Fedorov, O. Y., Dowler, E. F., Higman, V. A., Hutsell, S. Q., Sundström, M., Doyle, D. A., and Siderovski, D. P. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 6457–6462
10. Kimple, R. J., Kipple, M. E., Betts, L., Sonde, J., and Siderovski, D. P. (2002) Nature 416, 878–881
11. Kimple, A. J., Yasar, A., Hughes, M., Jadav, A., Willard, F. S., Muller, R. E., Austin, C. P., Inglese, I., Ibeanu, G. C., Siderovski, D. P., and Simeonov, A. (2008) Comb. Chem. High Throughput Screen. 11, 396–409
12. Sjogren, B., Blazer, L. L., and Neubig, R. R. (2010) Prog. Mol. Biol. Transl. Sci. 91, 81–119
13. Le-Niculescu, H., Niesman, I., Fischer, T., DeVries, L., and Farquhar, M. G. (2005) J. Biol. Chem. 280, 22012–22020
14. Garcia-Marcos, M., Ghosh, P., and Farquhar, M. G. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 3178–3183
15. Johnston, C. A., Willard, F. S., Jezeky, M. R., Fredrickks, Z., Bodor, T. E., Jones, M. B., Blaesius, R., Watts, V. J., Harden, T. K., Sondek, J., Ramer, I. K., and Siderovski, D. P. (2005) Structure 13, 1069–1080
16. Garcia-Marcos, M., Jung, B. H., Ear, J., Cabrera, B., Carethers, J. M., and Ghosh, P. (2011) FASEB J. 25, 590–599
17. Ghosh, P., Garcia-Marcos, M., Bornheimer, S. J., and Farquhar, M. G. (2008) J. Cell Biol. 182, 381–393
18. Anai, M., Shojima, N., Katagiri, H., Ogihara, T., Sakoda, H., Onishi, Y., Ono, H., Fujishiro, M., Fukushima, Y., Horike, N., Viana, A., Kikuchi, M., Noguchi, N., Takahashi, S., Takata, K., Oka, Y., Uchijima, Y., Kurihara, H., and Asano, T. (2005) J. Biol. Chem. 280, 18525–18535
19. Weng, L., Enomoto, A., Ishida-Takagishi, M., Asai, N., and Takahashi, M. (2010) Cancer Sci. 101, 836–842
20. Morel-Huaux, V. M., Pypaert, M., Wouters, S., Tartakoff, A. M., Jurgan, U., Gevaert, K., and Courtoy, P. J. (2002) Eur. J. Cell Biol. 81, 87–100
21. Lin, P., Le-Niculescu, H., Hofmeister, R., McCaffery, J. M., Jin, M., Hennen, H., McQuistan, T., De Vries, L., and Farquhar, M. G. (1998) J. Cell Biol. 141, 1515–1527
22. Lin, P., Yao, Y., Hofmeister, R., Tsien, R. Y., and Farquhar, M. G. (1999) J. Cell Biol. 145, 279–289
23. Miura, K., Kurosawa, Y., and Kanai, Y. (1994) Biochem. Biophys. Res. Commun. 199, 1388–1393
24. Gilchrist, A., Au, C. E., Hiding, J., Bell, A. W., Fernandez-Rodriguez, I., Lesimple, S., Nagaya, H., Roy, L., Goscine, S. J., Hallett, M., Paient, I., Kearney, R. E., Nilsson, T., and Bergeron, I. J. (2006) Cell 127, 1265–1281
25. Lin, P., Fischer, T., Weiss, T., and Farquhar, M. G. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 674–679
26. Weiss, T. S., Chamberlain, C. E., Takeda, T., Lin, P., Hahn, K. M., and Farquhar, M. G. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 14961–14966
27. Lin, P., Fischer, T., Lavoie, C., Huang, H., and Farquhar, M. G. (2009) Mol. Neurodegener. 4, 15
28. Gump, J. M., June, R. K., and Dowdy, S. F. (2010) J. Biol. Chem. 285, 1500–1507
29. Garcia-Marcos, M., Ghosh, P., Ear, J., and Farquhar, M. G. (2010) J. Biol. Chem. 285, 12765–12777
30. Stols, L., Gao, M., Dieckman, L., Raffen, R., Collart, F. R., and Donnelly, M. I. (2002) Protein Expr. Purif. 25, 8–15
31. Studier, F. W. (2005) Protein Expr. Purif. 41, 207–234
32. De Vries, L., Elenko, E., McCaffery, J. M., Fischer, T., Hubler, L., McQuistan, T., Watson, N., and Farquhar, M. G. (1998) Mol. Biol. Cell 9, 1123–1134
33. Austin, R. J., Ja, W. W., and Roberts, R. W. (2008) J. Mol. Biol. 377, 1406–1418
34. de Alba, E., and Tjandra, N. (2004) Biochemistry 43, 10039–10049
35. Mukhopadhyay, S., and Ross, E. M. (2002) Methods Enzymol. 345, 350–369
36. Cuppen, E., van der Linden, A. M., Jansen, G., and Plasterk, R. H. (2003) Comp. Funct. Genomics 4, 479–491
37. Welch, E. J., Jones, B. W., and Scott, J. D. (2010) Mol. Interv. 10, 86–97
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38. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) Science 265, 1405–1412
39. Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993) Nature 366, 654–663
40. Denker, B. M., Schmidt, C. J., and Neer, E. J. (1992) J. Biol. Chem. 267, 9998–10002
41. Woulfe, D. S., and Stadel, J. M. (1999) J. Biol. Chem. 274, 17718–17724
42. Shu, F. J., Ramineni, S., Amyot, W., and Hepler, J. R. (2007) Cell. Signal. 19, 163–176
43. Mittal, V., and Linder, M. E. (2004) J. Biol. Chem. 279, 46772–46778
44. Webb, C. K., McCudden, C. R., Willard, F. S., Kimple, R. J., Siderovski, D. P., and Oxford, G. S. (2005) J. Neurochem. 92, 1408–1418
45. McCudden, C. R., Willard, F. S., Kimple, R. J., Johnston, C. A., Hains, M. D., Jones, M. B., and Siderovski, D. P. (2005) Biochim. Biophys. Acta 1745, 254–264
46. Kimple, R. J., Willard, F. S., Hains, M. D., Jones, M. B., Nweke, G. K., and Siderovski, D. P. (2004) Biochem. J. 378, 801–808
47. Kapoor, N., Gupta, R., Menon, S. T., Folta-Stogniew, E., Raleigh, D. P., and Sakmar, T. P. (2010) J. Biol. Chem. 285, 31647–31660
48. Willard, F. S., and Siderovski, D. P. (2006) Biochem. Biophys. Res. Commun. 339, 1107–1112