Nucleobindin 1 Is a Calcium-regulated Guanine Nucleotide Dissociation Inhibitor of Goi1

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Nucleobindin 1 (NUCB1) is a widely expressed multidomain calcium-binding protein whose precise physiological and biochemical functions are not well understood. We engineered and heterologously expressed a soluble form of NUCB1 (sNUCB1) and characterized its biophysical and biochemical properties. We show that sNUCB1 exists as a dimer in solution and that each monomer binds two divalent calcium cations. Calcium binding causes conformational changes in sNUCB1 as judged by circular dichroism and fluorescence spectroscopy experiments. Earlier reports suggested that NUCB1 might interact with heterotrimeric G protein α subunits. We show that dimeric calcium-free sNUCB1 binds to expressed Goi1 and that calcium binding inhibits the interaction. The binding of sNUCB1 to Goi1 inhibits its basal rate of GDP release and slows its rate and extent of GTPγS uptake. Additionally, our tissue culture experiments show that sNUCB1 prevents receptor-mediated Goi-dependent inhibition of adenyl cyclase. Thus, we conclude that sNUCB1 is a calcium-dependent guanine nucleotide dissociation inhibitor (GDI) for Goi1. To our knowledge, sNUCB1 is the first example of a calcium-dependent GDI for heterotrimeric G proteins. We also show that the mechanism of GDI activity of sNUCB1 is unique and does not arise from the consensus GoLoco motif found in RGS proteins. We propose that cytoplasmic NUCB1 might function to regulate heterotrimeric G protein trafficking and G protein-coupled receptor-mediated signal transduction pathways.

Heterotrimeric guanine nucleotide-binding proteins, G proteins, couple to heptahelical cell surface G protein-coupled receptors (GPCRs) and participate in intracellular signaling events. The G protein heterotrimer is composed of the Go subunit and the Gβγ heterodimer. Upon ligand-mediated activation, GPCRs catalyze the exchange of GDP for GTP on Go leading to dissociation of the heterotrimer into Go-GTP and Gβγ subunits (1–3). These individual subunits then regulate downstream signaling cascades involving effector systems like adenyl cyclase, Ca2+ and K+ channels, phospholipase C isozymes, and cyclic nucleotide phosphodiesterases (4, 5). Thereafter, the intrinsic GTPase activity of Go reverts it back to the GDP-bound state, which can reassociate with Gβγ. This inhibits the interaction of G protein subunits with downstream effectors, which results in the turning-off of the signaling pathways. Hence, signaling by heterotrimeric G proteins is directly dependent on the lifetime of the GTP-bound state of Go. This lifetime is regulated by GTPase-accelerating proteins (GAPs), which catalyze the rapid hydrolysis of the Go-bound GTP to GDP and by guanine nucleotide dissociation inhibitors (GDIs), which inhibit the exchange of GDP for GTP in the catalytic pocket of Go (6).

Together, GAPs and GDIs exert a regulatory control on G protein signaling. In recent years, novel interacting partners of heterotrimeric G proteins called the regulators of G protein signaling or RGS proteins have been discovered that possess GAP activity (7, 8). Interestingly, RGS12 and RGS14 in addition to functioning as GAPs can also act as GDIs (9, 10). In 1999, Lanier and co-workers (11) used yeast two-hybrid analysis to identify distinct receptor-independent activators of G protein signaling or AGS proteins. Several members of this AGS family (AGS3–6) have been shown to function as GDIs of Goi subunits (12). The observed GDI activity of both AGS and RGS proteins toward Goi1α is attributed to a 19-amino acid consensus sequence called the GoLoco motif (9, 13, 14). Earlier in 1995, in an independent yeast two-hybrid screen, Mochizuki et al. (15) established the interaction of a novel Golgi-resident Ca2+/-binding protein Nucleobindin 1 or NUCB1, specifically with the heterotrimeric G protein α subunit, Goi1α. Subsequently, Lin et al. (16) demonstrated that NUCB1 interacts exclusively with the adenyl cyclase inhibitory (Goi1) and stimulatory (Goi2) classes of Go subunits. In a recent study, it was shown that overexpression of NUCB1 causes redistribution of only the Goi1α subunits and not the Gβγ subunits to the plasma membrane and regulated secretion granules (17). However, a role for NUCB1 in modulating Goi1α activation and the biochemistry of NUCB1-Goi1α interaction has not been reported.
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NUCB1 is a 55-kDa multidomain Ca\(^{2+}\)-binding protein that was first identified as a novel B cell growth and differentiation factor associated with lupus syndrome (18). NUCB1 derives its trivial name, Calnuc, from its Ca\(^{2+}\)-binding and DNA-binding ability (19). The DNA-binding domain of basic residues (172–218) lies at the N terminus following the signal sequence. The Ca\(^{2+}\)-binding domain is at the core of the protein sequence consisting of two EF hand motifs with an intervening acidic region (residues 253–316). The Ca\(^{2+}\)-binding domain is followed by a leucine zipper domain (residues 347–389), which has been postulated to induce NUCB1 dimerization (Fig. 1A) (20). The C-terminal (CT) region following the leucine zipper domain is predicted to be intrinsically disordered and unstructured. Intriguingly, NUCB1 is strongly conserved from flies to humans (21) and is widely distributed among Golgi (16, 22), nucleus (19, 23), endoplasmic reticulum (24, 25), and cytoplasm (17). The N-terminal signal sequence of NUCB1 targets it to different membrane compartments and its deletion renders NUCB1 cytosolic.

Recently, Brodeur et al. (26) reported the role of NUCB1 in LDL receptor-related protein 9 (LRP9) trafficking where the cytosolic NUCB1 fraction helps in LRP9 endosomal sorting and prevents its delivery to lysosomes. The ubiquitous expression of NUCB1 in various cell and tissue types results in a diverse interplay, including interacting partners like G protein \( \Gamma \) subunits, cyclooxygenases, and amyloid precursor protein (16, 17). Because G \( \Gamma \) subunits have been shown to interact with NUCB1 (16, 25–27). Several classes of G \( \Gamma \) subunits are ubiquitously involved in diverse signaling pathways. NUCB1 interacts with G \( \Gamma \) subunits, cyclooxygenases, and amyloid precursor protein (16, 25–27). Several classes of G \( \Gamma \) subunits have been shown to interact with NUCB1 (16, 25–27). Yeast two-hybrid experiments performed with various deletion constructs of either G\( \Gamma_{i3} \) or NUCB1 mapped the C-terminal \( \alpha \) helix domain of the G protein and the acidic region of NUCB1 (residues 264–305) to be necessary for interaction (28). Immunofluorescence-based studies showed that NUCB1 and G\( \Gamma \) protein subunits co-localize on the Golgi lumen and regulated secretion granules (17). Because G\( \Gamma \) subunits are ubiquitously involved in numerous signal transduction pathways in different tissue types, their interaction with NUCB1 might regulate downstream signaling events.

We are interested in understanding the role of the \( \alpha \) helix of the G protein \( \alpha \) subunit in regulating nucleotide exchange rates (29–31). Because the \( \alpha \) helix of G\( \Gamma \) is involved in interaction with NUCB1, we characterized and studied the interaction of NUCB1 with G\( \Gamma_{i1} \) in detail. In this study, we present the detailed biological and biochemical characterization of the Ca\(^{2+}\)-binding ability and the oligomeric state of a heterologously expressed N-terminally truncated soluble form of NUCB1, termed sNUCB1 (Fig. 1A). We show that sNUCB1 binds Ca\(^{2+}\) and exists as a dimer in solution. Ca\(^{2+}\)-free sNUCB1 preferentially binds G\( \Gamma_{i1} \) and inhibits its GDP release as demonstrated by several independent assays. We have shown that overexpression of sNUCB1 in HEK293 cells markedly decreases ligand-dependent receptor-mediated G\( \Gamma \) inhibition of adenyl cyclase. We conclude that sNUCB1 serves as a novel Ca\(^{2+}\)-regulated GDI of G\( \Gamma_{i1} \). Our findings suggest that NUCB1 is involved in regulating cytoplasmic G protein signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Reagents**

GDP sodium salt and GTP\( \gamma \)S tetralithium salt were purchased from Sigma. The fluorescent GTP\( \gamma \)S analogue, mant\( (2',3'-O-(N-ethyl-anthranioloyl))-\)GTP\( \gamma \)S was purchased from Jena Biosciences (Jena, Germany), and BODIPY-FL-GTP\( \gamma \)S was purchased from Molecular Probes (Invitrogen). The His\( \delta \)tagged mutants of WT G\( \Gamma_{i1} \) were generated by site-directed mutagenesis using the high fidelity thermostable DNA polymerase Pfu (Stratagene). The cAMP dynamic 2 kit was purchased from CisBio (Bedford, MA) for cell-based studies. All reagents and chemicals used were of highest available purity.

**Heterologous Expression of sNUCB1 and Heterotrimeric G Protein \( \alpha \)-Subunit G\( \Gamma_{i1} \)**

The cDNA clones for human NUCB1 and rat G\( \Gamma_{i1} \) were obtained from the ATCC. The DNA fragment for the soluble form of NUCB1 or sNUCB1, corresponding to residues 31–461 of the human NUCB1 protein without the N-terminal signal sequence (residues 1–31), was cloned into the pET28a(+) expression vector (Amersham Biosciences). Similarly, the DNA fragment corresponding to full-length WT rat G\( \Gamma_{i1} \) subunit (residues 1–354) was cloned into the pET28a(+) expression vector. The vector was used to transform BL21 (DE3) cells to express an N-terminal His\( \delta \) tag protein containing a PreScission protease site directly after the His\( \delta \) tag. The expressed construct for sNUCB1 served as a template for introducing site-specific mutations using the QuickChange system (Stratagene, La Jolla, CA) to generate sNUCB1(W232A/W333A) or for introducing a missense mutation to generate the truncated version of the protein, namely sNUCB1(W333T). All proteins were expressed in BL21 (DE3) cells grown in the presence of 50 \( \mu \)g/ml kanamycin. Cells were grown at 37 °C to a\( A_{600 \text{ nm}} \) of 0.7 and then induced with 500 \( \mu \)M isopropyl-\( \beta \)-D-galactopyranoside (United States Biological). Post-induction, the culture was grown overnight at 17 °C and subsequently harvested. The resulting pellets were resuspended in a buffer containing 50 mm Tris–HCl, pH 8.0, 150 mm NaCl, 50 mm \( \beta \)-mercaptoethanol, bovine lung aprotinin (20 mg/ml), 2 mm phenylmethylsulfonyl fluoride (PMSF), and complete EDTA-free protease inhibitor mixture tablets (Roche Applied Science). Thereafter, the cells were lysed, and the expressed His\( \delta \) tag protein was purified from crude extract by affinity chromatography using nickel-nitritotriacetic acid column pre-equilibrated with buffer A (50 mm Tris, pH 8.0, 150 mm NaCl, 50 mm \( \beta \)-mercaptoethanol). The fluorescent GDP sodium salt and GTP\( \gamma \)S tetralithium salt were purchased from Sigma. The fluorescent GTP\( \gamma \)S analogue, mant(2',3'-O-(N-ethyl-anthranioloyl))-GTP\( \gamma \)S was purchased from Jena Biosciences (Jena, Germany), and BODIPY-FL-GTP\( \gamma \)S was purchased from Molecular Probes (Invitrogen). The His\( \delta \)tagged mutants of WT G\( \Gamma_{i1} \) were generated by site-directed mutagenesis using the high fidelity thermostable DNA polymerase Pfu (Stratagene). The cAMP dynamic 2 kit was purchased from CisBio (Bedford, MA) for cell-based studies. All reagents and chemicals used were of highest available purity.

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mm Tris, pH 8.0, 150 mM NaCl, 2 mM MgCl₂•6H₂O, 10 μM GDP, 1 mM DTT) for WT Gα₁₁ to obtain homogeneously pure protein. The typical yield for sNUCB1 and its variants was ~5 mg/liter and for WT Gα₁₁ ~12–15 mg/liter. The purity of the proteins was assessed by Coomassie Brilliant Blue staining after the proteins were separated through SDS-PAGE. The purity of all proteins was greater than 95%.

**Isothermal Titration Calorimetry (ITC)**

ITC was performed at 25 °C (298 K) using a MicroCal VP-ITC (MicroCal, Northampton, MA) calorimeter. To measure quantitatively the binding of Ca²⁺ or WT Gα₁₁-GDP to sNUCB1, 2 ml of 50 μM sNUCB1 (Ca²⁺-free) and 700 μl of either 500 μM CaCl₂•6H₂O (prepared in 50 mM Tris, pH 8.0, 150 mM NaCl) or 600 μM WT Gα₁₁-GDP (prepared in 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM MgCl₂•6H₂O, 10 μM GDP, 1 mM tris(2-carboxyethyl)phosphine) was used and thoroughly degassed. Contents of the sample cell were stirred continuously at 280 rpm during the experiment. A typical titration of sNUCB1 involved 50 injections each of 5 μl of CaCl₂•6H₂O (500 μM) or WT Gα₁₁-GDP (600 μM) into a sample cell containing 1.4 ml of sNUCB1 (50 μM). The heat of dilution of the titrant (CaCl₂•6H₂O or WT Gα₁₁-GDP) was subtracted from the titration data for base-line correction. The base line-corrected data were analyzed with MicroCal Origin™ 6.0 software to determine the change in enthalpy (ΔH) and association constant (Ko). Thermal titration data fit showed two sets of binding sites for Ca²⁺ to sNUCB1. The parameters were calculated based on three or more independent titration experiments. Each numerical value reported is the mean ± S.E. among the independent data sets.

**Size-exclusion chromatography**

Complex Formation between sNUCB1 and WT Gα₁₁—Complex formation between sNUCB1 and Gα₁₁ was monitored using SEC. 150 μM sNUCB1 (−Ca²⁺/+Ca²⁺) was incubated with 256 μM Gα₁₁-GTPγS in 20 mM Tris, pH 8.0, 100 mM NaCl, 10 mM MgCl₂•6H₂O, 10 μM GDP, and 1 mM DTT at 30 °C for 20 min with minimal shaking to enable saturation binding. WT Gα₁₁ in the GTPγS-bound state was generated by incubating WT Gα₁₁-GDP with 50 mM GDP, 1 mM DTT for 10 min with minimal shaking to enable saturation binding. Complete exchange of nucleotide. Furthermore, the complete exchange was confirmed by the AlF₄⁻ exchange assay. About 200 μl of the reaction mixture was injected onto Superdex200 10/30 GL column attached to AKTA FPLC (GE Healthcare) and run at 4 °C. Elution was done with the incubation buffer at a flow rate of 0.5 ml/min with 0.35-ml fractions collected. Peak fractions were analyzed through SDS-PAGE and Coomassie-stained to analyze the complex formation.

Estimation of sNUCB1 Molecular Mass Using Heavy Molecular Weight Standards—Purified sNUCB1 or the heavy molecular weight standards (GE Healthcare) were injected onto a Superdex200 10/30 GL column pre-equilibrated with buffer S, and the normalized absorbance of the eluting peak was plotted. A calibration curve of Kₒ ν (Vₑ − V₀/Vₑ − Vₒ) versus log₁₀(molecular weight) was plotted, where Vₑ is the elution volume; Vₒ is the column void volume corresponding to the elution of blue dextran, and Vₑ is the geometric column volume. The data were fit to a straight line curve like Y = A + Bx, where A = 1.915 and B = 0.311 were obtained from the fit. Therefore, for a Y = Kₒ ν value, the corresponding value of log₁₀(molecular weight) was obtained to estimate the molecular mass of sNUCB1 and sNUCB1(W333Ter).

**Light Scattering**

Multiangle Light Scattering (MALS) and Dynamic Light Scattering (DLS)—The light scattering data were collected using a Superdex200 10/30, HR SEC column (GE Healthcare), connected to an HPLC system (Agilent 1200, Agilent Technologies, Wilmington, DE) equipped with an autosampler. The elution from SEC was monitored by a photodiode array UV-visible detector (Agilent Technologies), differential refractometer (OPT-Lab Ex, Wyatt Corp., Santa Barbara, CA), static and dynamic, multiangle laser light scattering detector (HELEOS II with QELS capability, Wyatt Corp.). The SEC-UV/LS/RI system was equilibrated in buffer (50 mM Tris, pH 8.0, 150 mM NaCl) for sNUCB1 alone and in buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 10 μM GDP, 1 mM DTT) for WT and truncated sNUCB1/G protein complex at the flow rate of 1.0 ml/min. Two software packages were used for data collection and analysis; the Chemstation software (Agilent Technologies) controlled the HPLC operation and data collection from the multiwavelength UV-visible detector, and the ASTRA software (Wyatt Corp., Santa Barbara, CA) collected data from the RI detector and the LS detectors and recorded the UV trace at 280 nm sent from the photodiode array detector. The weight average molecular masses, Mₒ, were determined across the entire elution profile in the intervals of 1 s from static LS measurements using ASTRA software as described previously (32). The approach uses a Rayleigh-Debye-Gans light scattering model (Equation 1), which relates the amount of scattered light to the concentration and weight average molecular weight of solute and second virial coefficient,

$$\frac{R(\theta)}{\theta} = \frac{1}{M_o} + 2A_2c \tag{1}$$

where $R(\theta)$ is the intensity of excess scattered light at angle $\theta$; $c$ is the concentration of the solute, $M_o$ is the weight average molecular weight of the solute; $A_2$ is the second virial coefficient; $K_o$ is an optical parameter equal to $4\pi^2n^2 \left(\frac{dn/dc}{d_\lambda}\right)^2 / (\lambda^2N_A)$, where $n$ is the refractive index; $dn/dc$ is the refractive index increment for the solute; $N_A$ is Avogadro’s number, and $\lambda$ is the wavelength of the scattered light.

DLS measurements were made “on line” at an angle of 100° with a 2-s collection time. Time-resolved scatter intensity fluctuations were analyzed using Astra software (Wyatt Corp.), which implements the cumulants method (33) to determine the time dependence of diffusive motion also referred to as the intensity correlation function, $G(T)$, as shown in Equation 2 (34),

$$G(T) = B\left[1 + \alpha(exp(-D_\tau^2t))^2\right] \tag{2}$$

where $B$ is the average base-line intensity; $\alpha$ is an instrument-specific correction factor; $D_\tau$ is the concentration-dependent translational diffusion constant of the solute; $t$ is a delay time,
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and q is the scattering vector equal to (4π/λ)sinθ/2, where n is the refractive index of the solvent; λ is the wavelength of the scattered light, and θ is the scattering angle. Equation 2 describes the relationship between the time dependence of fluctuation in scatter intensity and the translational diffusion coefficient. The value of D_t can be used to estimate the apparent hydrodynamic radius of an equivalent sphere using Stokes-Einstein relationship shown in Equation 3,

\[ R_h = \frac{kT}{6\pi\eta D_t} \]  

(Eq. 3)

where k is the Boltzmann constant; T is the absolute temperature, and η is the temperature-corrected viscosity of the solution.

**Determination of Dimerization Equilibrium Constant from SEC-LV/LS/RI**—The concentration-dependent changes in the weight average molecular mass determined for the apex of the eluting peak for sNUCB1 were used to determine the dimerization constant. The weight average molecular weights were plotted as a function of protein concentration for sNUCB1; error bars represent ±3% of \( \bar{M}_w \) for concentrations above 100 μg/ml, ±5% of \( \bar{M}_w \) for concentrations between 1 and 100 μg/ml, and ±10% of \( \bar{M}_w \) for concentrations below 1 μg/ml. Lines represent the nonlinear least square fits to a monomer-dimer association model for sNUCB1 dimerization.

**Determination of K_d of Complex Formation from SEC-LV/LS/RI**—The concentration-dependent changes in the \( \bar{M}_w \) determined for the apex of the eluting peak were used to determine the complexation of Gα_{11} with sNUCB1/sNUCB1(W333Ter). The \( \bar{M}_w \) values were plotted as a function of total protein concentration for complexation; error bars represent ±3% of \( \bar{M}_w \) for concentrations above 100 μg/ml, ±5% of \( \bar{M}_w \) for concentrations between 1 and 100 μg/ml, and ±10% of \( \bar{M}_w \) for concentrations below 1 μg/ml. Lines represent the nonlinear least square fits to a stimulated complexation model using Origin 6.0.

**Fluorescence Spectroscopy**

**Steady-state Trp Fluorescence upon Ca²⁺ Binding**—Steady-state fluorescence measurements for sNUCB1 were carried out in a SPEX \( \tau 3 \) spectrofluorimeter (Jobin-Yvon Instruments) at 25 °C. The excitation wavelength was set at 295 nm specifically for Trp residues, although emission wavelength was set at 340 nm. The excitation and emission slit width were 5 nm each.

**AIF₃ Uptake Assay**—WT Gα_{11} was incubated at 25 °C in reaction buffer K (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl₂, and 1 mM DTT) with increasing amounts of sNUCB1(W232A/W333A) for 10 min with constant stirring. Thereafter, for AIF₃-dependent activation of Gα_{11}, a premixed solution of AlF₄⁻ (20 mM) and NaF (1 mM), respectively, was injected at 180 s. A time course of enhancement in Trp fluorescence coupled to activation was monitored in the presence of increasing concentrations of sNUCB1(W232A/W333A) in the reaction mixture.

**GTPγS Nucleotide Exchange Assay**—WT Gα_{11} was incubated at 25 °C with increasing concentrations of sNUCB1(W232A/W333A) in reaction buffer K for 10 min with constant stirring. The base-line fluorescence was monitored for 200 s after which GTPγS (20 μM) was injected into the reaction mixture, and the relative increase in intrinsic Trp fluorescence (\( \lambda_{ex} = 295 \) nm, \( \lambda_{em} = 340 \) nm) was measured as a function of time for each concentration of sNUCB1(W232A/W333A).

**BODIPY-FL-GTPγS Nucleotide Exchange Assay**—WT Gα_{11} (20 μM) was incubated at 25 °C in reaction buffer K with increasing concentrations of Ca²⁺-free sNUCB1 for 10 min with constant stirring. BODIPY-FL-GTPγS (20 μM) was added to the reaction mixture, and the relative increase in intrinsic fluorescence (\( \lambda_{ex} = 485 \) nm, \( \lambda_{em} = 512 \) nm) was measured for each concentration of Ca²⁺-free sNUCB1 as a function of time.
BODIPY-FL-GTPγS Nucleotide Binding Assay—WT Gα11 (20 μM) was incubated alone and with Ca²⁺-free sNUCB1 (100 μM) at room temperature in reaction buffer K. 10-μl samples were withdrawn from each reaction mixture at various time points and loaded onto a pre-equilibrated Zeba™ micro-desalt spin column to remove the unbound nucleotide. The columns were spun at 1000 × g for 1 min, and the flow-through was collected. The amount of bound nucleotide in each flow-through was monitored to ensure that the same amount of protein was being collected in each flow-through sample.

Radioligand [35S]GTPγS-based Nucleotide Exchange Assay

The effect of Ca²⁺-free sNUCB1 or Ca²⁺-free sNUCB1-(W333Ter) binding to WT Gα11-GDP on the nucleotide exchange was monitored using [35S]GTPγS exchange assay. 20 μM WT Gα11-GDP alone or with 100 μM Ca²⁺-free sNUCB1/sNUCB1(W333Ter) was incubated in buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 2 mM DTT) for 20 min at 30 °C. After incubation, [35S]GTPγS solution diluted with GTPγS was added to the reaction mixtures such that the final GTPγS concentration in each reaction mixture was 100 μM with an associated radioactivity of 50 nCi/μl of reaction volume. Thereafter, 8-μl samples (50 nCi) were withdrawn from each reaction mixture and added to buffer-equilibrated nitrocellulose filters. Subsequently, the filters were extensively washed with Wash buffer (50 mM Tris, pH 7.5, 5 mM MgCl₂) to remove excess radioligand. For data analysis, the counts were recorded, and the value for buffer alone was subtracted from each reading. Each individual data point was scaled to the value of radioactivity within 8 μl of reaction volume to get the picomoles of radioligand bound to WT Gα11 in each reaction. As a positive control, the C-terminal GoLoco motif peptide from RGS14, namely R14GL(496–531), was used in an identical experimental setup.

Circular Dichroism Spectroscopy

Wavelength Scan—All CD experiments were performed using an Aviv 62A DS CD spectrophotometer. Far-UV CD spectra were recorded at the end of each kinetic run. Spectra were recorded over the wavelength range of 190–250 nm at 1-nm intervals with an averaging time of 3 s using a 0.1-cm path length cell. Background spectrum was subtracted from each of the collected data sets. Each spectrum obtained was an average of three scans. The recorded spectra in millidegrees of ellipticity was converted to mean residue ellipticity in degrees cm² dmol⁻¹ using Equation 5,

\[
\theta = \frac{\theta}{10 \times c \times l \times n}
\]

where \([\theta]\) is the mean residue ellipticity in degrees cm² dmol⁻¹; \(\theta\) is the ellipticity in millidegrees; \(c\) is the concentration in M; \(l\) is the path length in cm, and \(n\) is the number of peptide bonds.

Thermal Unfolding—The unfolding of the protein (8 μM) with temperature was monitored using CD at a wavelength of 222 nm, which is characteristic of an α-helix. The data points were averaged over 30 s for every unit increment in temperature. A plot of CD signal versus temperature was fit to Equations 6 and 7,

\[
f(T) = \frac{\alpha_N + \beta_N \times T + (\alpha_D + \beta_D \times T) \cdot e^{-\Delta G^0_{D-N}(T)/R T}}{1 + e^{-\Delta G^0_{D-N}(T)/R T}}
\]

where,

\[
-\Delta G^0_{D-N}(T) = -\Delta H^0_{D-N}(T_m) \cdot \left(1 - \frac{T}{T_m}\right) - \Delta C_p \cdot \left(\frac{T_m - T}{T_m}\right) + T \cdot \ln(T_m)
\]

Analytical Ultracentrifugation (AUC)

Sedimentation equilibrium studies were carried out at different centrifugal speeds and protein concentrations for sNUCB1 and sNUCB1(W333Ter). AUC experiments for sNUCB1 (+/−Ca²⁺) were done at 25 °C with protein concentrations of 50, 100, and 150 μM each at speeds of 20,000, 30,000, and 40,000 rpm. Similarly for sNUCB1(W333Ter), AUC experiments were done with 32, 70, and 150 μM protein concentrations each at speeds of 25,000, 30,000, and 40,000 rpm. The individual protein samples were run for sufficiently long periods of time to allow for the equilibrium to generate a time invariant concentration gradient balanced by diffusion of the macromolecular species. The speeds were decided based on the molecular weight of the protein. Under no net transport conditions, the following correlation (Equations 8 and 9) should be observed between concentration and the radial distance (35),

\[
Cr = Cr_o \cdot e^{(\sigma \cdot r)} \cdot (r^2 - r_o^2)
\]

where,

\[
\sigma = M(1 - \nu \rho) \cdot \frac{\omega^2}{RT}
\]

Cr is the concentration of macrosolute at any radial distance \(r\); \(Cr_o\) is the concentration of the macrosolute at the reference radial distance \(r_o\); \(\nu\) is the partial specific volume; \(\omega\) is the angular velocity; \(\rho\) is the density; \(R\) is the gas constant; \(T\) is the absolute temperature; and \(M\) is the molecular mass. The data were analyzed using Optima™ XL-A/XL-I data analysis software (Beckman, 2001).
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Western Blot

HEK293 cells were maintained in Dulbecco’s modified Eagle medium (Invitrogen) with 10% (v/v) fetal bovine serum (FBS) at 37 °C and 5% CO2. For Western blot analysis, cells were plated in 6-well plates and transiently transfected with 4 μg of total DNA (3.0 μg of pcDNA3.1(+) + 1.0 μg of WT Gαi1/sNUCB1/CXCR4), and cells were harvested after 48 h. Thereafter, cells were lysed, and the heat-denatured supernatant was subjected to SDS-PAGE analysis. Subsequently, the separated protein fractions were transferred to a PVDF membrane using semi-dry transfer procedures and probed with either 1D4 antibody to detect CXCR4, which has a C-terminal 1D4 tag, or with the respective primary antibodies for NUCB1 or Gαi1. Finally, the excess antibody was washed away, and the membrane was probed with a horseradish peroxidase-conjugated secondary antibody, and the chemiluminescence upon addition of the substrate was recorded.

cAMP Assay

cAMP dynamic2 assay kit was purchased from CisBio. HEK293 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% (v/v) fetal calf serum (FCS) at 37 °C and 5% CO2. For cAMP assay, 800,000 cells were plated in a 6-well plate and transiently transfected with pcDNA3.1(+) control, WT Gαi1, sNUCB1, or CXCR4 expression constructs using Lipofectamine 2000 (Invitrogen). Briefly, 4.0 μg of total DNA (3.0 μg of pcDNA3.1(+) + 1.0 μg of WT Gαi1/sNUCB1/CXCR4) was complexed with 12 μl of Lipofectamine 2000 reagent and incubated for 20 min at room temperature before addition of the DNA/lipid mixture to the cells. Cells were plated 24 h after transfection on a 384-well plate. 48 h after transfection, cells were left untreated or stimulated with forskolin alone or forskolin (10 μM) + isobutylmethylxanthine (IBMX) (10 mM), which is an inhibitor of phosphodiesterase, namely isobutylmethylxanthine (3,7-dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione), for 15 min at 37 °C and 45 min at room temperature. After an hour of stimulation, lysis buffer alone was added to the untreated cells and lysis buffer with cAMP-d2 was added to the stimulated cells. Thereafter, cryptate-conjugated cAMP antibody was added to all cells and incubated for 1–2 h. Homogeneous time-resolved fluorescence was recorded with excitation at 320 nm and emission at 620 and 665 nm. A standard curve was prepared by varying the amount of cAMP-d2 and probing it with cryptate-conjugated cAMP antibody using homogeneous time-resolved fluorescence. The normalized signal from treated cells with respect to untreated cells was converted to the cAMP concentration using the standard curve.

RESULTS

Ca2+ Binding to EF Hand Domains of sNUCB1 Induces Conformational Changes—A soluble form of NUCB1 was engineered, expressed in Escherichia coli, and purified to homogeneity (Fig. 1A and supplemental Fig. S1A). The domain architecture of sNUCB1 shows two EF hand Ca2+-binding regions sandwiched between an N-terminal DNA binding domain and a C-terminal leucine zipper (20). The thermodynamics of Ca2+ binding to sNUCB1 were studied using ITC. The Ca2+ binding was exothermic with ΔH° = −2.1 ± 0.06 kcal mol−1 and ΔS° = 16.5 cal mol−1 K−1 for the first EF hand and ΔH° = −0.62 ± 0.31 kcal mol−1 and ΔS° = 17.1 cal mol−1 K−1 for Ca2+ binding to the second EF hand, suggesting a spontaneous uptake of Ca2+ by Ca2+-free sNUCB1. The recorded data were best fit to a “two-set binding sites” model giving Kd values of 6.3 and 73.5 μM for Ca2+ binding (Fig. 1B). The difference in binding affinities arises due to the presence of a noncanonical Arg residue substituting for the canonical Gly at the sixth position of the 12-residue Ca2+ binding loop of the second EF hand. Our results show that the two EF hands in sNUCB1 bind to Ca2+ differentially with an order of magnitude difference in their Kd values. In 2004, de Alba and Tjandra (36) heterolously expressed and purified only the EF hand domain (residues 228–326) of NUCB1 and also reported two Ca2+-binding sites with different affinities. The EF hand regions undergo conformational changes upon Ca2+ binding that can be monitored through change in sNUCB1 Trp fluorescence. sNUCB1 has two Trp residues at positions 232 and 333 lying sequentially before and after the EF hand domain, respectively. Steady-state fluorescence measurements show a blue shift of 15 nm and an enhancement in fluorescence intensity as more Ca2+ was added to Ca2+-free sNUCB1 (supplemental Fig. S1B). Thus, Ca2+ binding causes a conformational change in sNUCB1 causing reorientation of the Trp residues from a solvent-exposed polar to a hydrophobic environment. Fluorescence quenching experiments with acrylamide and iodide were performed to probe the conformational changes around the Trp residues upon Ca2+ binding. Acrylamide is a diffusible neutral quencher of Trp fluorescence unaffected by the charges surrounding the fluorophore. 1+, on the other hand, is an anionic quencher for surface-exposed fluorophores only. Quenching experiments show that Trp residues are more accessible to acrylamide than 1− for quenching. On binding to Ca2+, acrylamide quenching decreases much more than 1− quenching (supplemental Fig. S1C). F0/F versus quencher concentration gave a straight line indicative of a dynamic quenching rather than static quenching. These observations suggest that conformational changes associated with Ca2+ binding make Trp residues less accessible by reorienting them into a hydrophobic environment.

The binding of Ca2+ to an EF hand motif forms an octahedral coordination sphere, which can cause structural rearrangement in the protein (38). This secondary structural change can in principle be followed by CD spectroscopy. Ca2+ binding to sNUCB1 causes only a slight increase in helicity. Intriguingly, the far-UV CD spectra of sNUCB1 show a smooth structural transition from an α-helical secondary structure at lower concentrations to a predominant β-sheet structure at higher concentrations. These concentration-dependent structural changes were observed both in the absence and presence of Ca2+ (supplemental Fig. S2A). Furthermore, the thermal unfolding data for sNUCB1 showed an increase in apparent Tm of 4 °C on binding to Ca2+. The folding of sNUCB1 was observed to be noncooperative and irreversible both in the absence and presence of Ca2+ (supplemental Fig. S2B). The increase in stabilization on binding to Ca2+ suggests a structural ordering of sNUCB1.
NUCB1 Is a Dimer—The domain diagram of NUCB1 reveals the presence of a C-terminal domain leucine zipper, which is a canonical structural motif known to cause dimerization (39). The oligomeric state of NUCB1 was evaluated through sedimentation equilibrium experiments using AUC. This experiment is insensitive to the shape of the molecule and directly reports on the molar mass of the sedimenting species characterized by the Boltzmann distribution. Sedimentation equilibrium measurements were performed with Ca\(^{2+}\)-free sNUCB1 for concentrations varying from 50 to 150 \(\mu\)M. The data could be well fit to a monomer-\(n\)-mer model, which gave a molecular mass of \(\sim 100\) kDa corresponding to a dimer of sNUCB1 in solution (Fig. 2A). AUC analysis of Ca\(^{2+}\)-bound sNUCB1 indicated that it is also primarily dimeric in solution (Fig. 2A, inset). To confirm that the dimerization of sNUCB1 is caused by the leucine zipper domain, a C-terminal truncation mutant, sNUCB1(W333Ter), lacking the leucine zipper was designed and heterologously expressed. The far-UV CD spectrum of sNUCB1(W333Ter) showed an \(\alpha\)-helical secondary structure even at high protein concentrations in contrast to the concentration-dependent transition observed for sNUCB1 (supplemental Fig. S2C). AUC analysis of sNUCB1(W333Ter) revealed a monomeric species in solution over the concentration range of 32 to 150 \(\mu\)M (Fig. 2B). Similar to sNUCB1, the association state of sNUCB1(W333Ter) was unaffected upon binding to Ca\(^{2+}\) (data not shown). The experiments with the truncation mutant confirm that the leucine zipper domain of sNUCB1 is essential for the dimeric state of the protein in solution.

The thermodynamics of the dimerization of sNUCB1 was further investigated using MALS. In a series of SEC experiments, Ca\(^{2+}\)-free sNUCB1 was analyzed by gel filtration using a Superdex200 10/30 HR column. The eluted protein peak was fractionated and subjected to MALS analysis at seven different angles to estimate the molecular mass of the eluting species. As shown in Fig. 2C, a gradual increase in the weight-average molecular weight of the eluting species was observed with increasing concentrations of the protein. At concentrations around 10 nm, sNUCB1 exists as a monomer. As the concentration is gradually increased, a monomer-dimer equilibrium was
observed with dimeric sNUCB1 dominating at concentrations greater than 1 μM. The data collected at different angles for each run were fit to a monomer-dimer equilibrium model giving a dissociation constant, $K_d$, of 0.26 ± 0.12 μM. sNUCB1(W333Ter) in MALSS measurements continued to exist as a monomer even at high protein concentrations. Thus, the leucine zipper region is responsible for the dimeric state of sNUCB1 in solution under physiological conditions.

sNUCB1 Is Structurally Asymmetric with an Elongated C Terminus—Ca$^{2+}$-free sNUCB1 in SEC experiments elutes at a much higher volume than a globular protein with a molecular mass similar to that of a sNUCB1 monomer or a dimer (supplemental Fig. S3A). A plot of the elution parameter, $K_{av}$, versus log(molecular mass) for a number of globular protein standards was utilized to estimate an apparent molecular mass of 300 kDa for sNUCB1 (supplemental Fig. S3B). This implies that either sNUCB1 exists as a trimer of dimers ($3 \times 100$ kDa) or is asymmetric in its shape. Because AUC and MALSS analysis convincingly ruled out any association of sNUCB1 dimers into higher order structures, we proceeded to derive structural information on sNUCB1 through DLS. DLS analysis of Ca$^{2+}$-free sNUCB1 yields a hydrodynamic radius ($R_h$) of 6.2 nm and a frictional coefficient ($f/f_0$) of 2.03 (Fig. 2D). The frictional coefficient of globular proteins usually lies in the range of 1.1–1.25 (40). A value of 2.03 for sNUCB1 indicates an asymmetric shape for the dimer, deviating considerably from a sphere. Interestingly, Ca$^{2+}$-free sNUCB1(W333Ter) has an $R_h$ of 3.3 nm and a
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In both the GDP- and GTPγS-bound states is absent when sNUCB1 is bound to Ca^{2+} as shown in the lower panels of Fig. 3. The two protein subunits elute out as individual components as the complex is disrupted in the presence of Ca^{2+}. The inhibition of the complex formation between sNUCB1 and Gα11 in the presence of Ca^{2+} can be explained by the structural rearrangement of EF hands upon Ca^{2+} binding. Yeast two-hybrid analysis for interaction of Gα with deletion mutants of NUCB1 as bait proposed the EF hand intervening acidic region of NUCB1 to be involved in G protein binding (16). The conformational change upon Ca^{2+}-binding can possibly result in the masking of the acidic region sandwiched between the two EF hands, thereby making it inaccessible for G protein binding.

The thermodynamics of Ca^{2+}-free sNUCB1 complex formation with Gα11-GDP was investigated using ITC. The exothermic nature of the reaction shows a thermodynamically favorable binding event between the two protein subunits with ΔH = −1.45 ± 0.17 kcal mol^{-1} and ΔS = 16.9 cal mol^{-1} K^{-1}. The data were best fit to a “one-set binding sites” model with ff₀ of 1.59 showing that the deletion of the C-terminal domain reduces the radius of hydration by almost 50% (Fig. 2E). This suggests that although the N terminus of sNUCB1 is relatively more compact and globular, the C-terminal domain of the protein is elongated, contributing to the observed asymmetry in the shape of sNUCB1.

sNUCB1 Preferentially Interacts with GDP-bound WT Gα11 in the Absence of Ca^{2+} — sNUCB1 is a Ca^{2+}-binding protein that has been shown to interact with G₁ and G₂ classes of α subunits (16). Physiologically, Gα subunits can individually exist both in the “inactive” or GDP-bound state and the “active” or GTP-bound state. To investigate the interaction of sNUCB1 with WT Gα11, sNUCB1 was incubated with WT Gα11-GDP (Fig. 3A) and WT Gα11-GTPγS (Fig. 3B), respectively, and the complex formation was analyzed by SEC using a Superdex200 10/30 HR column. As shown in the top panels of Fig. 3, the Coomassie-stained gels of the fractionated peaks show elution of the complex containing both sNUCB1 and Gα11, with excess Gα11 fractionating at higher elution volume. The amount of Gα11 eluting with sNUCB1 was significantly higher when Gα11 was present in the GDP-bound state rather than in the GTPγS-bound state. Thus, Ca^{2+}-free sNUCB1 preferentially interacts with Gα11 in the GDP-bound form. Interestingly, the interaction with Gα11 dimeric sNUCB1 and monomeric G protein as the interacting partners. The fit provides a K_d value of 18.3 ± 1.45 μM suggesting that sNUCB1 interacts relatively weakly with GDP-bound Gα11 (Fig. 4A). The association between Ca^{2+}-free sNUCB1 and Gα11-GDP was also monitored using light scattering. Protein samples with increasing concentrations of sNUCB1-Gα11-GDP complex were injected onto a Superdex200 10/30 HR column, and MALS was done on the complex elution peak. The analysis shows that binding of Gα11-GDP to sNUCB1 initiates only after dimerization of Ca^{2+}-free sNUCB1. At lower concentrations, dimerization of sNUCB1 from molecular mass of 50–00 kDa was traced. Once the dimer was formed, binding of one Gα11-GDP subunit to sNUCB1 was evident from an increase in molecular mass to 140 kDa (Fig. 4B). The trace observed for sNUCB1-Gα11-GDP complex formation can be understood from the experimental K_d values. The K_d value for sNUCB1 dimerization is much smaller than the K_d value for binding to Gα11-GDP, resulting in sNUCB1 dimerization prior to Gα11-GDP binding. However, sNUCB1-Gα11-GDP association curve does not rule out the ability of sNUCB1 monomer to interact with Gα11-GDP.

MALS experiments were also conducted with the monomeric truncation mutant sNUCB1(W333Ter). A plot of the

![Figure 3](image-url)
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**A**

![Graph showing the binding of Ca^{2+}-free sNUCB1 to Ga11-GDP. The heat released per injection of aliquots of a solution of Ga11-GDP (200 μM) into a buffered solution of Ca^{2+}-free sNUCB1 (50 μM) was recorded, and the area under the curve was integrated. The heat of dilution for the addition of Ga11-GDP to buffer alone was subtracted. A nonlinear least squares fit of the calculated values using the C-terminally linked sNUCB1(W333Ter) sequence.](image)

**B**

![Graph showing the calculation of Kd (sNUCB1 dimerization) = 0.26 ± 0.12 μM](image)

**C**

![Graph showing the calculation of Kd = 1.99 ± 0.26 μM](image)

**FIGURE 4. Interaction of sNUCB1 and Ga11-GDP.** A, we used ITC to measure the binding of Ca^{2+}-free sNUCB1 to Ga11-GDP. The heat released per injection of aliquots of a solution of Ga11-GDP (200 μM) into a buffered solution of Ca^{2+}-free sNUCB1 (50 μM) was recorded, and the area under the curve was integrated. The heat of dilution for the addition of Ga11-GDP to buffer alone was subtracted. A nonlinear least squares fit of the calculated values using the C-terminally linked sNUCB1(W333Ter) sequence. Farquhar and co-workers (28) have shown that NUCB1 possibly interacts with the C-terminal α5 helix region of Ga13. We evaluated the effect of this interaction on G protein activation. We measured the nucleotide exchange on Ga in the presence of Ca^{2+}-free sNUCB1. The concentrations of both sNUCB1 and Ga11 were kept above the determined Kd value for sNUCB1-Ga11 complex formation. Steady-state and time-based fluorescence experiments were performed by monitoring the enhancement in Trp fluorescence, which is coupled to activation of Ga11. Any potential contribution from the Trp residues of sNUCB1 was eliminated by mutating both Trp-232 and Trp-333 to Ala, thereby generating sNUCB1(W232A/W333A) (supplemental Fig. S4A). The binding of sNUCB1(W232A/W333A) to Ga11-GDP was confirmed using SEC and SDS-PAGE analysis. The interaction of sNUCB1(W232A/W333A) with Ga11-GDP was present only in the absence of Ca^{2+} (supplemental Fig. S4B). Both AlF₄⁻ uptake (supplemental Fig. S4C) and GTPγS exchange (supplemental Fig. S4D) by Ga11-GDP alone shows an increase in Trp fluorescence as Ga11 molecules were acti-
vated with time. However, supplemental Fig. S4, C and D, shows that binding to sNUCB1(W232A/W333A) reduces the relative enhancement of Trp fluorescence coupled to \( \alpha_{i1} \) activation. Furthermore, to simultaneously monitor the activation-associated conformational change in \( \alpha_{i1} \) and the uptake of nucleotide, a mGTPY\( \gamma \)S-based FRET assay was performed. The binding of mGTPY\( \gamma \)S in the catalytic pocket of \( \alpha_{i1} \) brings it in close vicinity of Trp-211 on the switch II region of \( \alpha_{i1} \). Hence, FRET can be observed between the Trp and mant fluorophores upon \( \alpha_{i1} \) activation (43). As shown in supplemental Fig. S4E, the FRET intensity for \( \alpha_{i1} \) alone at each time point is significantly higher in comparison with the FRET intensity for \( \alpha_{i1} \) associated with Ca\(^{2+}\)-free sNUCB1(W232A/W333A). These experiments suggest that sNUCB1(W232A/W333A) might inhibit nucleotide exchange by \( \alpha_{i1} \).

G protein activation was also monitored using a fluorescent nucleotide analogue BODIPY FL-GTP\( \gamma \)S, which absorbs at 504 nm on binding to \( \alpha_{i1} \). We isolated the BODIPY FL-GTP\( \gamma \)S-bound fraction of \( \alpha_{i1} \) in the absence and presence of sNUCB1 and calculated the amount of bound BODIPY FL-GTP\( \gamma \)S in each case (Fig. 5). The supplemental Fig. S5 shows that binding of sNUCB1 to \( \alpha_{i1} \) does not affect the \( \lambda_{\text{max}} \) of bound BODIPY FL-GTP\( \gamma \)S. However, binding of sNUCB1 to \( \alpha_{i1} \) reduces the amount of nucleotide exchange by \( \alpha_{i1} \), as seen by a decrease in the BODIPY absorbance. Thus, measuring absorbance of bound BODIPY FL-GTP\( \gamma \)S provides a direct monitor of the number of bound nucleotides on \( \alpha_{i1} \) in the presence or absence of sNUCB1. Fig. 5A shows BODIPY FL-GTP\( \gamma \)S nucleotide exchange by \( \alpha_{i1} \) alone (blue) and by \( \alpha_{i1} \) in complex with sNUCB1 (orange). The results show a significant reduction in the number of bound BODIPY FL-GTP\( \gamma \)S nucleotides to \( \alpha_{i1} \) when complexed with Ca\(^{2+}\) -free sNUCB1. This shows that binding of sNUCB1 to \( \alpha_{i1} \) causes inhibition of nucleotide exchange.

To further substantiate the effect of sNUCB1 on nucleotide exchange by WT \( \alpha_{i1} \), we monitored the exchange of radioligand [\(^{35}\)S]GTP\( \gamma \)S by \( \alpha_{i1} \) in the presence and absence of sNUCB1. Fig. 5B shows the kinetics of exchange of GDP for [\(^{35}\)S]GTP\( \gamma \)S by \( \alpha_{i1} \) alone (blue). However, in the presence of sNUCB1 (Fig. 5B, green), the rate and extent of [\(^{35}\)S]GTP\( \gamma \)S exchange are significantly reduced. Thus, sNUCB1 on binding to \( \alpha_{i1} \) influences the nucleotide exchange rate by substantially inhibiting GDP release and subsequently the GTP uptake.

**GDI Activity of sNUCB1 Does Not Arise from a Canonical GoLoco Motif**—Earlier work by Siderovski and co-workers (9, 14, 44—46) has unraveled several interacting partners of \( \alpha_{i1} \)-GDP, namely RGS12, RGS14, AGS3, GPSM2, PCP-2, and G18, all of which possess GDI activity. The GDI activity was attributed to the presence of a single or tandem repeats of a 19-amino acid-long conserved GoLoco motif present in each of these proteins. The crystal structure of \( \alpha_{i1} \)-GDP complexed to RGS14-derived GoLoco peptide showed that the conserved C terminus of the 19-amino acid GoLoco motif makes contacts with both the switch I region and the bound nucleotide. Each GoLoco motif ends in a conserved triad of (Asp/Glu)-Gln-Arg. On binding to \( \alpha_{i1} \), the Arg side chain extends into the catalytic pocket to interact directly with the bound GDP and stabilize it.

In addition, the highly conserved Asp/Glu and Gln residues are absolutely essential for binding to G protein \( \alpha \) subunit as they are involved in both intra- and intermolecular H-bonding interactions (47). A sequence alignment of C-terminal residues of NUCB1(381—399) with GoLoco motifs of several GDIs of \( \alpha_{i1} \) subunits reveals that NUCB1 also possesses the highly conserved triad of Glu-Gln-Arg and the Gln conserved at the 11th position of the GoLoco motifs (supplemental Fig. S6, A and B). The other residues in the aligned sequence of NUCB1 show relatively poor conservation across the GoLoco motif.

We synthesized the NUCB1 peptide corresponding to residue 381—419, namely NUCB1(381—419) (supplemental Fig. S6A). We first performed an ITC experiment to evaluate the interaction of the NUCB1(381—419) with \( \alpha_{i1} \). The supplemental Fig. S6C shows that NUCB1(381—419) does not interact with \( \alpha_{i1} \), suggesting that this C-terminal region of NUCB1 alone is not responsible for the observed GDI activity of sNUCB1. To identify further the region of sNUCB1 responsible for its GDI activity, we tested the effect of C-terminal truncation mutant sNUCB1(W333Ter) on the nucleotide exchange by \( \alpha_{i1} \) using [\(^{35}\)S]GTP\( \gamma \)S exchange assay. Fig. 5B illustrates the ability of sNUCB1(W333Ter) to inhibit nucleotide exchange on \( \alpha_{i1} \) in a manner similar to sNUCB1. This suggests that the GDI activity of sNUCB1 lies in the N-terminal part of the protein containing the two EF hands. Thus, the GDI activity of sNUCB1 may not depend on a peptide sequence that is similar to a canonical GoLoco sequence.

As a positive control, we also monitored the [\(^{35}\)S]GTP\( \gamma \)S uptake by \( \alpha_{i1} \) in the presence of RGS14 GoLoco peptide RGS14(496—531). RGS14(496—531) completely inhibits the nucleotide exchange by \( \alpha_{i1} \), as reported by Siderovski et al. (54). Furthermore, using ITC, we tested the binding of RGS14(496—531) to \( \alpha_{i1} \), both in the absence and presence of sNUCB1. As shown in supplemental Fig. S7A, RGS14(496—531) (200 \( \mu \)M) binds to \( \alpha_{i1} \) (20 \( \mu \)M) alone with an affinity of 268.8 nM. However, when \( \alpha_{i1} \) was complexed to sNUCB1, RGS14(496—531) did not show any binding to \( \alpha_{i1} \) (supplemental Fig. S7B) even at a concentration of 500 \( \mu \)M. Similar results were obtained when \( \alpha_{i1} \) was complexed to sNUCB1(W333Ter) (supplemental Fig. S7C). RGS14(496—531) has been postulated to compete off \( \beta\gamma \) for \( \alpha_{i1} \) binding (47). In our experiments, RGS14(496—531) was not able to bind to \( \alpha_{i1} \) in the presence of sNUCB1 suggesting that both RGS14(496—531) and sNUCB1 may bind to the same site on \( \alpha_{i1} \). These results demonstrate that the GDI activity of sNUCB1 does not originate from the conventional GoLoco motif but that it competes for the same general binding site on \( \alpha_{i1} \).

**Physiological Relevance of GDI Activity of sNUCB1**—To test the physiological relevance of the observed GDI activity of sNUCB1, we expressed the human WT \( \alpha_{i1} \) in the HEK293 cells in the absence and presence of sNUCB1. We first measured forskolin-stimulated intracellular cAMP levels for each transfection. Forskolin directly activates adenyl cyclase (AC), which converts adenosine monophosphate to cAMP. Thus the amount of cAMP production can evaluate the ability of \( \alpha_{i1} \) or \( \alpha_{i1} \) to stimulate or inhibit cellular AC activity. We used the cAMP assay to measure the effect of GDI activity of sNUCB1 on
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However, when sNUCB1 was over-expressed along with CXCR4 and cells were stimulated with SDF-1α, no dose-dependent inhibition was observed. The amount of cAMP produced does not diminish upon stimulation with increasing concentrations of SDF-1α (Fig. 6.).

DISCUSSION

NUCB1 is a ubiquitously expressed multidomain Ca2+-binding protein whose physiological role is not well understood. Our results provide the first direct evidence that sNUCB1 is a Ca2+-regulated GDI for Gα11. SEC experiments showed that Ca2+-free sNUCB1 interacts primarily with the GDP-bound state of Gα11 and that no interaction occurs when sNUCB1 is bound to Ca2+ (Fig. 3). The role of Ca2+ binding as a regulatory switch for the association of sNUCB1 with Gα11 can be explained structurally through the involvement of the acidic region of sNUCB1. The acidic region has been previously shown to be a G protein interaction site (28). Thus binding of Ca2+ may cause structural rearrangement that can occlude the G protein interaction site on sNUCB1. In addition, yeast two-hybrid experiments have suggested that the C-terminally linked α5 helix of G protein interacts with NUCB1 (28). Interestingly, the α5 helix is conformationally identical in both the Gα11-GDP and Gα11-GTPγS structures. However, as Gα undergoes transition from the inactive to the active state, the structurally dynamic switch regions (switch I to IV) of Gα subunits change conformation (48). Thus, the preferential binding of Ca2+-free sNUCB1 to Gα11-GDP, which can modulate G protein activation, points toward a possible participation of switch regions in this interaction.

Analysis of the dimer of sNUCB1 using SEC (supplemental Fig. S3) and dynamic light scattering (Fig. 2D) revealed that it is considerably asymmetric in its shape with a long axis of 12.4 nm. This elongated shape may facilitate simultaneous interaction of sNUCB1 with both the α5 helix and the switch regions of Gα11-GDP. Interestingly, deletion of the leucine zipper domain along with the C-terminal region of sNUCB1 increased the binding affinity of the monomeric truncation

Gα1 signaling (Fig. 6, inset). Intriguingly, the results show that sNUCB1 decreases Gα1-mediated AC inhibition after forskolin stimulation.

We further evaluated the effect of sNUCB1 on GPCR-mediated signaling. We transfected HEK293 cells with CXCR4 and monitored cAMP production as a function of SDF-1α concentration. SDF-1α is an agonist ligand of CXCR4, which activates the receptor to facilitate nucleotide exchange on Gα1. CXCR4-transfected cells show a dose-response curve for cAMP production upon SDF-1α stimulation with an EC₅₀ value of 1 nm.

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\text{DISCUSSION}
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**, G proteins are widely expressed in different tissue types and are involved in various signal transduction pathways interacting with a number of binding partners. The interaction of NUCB1 with Gαi1 has been previous established, but no guanine nucleotide exchange factor or GDI activity was observed because the in vitro experimental concentrations used were below the Kd values of association. Our results show that sNUCB1 inhibits G protein activation and that Ca2+ binding regulates interaction of Gαi1 with sNUCB1. Furthermore, to investigate the physiological importance of this interaction, we performed tissue culture-based experiments. Our results show that upon expression in HEK293 cells, sNUCB1 significantly decreases Goi-mediated AC inhibition. In addition, even receptor-mediated inhibition of AC was prevented by overexpression of sNUCB1. This can be explained through the possible sequestration of Gαi subunits by sNUCB1, thereby alleviating inhibition of AC activity. Together with the biophysical studies, this result suggests that sNUCB1 and possibly NUCB1 can affect and regulate downstream signaling events involving heterotrimeric G proteins. In 2009, Lin et al. (17) showed that NUCB1 affects the dynamic distribution of the Gα subunits to the plasma membrane. It is possible that the association of the two proteins due to colocalization on the secretory granules may facilitate inhibition of G protein activation, which would tend to promote delivery of the Gα protein subunit primarily in the GDP-bound state to the plasma membrane for heterotrimer formation with Gβγ. Thus the cytosolic pool of NUCB1 may exclusively regulate G protein activation, whereas the membrane-bound pool would regulate both the activation and trafficking to facilitate receptor association upon heterotrimer formation.

In addition, NUCB1 also has a nuclear localization signal sequence within the DNA binding region. Recently, its role in

which is a variant of NUCB1 that lacks the N-terminal signal sequence and represents the cytosolic pool of NUCB1.

Our nucleotide-exchange assays with GTPγS and its fluorescent analogues (supplemental Fig. 5a and supplemental Fig. 5b) together show that Ca2+-free sNUCB1 significantly inhibits nucleotide exchange by Goi1-GDP. The GDI activity of sNUCB1 was established by monitoring exchange of radiolabeled [35S]GTPγS. Our results clearly show that sNUCB1 significantly inhibits Goi3 activation. Interestingly, this ability was unaffected upon deletion of the C-terminal domain of sNUCB1, showing that the GDI ability of sNUCB1 does not depend on a region of the proteins that resembles a GoLoco motif. Thus, the mechanism of GDI activity of sNUCB1 is likely to be different from the GoLoco motif peptides derived from RGS proteins. The RGS peptides generally have nanomolar binding affinity for Goi1-GDP in comparison with micromolar binding affinities observed for GSPM2, GPC2, or G18-derived GoLoco motif peptides (44–46). Unlike these peptide-based studies, our in vitro assays report the binding affinity of 18.3 μM for a 99-kDa sNUCB1 dimer with Gαi1. Yeast two-hybrid analysis by Lin et al. (16) showed that NUCB1 preferentially interacts with Goi1 and Goi2 with higher affinity than with Goi12 and Goi4. Further structural studies now underway should reveal the mechanism of GDI activity of sNUCB1 and its relevance to other classes of G protein subunits.

G proteins are widely expressed in different tissue types and are involved in various signal transduction pathways interacting with a number of binding partners. The interaction of NUCB1 with Goi1 has been previous established, but no guanine nucleotide exchange factor or GDI activity was observed because the in vitro experimental concentrations used were below the Kd values of association. Our results show that sNUCB1 inhibits G protein activation and that Ca2+ binding regulates interaction of Goi1 with sNUCB1. Furthermore, to investigate the physiological importance of this interaction, we performed tissue culture-based experiments. Our results show that upon expression in HEK293 cells, sNUCB1 significantly decreases Goi1-mediated AC inhibition. In addition, even receptor-mediated inhibition of AC was prevented by overexpression of sNUCB1. This can be explained through the possible sequestration of Goi subunits by sNUCB1, thereby alleviating inhibition of AC activity. Together with the biophysical studies, this result suggests that sNUCB1 and possibly NUCB1 can affect and regulate downstream signaling events involving heterotrimeric G proteins. In 2009, Lin et al. (17) showed that NUCB1 affects the dynamic distribution of the Gα subunits to the plasma membrane. It is possible that the association of the two proteins due to colocalization on the secretory granules may facilitate inhibition of G protein activation, which would tend to promote delivery of the Gα protein subunit primarily in the GDP-bound state to the plasma membrane for heterotrimer formation with Gβγ. Thus the cytosolic pool of NUCB1 may exclusively regulate G protein activation, whereas the membrane-bound pool would regulate both the activation and trafficking to facilitate receptor association upon heterotrimer formation.

In addition, NUCB1 also has a nuclear localization signal sequence within the DNA binding region. Recently, its role in
sNUCB1 Is a Calcium-dependent GDI of \( \alpha_{i1} \)

affecting the biogenesis of amyloid precursor protein was reported (27). Such a potential for diverse functionality makes the comprehensive understanding of the physiological role of NUCB1 difficult. Based on our current knowledge, NUCB1 seems to be neither an RGS nor an AGS protein. Future experiments, including structural studies, will unravel the functional relevance of the interaction of NUCB1 with other \( \alpha \) subunits. Understanding the role of NUCB1 in the context of different \( \alpha \) subunits will highlight the signaling pathways modulated by NUCB1.

In summary, our results show that sNUCB1 is a physiological dimer that interacts with the GDP-bound form of \( \alpha_{i1} \). The binding of sNUCB1 to \( \alpha_{i1} \) inhibits GDP release and suggests that NUCB1 is a GDI. Ca\(^{2+}\)-binding to sNUCB1 regulates sNUCB1-\( \alpha_{i1}\)-GDP complex formation and possibly modulates this interaction in different cellular compartments. We conclude that NUCB1 is a novel Ca\(^{2+}\)-binding GDI. The elucidation of the Ca\(^{2+}\)-regulated GDI activity of NUCB1 adds a new level of understanding to the role of NUCB1 and to the complexity of G protein-mediated signal regulation.

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