Interaction of the protein nucleobindin with $G_{\alpha i2}$, as revealed by the yeast two-hybrid system

Naoki Mochizuki[a], Masahiko Hibi[a], Yoshiyuki Kanai[b], Paul A. Insel[a,*]

[a]*Department of Pharmacology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0636, USA*
[b]*Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan*

Received 18 August 1995

Abstract **The heterotrimeric G protein, $G_{\alpha i2}$, transduces signals from seven membrane spanning receptors to effectors such as adenylyl cyclase and ion channels. The purpose of this study was to identify these or other cellular proteins that interact with $G_{\alpha i2}$ by use of the yeast two-hybrid system. A human B cell cDNA library was screened by this system using full length $G_{\alpha i2}$. Four positive colonies were obtained. Two of the four were identified as nucleobindin, a calcium binding protein and a putative antigen to which anti-nuclear antibodies are generated in mice with a disorder that resembles systemic lupus erythematosus. Nucleobindin has a leucine zipper, EF hands, and a signal peptide sequence and is thought to localize to the nucleus as well as being secreted. The specificity of intehraction between $G_{\alpha i2}$ and nucleobindin was confirmed by an in vitro binding assay using recombinant proteins. Transfection of $G_{\alpha i2}$ and nucleobindin in COS cells increased $G_{\alpha i2}$ expression relative to cells transfected with $G_{\alpha i2}$ and mock vector. Our results indicate that the yeast two-hybrid system provides a means to identify novel proteins that interact with G_{α} proteins. Nucleobindin appears to represent one of those proteins.**

Key words: G protein; GTP-binding protein; Nucleobindin; Yeast two-hybrid

1. Introduction

Heterotrimeric guanine nucleotide binding proteins (G proteins) transduce signals derived from receptor interaction of stimuli, such as peptide hormones, neurotransmitters, light and odorants, to various cellular effectors [1]. Pertussis toxin sensitive G_{α} subunits of the $G_{\alpha i}$ family ($G_{\alpha i}$, $G_{\alpha o}$, and $G_{\alpha z}$) have been shown to regulate adenylyl cyclases, K^+ channels, and Ca^{2+} channels [2–4]. One of these proteins, $G_{\alpha i3}$, has been localized not only to the plasma membrane but also to the Golgi apparatus, where it appears to be involved in the secretion of proteins [5]. $G_{\alpha i2}$, which interacts with adenylyl cyclase, causes neoplastic transformation of some types of cells when mutated to a constitutively active state [6]. $G_{\alpha i2}$ may also be involved in the regulation of cell differentiation and in transmission of signals from α_2-adrenergic receptors to the p21 ras-mitogen activated protein kinase pathway [7,8]. These findings suggest that $G_{\alpha i}$ proteins not only act as transducers of signals from the plasma membrane but also may play roles in unrecognized signalling pathways in other cellular compartments [9]. Moreover, other data have suggested that $G_{\alpha i}$ can be identified in the nuclei of cells [10].

We have used the yeast two-hybrid system [11] as a means to seek for previously unrecognized targets that could couple to $G_{\alpha i2}$. In this report, we identify one protein that appears to interact with $G_{\alpha i2}$: the human homolog of nucleobindin, a previously cloned protein that was originally identified as a putative auto-antigen in a model of systemic lupus erythematosus [12].

2. Materials and methods

2.1. Growth and handling of yeast strain and Escherichia coli strains

The yeast strain used in this work was provided by S. Elledge (Baylor College of Medicine, Houston). The strain was Y190 (MATα *gal*4 *gal*180 *his*3 *trp*1–190 *ade*2–101 *ura*3–52 *leu*2–3,-112 *URA*3::*GAL-lacZ*, *LYS*2::*GAL(UAS)-HIS*3 *cyhr*). Yeast were grown in YPD (1% yeast extract/2% peptone/2% glucose, pH 5.8) or in the SD synthetic medium (0.67% yeast nitrogen base/2% glucose with appropriate auxotrophic amino acids). *E. coli* strains of XL-1 blue (Strategene) and HB101 (Promega) were used for the purification of plasmids. Transformation of the yeast strain with vectors was undertaken by using modified instructions in the MATCHMAKER Two Hybrid System (Clontech).

2.2. Plasmid construction and library for the yeast two-hybrid system

In the yeast two-hybrid system, proteins linked to the amino-terminal domain that mediate DNA binding join to proteins linked to the carboxy-terminal domain involved in activation of GAL4 transcription [11]. Yeast cloning vectors containing the GAL4 DNA binding domain, pGBT9 and other control vectors, pVA3 containing the p53/GAL4 DNA-binding domain, pTD1 containing SV40 large T-antigen/GAL4 activation domain, and pLAM5' containing a human lamin C/GAL4 DNA-binding domain were obtained from Clontech. pACT containing the GAL4 activation domain and pACT into which a human B cell cDNA library was cloned were kind gifts from S. Elledge. pGBT-G_q, which contains the entire coding sequence of $G_{\alpha q}$, was inserted in-frame to pGBT9. The entire coding sequence was amplified by PCR using the P3 vector (from M. Simon, Caltech) containing the entire coding sequence of mouse $G_{\alpha q}$ cDNA as a template. pGBT-G_{i2} contained in-frame the entire coding sequence of rat $G_{\alpha i2}$ cDNA amplified from pG113 obtained from Y. Kaziro, DNAX. The following oligonucleotides were used for the amplification of $G_{\alpha q}$ and $G_{\alpha i2}$, $G_{\alpha q}$ forward primer: 5'-CCCGGGGATCCGTATGACTCTGGAGTCCATCAT-G-3' including the *Bam*HI site, $G_{\alpha q}$ reverse primer: 5'-AAGTCGACGGATCCTTAGACCAGATTGTACTCCTTG-3' including the *Bam*HI site, $G_{\alpha i2}$ forward primer: 5'-TCTCGAGAATTC-ATGGGCTGCACCGTGAGC-3' including the *Eco*RI site before the initiation codon, $G_{\alpha i2}$ reverse: 5'-AAGCTTGTCGACTCAGAAGA-GGCCACAGTCCTT- 3' including the *Sal*I site before the termination codon. PCR conditions were as follows: first denaturation: 3 min at 95°C, 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 30 s at 72°C. PCR products of $G_{\alpha q}$ and $G_{\alpha i2}$ were digested with appropriate restriction enzymes, purified from agarose gel and ligated into pGBT9.

pGST G_{i2} contain the entire coding sequence of $G_{\alpha i2}$ expressed as a fusion protein to GST. The coding sequence was amplified by the set of forward and reverse primers; 5'-GGTGGAATTCTAATGGGCTG-CACCGTGAGCGCC-3' and 5'-AAGCTTGTCGACTCAGAAGA-

*Corresponding author. Fax: (1) (619) 534-6833.

Abbreviations: G protein, guanine nucleotide-binding protein; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thio galactopyranoside.

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SSDI 0014-5793(95)01031-9

GGCCACAGTCCTT-3′. The amplified products were digested with *Eco*RI and *Sal*I, and ligated in-frame into pGEX-KG, which is a derivative of pGEX-2T (Pharmacia). pBluescript (pBS)-Nuc contained human nucleobindin cDNA. pcDNA-G_{i2} was made by ligation of pcDNA3 (Invitrogen) digested with *Eco*RI and the *Eco*RI fragment from pGI13 that contained rat $G_{\alpha i2}$ cDNA, including the 5′ non-coding region and the entire coding sequence digested with *Eco*RI. pcDNA-Nuc was constructed with the ligation of pcDNA digested with *Eco*RI and the *Eco*RI fragment from pBS-Nuc. All of the plasmids used in this experiment were sequenced in both directions by the Sequenase version 2.0 kit (U.S. Biochemical Corp) and/or analyzed by combinations of restriction enzyme digestion.

2.3. Library screening with the yeast two-hybrid system

Y190 was transformed with pGBT-G_{i2} using the lithium acetate procedure and grown on the SD plate in the absence of Trp. Colonies that appeared on the plate did not have transcription activation activity by themselves. Transformants with pGBT-G_{i2} were grown in the SD medium and sequentially transformed by 200 μg of the human B cell library inserted into pACT. Transformants were incubated on SD plates with 50 mM of 3-aminotriazole in the absence of Ura, Leu, Trp and His. Clones that could grow in the absence of His and that could activate the *lac*Z reporter gene were isolated. Plasmids containing an activation domain and protein that interacted with $G_{\alpha i2}$ in the yeast were purified from *lac*Z positive colonies. HB101 transformed with plasmids purified from yeast that could grow in the absence of Leu on the M9 plates possessed the activation domain plasmids.

2.4. Qualitative and quantitative β-galactosidase assays

A filter assay for qualitative analysis of β-galactosidase and liquid culture assay for β-galactosidase were performed according to the instructions of the MATCHMAKER Two-Hybrid System. Data for quantitative assays are mean ± S.D. of triplicate assays representing Miller's units.

2.5. In vitro binding studies using GST fusion proteins

XL-1 blue transformed pGEX-KG or pGEX-G_{i2} were grown in the 5 ml of LB broth in the presence of ampicillin overnight, diluted to 250 ml and incubated with IPTG for 7 h. Bacterial pellets were suspended in 5 ml of phosphate buffered saline (PBS) containing protease inhibitors (pepstatin 5 μg/ml, leupeptin 10 μg/ml, benzamadine hydrochloride 2 mM, phenylmethylsulfonyl fluoride 5 mM, EDTA 3 mM, and aprotinin 50 KIU/ml). Cells were sonicated four times each for 30 s followed by addition of 0.5 ml of 10% Nonidet P(NP)-40. The lysates were centrifuged for 10 min at 5,000 × *g* at 4°C. Pellets were suspended with 5 ml of 1.5% *N*-lauroylsarcosine, 25mM triethanolamine, 1mM EDTA, pH 8.0, mixed for 10 min at 4°C and recentrifuged at 10,000 × *g* for 10 min at 4°C. NP-40 and $CaCl_2$ were added to the second supernatant to final concentrations of 2% and 1 mM, respectively. The first and second supernatants were combined, suspended in 50 ml PBS with protease inhibitors, added to 400 μl of glutathione agarose, and rotated at 4°C for 1 h. The agarose was washed five times with PBS containing 0.1% NP-40. GST-G_{i2} was electrophoresed on a 10% SDS-polyacrylamide gel (PAGE), transferred to Immobilon (Millipore) and detected by using anti-$G_{\alpha i2}$ antibody (AS7, DuPont-NEN). The blot was visualized by ECL (Amersham).

Nucleobindin was produced and labeled with [^{35}S]Met by an in vitro transcription/translation system TNT kit (Promega) under the control of the T7 and T3 promoters, respectively, using pBS-Nuc. GST-G_{i2} agarose containing 5 μg of GST-G_{i2} or GST agarose was incubated with 1 mM GDP or GTPγS in 50 mM Tris containing 50 mM NaCl, 5 mM EDTA without Mg^{2+} for 30 min at 30°C, followed by addition of 5 mM $MgCl_2$ at 4°C and washed by binding buffer (20 mM Tris pH 7.5, 150 mM NaCl, 3 mM EDTA, 0.1% NP-40) with 1 mM DTT and the previously mentioned protease inhibitors. Subsequently, GST-G_{i2} agarose was incubated with the in vitro translated products at 4°C for 12 h, washed 5 times with binding buffer and centrifuged. The pellet was eluted with SDS-PAGE sample buffer and subjected to 10% SDS-PAGE.

2.6. Coexpression of $G_{\alpha i2}$ and nucleobindin in COS cells

COS-1 cells were transfected with pcDNA/pcDNA-G_{i2} or pcDNA/pcDNA-Nuc or pcDNA-G_{i2}/pcDNA-Nuc, by the use of Lipofectamine (GIBCO). After 48 h, cells were solubilized in 20 mM Tris, 150 mM NaCl, 3 mM EDTA and 0.1% NP-40 with protease inhibitors, followed by 1 h incubation at 4°C. After centrifugation for 30 min at 5,000 × *g*, the supernatant was subjected to 10% SDS-PAGE and transferred to Immobilon. Immunodetection was performed using the AS-7 antibody.

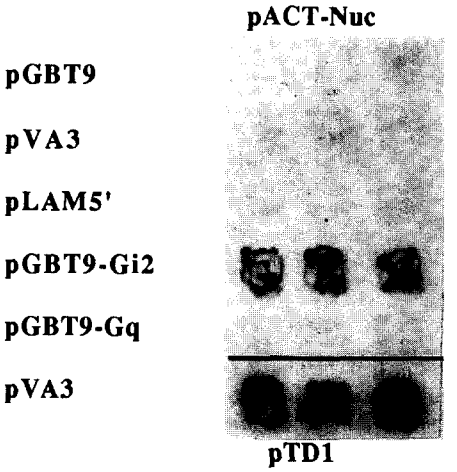


Fig. 1. Filter assay for β-galactosidase. Y190 was transformed with both vectors listed in the column and row. The result from yeast transformed with pVA3 and pTD1 used as a control is on the bottom line. The combinations of pGBT-G_{i2}/pACT-Nuc and the control combination of pVA3 and pTD1 showed positive reactions. β-Galactosidase activity measured in Miller's units (mean ± S.D.) with pACT-Nuc were as follows: pGBT9, 0.21 ± 0.06; pVA3, 0.13 ± 0.05; pLAM 5′, 0.11 ± 0.05; pGBT9-G_{i2}, 9.73 ± 3.88; pGBT9-Gq, 0.15 ± 0.08. Activity with pVA3 plus pTD1 was 136.4 ± 15.83. All values for pACT alone and together with other constructs were 0.10–0.13.

3. Results

Screening of a human B cell cDNA library by the yeast two-hybrid system showed that four of 1.4×10^6 double transformants displayed *lac*Z and were His positive. The library cDNAs from 4 doubly positive colonies were purified and sequenced. Two of the four transformants had the GAL4 activation domain followed by nucleotides that encoded a partial fragment of nucleobindin, a 461 amino acid protein isolated as a nuclear antigen in a murine model of systemic lupus erythematosus [12]. The sequence downstream of the GAL4 activation domain started from aa47 Gly in one and aa139 His in the other transformant encoding nucleobindin. We tested various combinations of control vectors and pGBT9-Gq. A filter assay for β-galactosidase activity showed that yeast transformed with the combination of pGBT9-G_{i2} and pACT-Nuc had the ability to induce the *lac*Z, but this was not the case for the combination of pGBT9-G_q and pACT-Nuc (Fig. 1). The GAL4 binding domain was unable to interact with either nucleobindin. p53 in pVA3 and lamin in pLAM5′ also could not interact with nucleobindin, nor did pACT-G_{i2} itself activate the reporter gene. The above results, observed with both filter assays and liquid culture assays (Fig. 1 legend), confirmed that interactions between $G_{\alpha i2}$ and nucleobindin are specific in this two-hybrid system.

In order to test the specificity of coupling of nucleobindin and $G_{\alpha i2}$ identified in the yeast two-hybrid system, we performed in vitro binding assays using a GST-G_{i2} fusion protein and nucleobindin produced by rabbit reticulocyte lysates. The GST-G_{i2} fusion protein, but not GST alone, bound [^{35}S]Met-

labelled nucleobindin. Neither GDP nor GTPγS changed the ability of $G_{\alpha i2}$ to bind nucleobindin (Fig. 2).

In order to examine possible interaction of the proteins during their expression, we transfected COS cells with the combination of pcDNA3/pcDNA-G_{i2}, pcDNA/pcDNA-Nuc, pcDNA-G_{i2}/pcDNA-Nuc, or mock/pcDNA-3. Solubilized fractions of transformants were subjected to the SDS-PAGE followed by immunodetection using anti-$G_{\alpha i2}$ antibody. The $G_{\alpha i2}$ protein expression level of the transformant produced by the combination of $G_{\alpha i2}$ and Nuc was greater than that produced by $G_{\alpha i2}$ and mock vector (Fig. 3).

4. Discussion

Although heterotrimeric G proteins couple to seven membrane-spanning receptors and their effectors, evidence has been presented regarding linkage of such G proteins to other types of molecules. For example, a G protein can couple to the insulin-like growth factor-II/mannose 6-phosphate receptor [13], $G_{\alpha o}$ can be associated with a nerve growth cone protein, GAP-43 [14,15], and a pertussis toxin-sensitive G protein has been implicated in neural growth via interaction with a neural cell adhesion molecule and N-cadherin [16]. $G_{\alpha i}$ has also been detected in organelles other than the plasma membrane (e.g. [9,17,18]).

The yeast two-hybrid system allowed us to demonstrate interaction between $G_{\alpha i2}$ and the protein nucleobindin. This interaction was also observed in an in vitro binding assay and transfection with nucleobindin in COS cells increased $G_{\alpha i2}$ expression. The binding activity of $G_{\alpha i2}$ and nucleobindin did not appear to depend on guanine nucleotide, as is also noted for receptor interaction with G_{α} proteins [19].

Nucleobindin was originally cloned as a nuclear antigen in a murine model of systemic lupus erythematosus [12,20]. Interestingly, it has a DNA-binding motif, leucine zipper, a signal peptide sequence, and an EF hand motif for Ca^{2+} binding activity [12,20–22]. Nucleobindin was independently cloned by investigators studying mineralized matrix of bovine bone and was detected in osteoid and around osteocytes of compact bone [22]. Among several tissues, the highest levels of protein were found in bone. It is intriguing that the action of several types

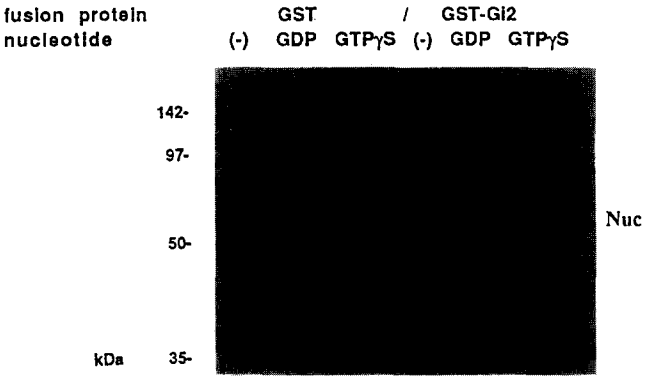


Fig. 2. In vitro binding assay of GST, GST-G_{i2} and in vitro translated nucleobindin. GST or GST-G_{i2} were incubated with [^{35}S]Met labelled nucleobindin in the presence or absence of GDP or GTPγS. After several washings, the precipitate was subjected to 10% SDS-PAGE, followed by drying and exposure to X-ray film. Nuc, nucleobindin.

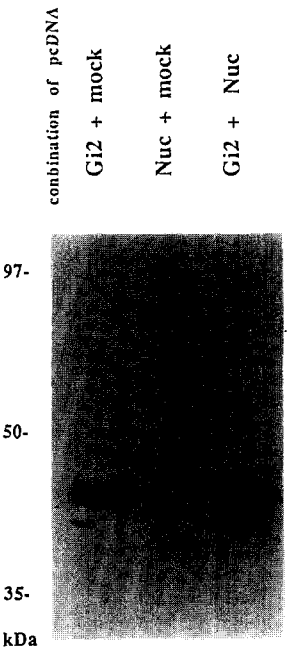


Fig. 3. $G_{\alpha i2}$ expression of transfected COS cells. Equal amounts of solubilized extract from transfectants expressing $G_{\alpha i2}$ obtained by transfection of pcDNA-G_{i2} alone or together with pcDNA-Nuc were subjected to 10% SDS-PAGE and immunoblotted with AS7. Numbers on the left indicate molecular weight (kDa).

of hormones that can influence bone formation or resorption are sensitive in inhibition by pertussis toxin (e.g. [23–26]).

The ability of coexpressed nucleobindin to enhance $G_{\alpha i2}$ expression suggests functional interaction between $G_{\alpha i2}$ and nucleobindin so as to influence transcription/translation of $G_{\alpha i2}$. Alternatively, the combination of these two-proteins may protect $G_{\alpha i2}$ from protease degradation. Whether $G_{\alpha i2}$ can influence nucleobindin expression will require further study.

Previous data suggest that G proteins can exist in the cell nucleus. G proteins that can be detected by anti-$G_{\alpha i}$ antibody exist in the nucleus of rat liver [10] and MDCK cells (M. Balboa and P.A. Insel, unpublished observations). $G_{\alpha s}$ has been detected in the nucleus of S49 lymphoma cells [25]. Such results suggest that $G_{\alpha i2}$ and nucleobindin may interact in the nucleus. Other data have demonstrated that heterotrimeric G proteins exist on the trans-Golgi network and can affect secretory vesicle formation [9]. Since nucleobindin has a signal peptide sequence and is secreted from murine cells, perhaps $G_{\alpha i2}$ is involved in the secretion of this molecule.

In summary, the current data show that use of the yeast two-hybrid system yields evidence of interaction of $G_{\alpha i2}$ and nucleobindin, a previously identified calcium binding protein and putative auto-antigen in an animal model of lupus erythematosus. It is intriguing to speculate that interaction of nucleobindin and $G_{\alpha i2}$ may have physiologic importance for bone and perhaps other tissues.

Acknowledgements: This investigation was supported by Research Grants GM31987, GM40781, HL35018 and HL53773 from the National Institutes of Health (to P.A.I). We thank Stephen J. Elledge for the human B cell library used for the yeast two-hybrid system and Akio Takada for preparing RNA from human tissues. The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank.

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