

Purification of the CaaX-modified, dynamin-related large GTPase hGBP1 by coexpression with farnesyltransferase[§]

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Abstract Over a hundred proteins in eukaryotic cells carry a C-terminal CaaX box sequence, which targets them for posttranslational isoprenylation of the cysteine residue. This modification, catalyzed by either farnesyl or geranylgeranyl transferase, converts them into peripheral membrane proteins. Isoprenylation is usually followed by proteolytic cleavage of the aaX tripeptide and methylation of the carboxyl group of the newly exposed isoprenylcysteine. The C-terminal modification regulates the cellular localization and biological activity of isoprenylated proteins. We have established a strategy to produce and purify recombinant farnesylated guanylate-binding protein 1 (hGBP1), a dynamin-related large GTPase. Our system is based on the coexpression of hGBP1 with the two subunits of human farnesyltransferase in *Escherichia coli* and a chromatographic separation of farnesylated and unmodified protein. **■** Farnesylated hGBP1 displays altered GTPase activity and is able to interact with liposomes in the activated state.—Fres, J. M., S. Müller, and G. J. K. Praefcke. **Purification of the CaaX-modified, dynamin-related large GTPase hGBP1 by coexpression with farnesyltransferase.** *J. Lipid Res.* 2010. 51: 2454–2459.

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Lipid modification is a mechanism to promote membrane interactions of cellular proteins and thus to regulate their localization and biological activities. Inside eukaryotic cells, three major types of lipid modification exist: N-terminal myristoylation, palmitoylation, and isoprenylation. Isoprenylation occurs on cysteine residues, where the attachment of either farnesyl- or geranylgeranylpyrophosphate yields a chemically stable thioether (1). This reaction is catalyzed either by farnesyltransferase (FTase), geranylgeranyltransferase

(GGTase) I or GGTase II. FTase and GGTase I are heterodimeric proteins, which share the same α subunit but have different β subunits (2). The X residues in the CaaX box determine the specificity for either FTase (X = S, A, Q, H, C), GGTase I (X = L, V), or both (X = T, I, F, M) but also the small and aliphatic “a” residues contribute to binding (3). A recent study suggested that there is more flexibility of substrate recognition in vivo than would be expected from biochemical and structural data (4). The dimeric GGTase II modifies both cysteines in CC and CXC sequences of Rab proteins when it is bound to the Rab escort protein and is therefore also called Rab GGTase (5, 6). Isoprenylated CaaX box proteins are subsequently processed by the C-terminal prenylprotein peptidases Ras and α -factor converting enzyme (Rce1p) and Ste24p (also α -factor converting enzyme Afc1p), which are multispinning transmembrane proteins in the endoplasmic reticulum. Finally, the carboxyl group of the now terminal isoprenyl cysteine is methylated by isoprenylcysteine carboxymethyltransferase, which also resides in the endoplasmic reticulum membrane and uses Sadenosylmethionine as donor for the methyl group (7).

Some proteins, e.g., members of the Src family protein kinases, G $_{\alpha}$ o and G $_{\alpha}$ z proteins, the SNARE protein Ykt6, or Ha-Ras and N-Ras, carry several different lipid modifications, which allow stronger membrane attachment (8, 9). Other proteins such as K-Ras, RhoA, Rac1, and Cdc42 carry a polybasic stretch of residues upstream of the CaaX box, which increases their affinity for negatively charged membranes and contributes to specific localization of the proteins in the cell (10). Whereas cellular proteins can undergo de- and repalmitoylation cycles to regulate the strength of their membrane association (11), isoprenylation is an irreversible process.

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Abbreviations: AlFx, aluminum fluoride; FTase, farnesyltransferase; GBP, guanylate-binding protein; GGTase, geranylgeranyltransferase; hGBP1, human GBP1; HIC, hydrophobic interaction chromatography.

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[§]The online version of this article (available at <http://www.jlr.org>) contains supplementary data in the form of one figure.

Several systems have been developed to obtain post-translationally modified CaaX box proteins for biochemical and structural studies. Isoprenylation can be achieved *in vitro* with recombinant FTase (12). Extraction from tissues or expression in eukaryotic cells such as SF9 cells offers the possibility to achieve CaaX box modification and also additional modifications by other lipids (13). Proteins with a strong membrane binding affinity, however, have to be extracted with detergents, which may have to be removed for certain assays. A different approach is the attachment of synthetic lipopeptides to C-terminally truncated CaaX box proteins by chemical coupling (14) or intein technology (15). These technologies offer the possibility of incorporating lipid moieties with different hydrophobicity, stability, site specificity or with a chemical label but the coupled product contains unnatural features, which may influence the activity of the protein (16).

Quantitative studies of the functional effects of isoprenylation are still sparse. Guanylate-binding proteins (GBPs) belong to the dynamin superfamily of large GTPases. These proteins have a high intrinsic GTPase activity compared with small regulatory GTP-binding proteins, and this can be further stimulated by oligomerization (17). Some GBPs are highly induced by interferon- γ and are recruited to pathogen-containing membrane compartments, implicating a role in innate immunity. Three of the 7 human and 3 of the 10 murine GBPs carry a C-terminal CaaX box (18, 19). In the case of hGBP1, the CTIS motif targets it for farnesylation (20, 21). This modification is essential for the recruitment of hGBP1 to the Golgi apparatus in cells treated with aluminum fluoride (AlFx) (22). Under these conditions, the protein forms a tetramer in complex with GDP*AlFx that mimics the transition state of the GTPase hydrolysis (23).

To characterize farnesylated hGBP1, we have established an *in vivo* coexpression system with the heterodimeric human FTase. Using liquid chromatography, we were able to purify the farnesylated and the unmodified hGBP1 to homogeneity in milligram amounts. Hence, our systems allowed us to investigate the influence of farnesylation on the enzymatic activity and also to reconstitute the intracellular dynamics of hGBP1 *in vitro*.

MATERIALS AND METHODS

Cloning

The pQE80L-hGBP1 construct for bacterial expression was a kind gift from C. Herrmann. The α and β subunits of the human FTase were cloned with PCR from RZPD (now BioGenes) clones IRATp970C0615DD6 and IRAUp969C0576D6 into the bicistronic pRSF-Duet1 coexpression vector (Novagen). FTase α was cloned with *EcoRI* and *BamHI* into the first multiple cloning site coding for an N-terminal His₆-tag and FTase β was cloned with *BglII* and *XhoI* into the second MCS without tag.

Expression and purification of unmodified and farnesylated hGBP1 expressed in *Escherichia coli*

The plasmid pQE80L-hGBP1 was transformed into competent Rosetta2 (DE3) *E. coli* cells (Novagen) containing the pRSF-FTase

α/β construct for coexpression. Cultures were supplemented with ampicillin (50 $\mu\text{g}/\text{ml}$) and induced with 100 μM isopropyl β -D-thiogalactopyranoside at an OD₆₀₀ of 0.6–0.8. Cells were cultured for 16 h at 20°C, collected by centrifugation at 6,000 *g* for 20 min, and resuspended in 50 mM Tris/HCl, pH 8.0, 300 mM KCl, 5 mM MgCl₂, 20 mM imidazole, 10 mM β -mercaptoethanol. The cells were disrupted by single passage through an Emulsi Flex (Avastin) at 100,000 kPa. Unbroken cells and large debris were removed by centrifugation at 60,000 *g* for 60 min at 4°C. The MRGSHis-tagged hGBP1 was further purified using Nickel-Sepharose 6 Fast Flow (GE Healthcare) and eluted with an imidazole gradient to 1,000 mM in 130 mM KCl. Farnesylated and unmodified proteins were separated by Butyl Sepharose High Performance or Butyl Sepharose 4B Fast Flow (GE Healthcare) using a decreasing salt gradient from 1.5 M (NH₄)₂SO₄ to 0 mM in 50 mM Tris/HCl, pH 8.0, 2 mM MgCl₂, 2 mM DTT. The removal of salt was carried out by gel filtration on Superdex 200 (GE Healthcare).

Mass spectrometry

Digestion in solution. Proteins were precipitated with chloroform methanol (24). Dried pellets were resuspended in 8 M urea, 50 mM Tris/HCl, pH 8.0, 10 mM DTT, and reduced by incubation at 60°C for 45 min. To S-alkylate reduced cysteine residues, iodoacetamide was added to a final concentration of 25 mM, and the reaction was allowed to proceed for 30 min in the dark. Prior to digestion, the samples were diluted 1:4 in 50 mM Tris/HCl, pH 8.0, and endoproteinase GluC (New England Biolabs) was added to a sample to protease ratio of 1:50. Samples were digested at 37°C overnight, and the digest was stopped by the addition of 0.1 vol of 1% trifluoroacetic acid.

MALDI-time of flight MS of modified proteins. Protein digests were desalted by micro-reversed phase chromatography on C18 ZipTips (Millipore). Then 1.0 μl aliquots of the desalted samples were mixed with 1.2 μl of 2.5 mg/ml 2,5-dihydroxybenzoic acid in 0.1% trifluoroacetic acid-acetonitrile (2:1) and spotted onto a 800 μm anchor target (Bruker Daltonics). Positive ion spectra were acquired on a Reflex IV MALDI-time of flight mass spectrometer (Bruker Daltonics) in the reflectron mode. A peptide calibration standard (Bruker Daltonics) was used for external calibration of the mass range from *m/z* 1,046 to *m/z* 3,147. The FlexAnalysis postanalysis software was used for optional internal recalibration on endoproteinase GluC autolysis peaks and the generation of peak lists. Biotools 3.0 (Bruker Daltonics) was used for the specific interpretation of mass spectra with regard to the expected full length or the C-terminally processed sequence of recombinant GBP1. Besides the optional oxidation of all methionines, carboxymethylation of the C terminus and farnesylation or carbamidomethylation of the very C-terminal cysteine were considered as optional modifications. All other cysteines were assumed to be carbamidomethylated. The protease was expected to cleave after aspartic acid and glutamic acid, but up to four missed cleavages were allowed with respect to the low cleavage rate after aspartic acid residues. The mass tolerance was set to 100 ppm.

Lipid vesicle preparation and cosedimentation assay

Folch fraction I brain lipid extract (Sigma-Aldrich) was dissolved in chloroform and stored at -80°C. Lipid solution was prepared in chloroform/methanol (19:1). Lipids were then dried into a film with a slow flow of argon gas and desiccated under vacuum for 30 min. Filtered and degassed buffer containing 50 mM Tris/HCl, pH 8.0, 130 mM KCl, 2 mM MgCl₂, and 2 mM DTT was added to yield a final lipid concentration of 1 mg/ml and used to hydrate the lipid film for 15 min at room temperature with occasional agitation. Lipids were resuspended to form

liposomes in a bath sonicator until the solution just began to clear. Two to five additional brief pulses with a small probe sonicator were applied until the solution became slightly cloudy. Finally, liposomes were extruded through 0.1 μm polycarbonate membrane filters (Whatman).

For liposome binding assays, freshly prepared liposomes (mg/ml), 2 μM of purified protein with or without nucleotide (200 μM guanine nucleotide and 300 μM AlCl_3 , 10 mM NaF if necessary) were incubated on ice for 15 min in the buffer described above. Ultracentrifugation at 100,000 g was carried out in a Beckman TLA 45 rotor for 20 min at 4°C. Supernatant and pellet were analyzed by SDS-PAGE and Coomassie staining.

GTP hydrolysis

The analysis of nucleotides was performed using HPLC with a hydrophobic C18 column (Chromolith Performance HPLC column 100–4.6 mm) in a triethylammonium carbonate buffer as previously described (25). The hydrolysis reaction in 50 mM Tris/HCl, pH 8.0, 130 mM KCl, 2 mM MgCl_2 , and 2 mM DTT with 1 mM GTP was started by adding the protein at the desired concentration at 37°C. Aliquots of 25 μl were taken after the appropriate time points and injected into the HPLC. Peak areas for each nucleotide were integrated to calculate the concentrations of GTP, GDP, and GMP. The rates derived from a linear fit to the initial rate of the reaction were plotted against protein concentrations and a binding model, which describes a concentration-dependent dimer formation of the protein, was fitted to the data as previously described (26).

RESULTS

Farnesylation in *E. coli*

Farnesylated hGBP1 was produced by coexpression with human FTase α and β in *E. coli*, which contain farnesylpyrophosphate as a precursor for ubiquinone and heme biosynthesis. The purification procedure is shown in Fig. 1A. After Nickel-NTA Sepharose affinity purification, the mixture contained His₆-tagged hGBP1 as well as His₆-tagged-FTase α and untagged FTase β (Fig. 1D). Mass spectrometric analysis revealed that the farnesylation was not quantitative (data not shown) and that the elution contained a mixture of farnesylated and unmodified hGBP1. The small differences in their biophysical properties prevented a separation of these two forms by ion-exchange, size exclusion chromatography, or isoelectric focusing (data not shown). Baseline purification of the farnesylated protein was made possible using a new hydrophobic interaction chromatography (HIC) resin, Butyl Sepharose HP (Fig. 1B). MALDI analysis showed no sign of unmodified protein in the final product and also no farnesylated protein in the pool of unmodified protein (Fig. 2). Furthermore, the copurified FTase (see Fig. 1D) was also removed by the size exclusion chromatography purification step (Fig. 1C). Using this method, we obtained approximately 60 mg (20 mg farnesylated/40 mg unmodified) of pure hGBP1 from 4l of bacterial culture.

Analysis of modified hGBP1

The elution profile from HIC shows that Butyl Sepharose HP allows for the baseline purification of farnesylated and unmodified hGBP1 (Fig. 1B). This had not been pos-

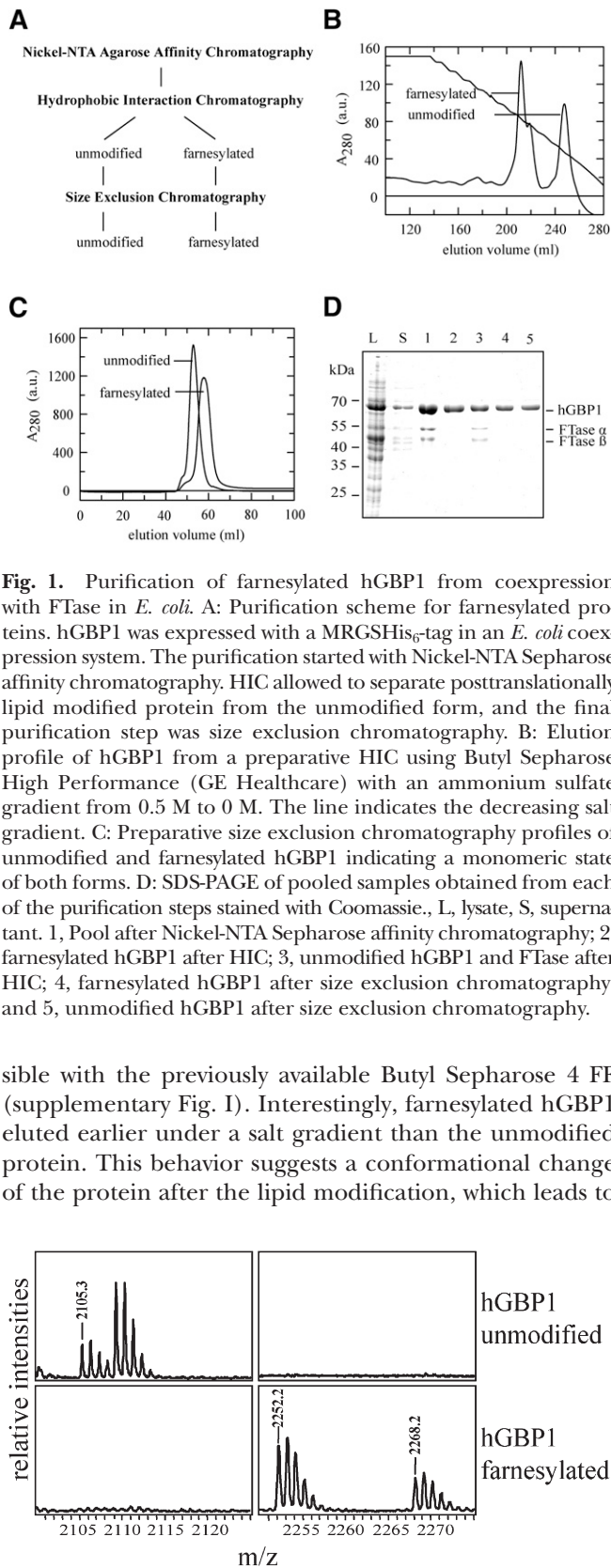


Fig. 1. Purification of farnesylated hGBP1 from coexpression with FTase in *E. coli*. A: Purification scheme for farnesylated hGBP1. hGBP1 was expressed with a MRGSHis₆-tag in an *E. coli* coexpression system. The purification started with Nickel-NTA Sepharose affinity chromatography. HIC allowed to separate posttranslationally lipid modified protein from the unmodified form, and the final purification step was size exclusion chromatography. B: Elution profile of hGBP1 from a preparative HIC using Butyl Sepharose High Performance (GE Healthcare) with an ammonium sulfate gradient from 0.5 M to 0 M. The line indicates the decreasing salt gradient. C: Preparative size exclusion chromatography profiles of unmodified and farnesylated hGBP1 indicating a monomeric state of both forms. D: SDS-PAGE of pooled samples obtained from each of the purification steps stained with Coomassie. L, lysate, S, supernatant. 1, Pool after Nickel-NTA Sepharose affinity chromatography; 2, farnesylated hGBP1 after HIC; 3, unmodified hGBP1 and FTase after HIC; 4, farnesylated hGBP1 after size exclusion chromatography; and 5, unmodified hGBP1 after size exclusion chromatography.

sible with the previously available Butyl Sepharose 4 FF (supplementary Fig. 1). Interestingly, farnesylated hGBP1 eluted earlier under a salt gradient than the unmodified protein. This behavior suggests a conformational change of the protein after the lipid modification, which leads to

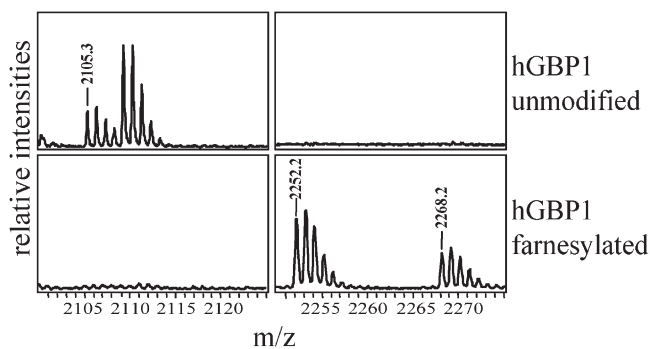


Fig. 2. MALDI-time of flight analysis of the modification status of hGBP1. The protein samples from the purification contained two identifiable masses listed bold in the range that correspond to the following: 2,105.3 m/z , unmodified hGBP1 with expected mass of 2,105.1; 2,252.2 m/z , farnesylated hGBP1 with expected mass of 2,252.3.

a shielding of the hydrophobic modification from the solvent. Size exclusion chromatography showed no significant change of elution for the farnesylated hGBP1, indicating a well-defined monomer as previously seen for the unmodified protein (Fig. 1C).

Nucleotide-dependent lipid interaction of hGBP1 proteins

To investigate the function of farnesylation on membrane binding, we analyzed the sedimentation of the farnesylated and unmodified hGBP1 with liposomes made from folch type I brain lipid extracts (Fig. 3). Farnesylated hGBP1 displayed a specific interaction with liposomes only in the presence of GDP*AlFx, which serves as a mimic of the transition state of the GTPase hydrolysis and not in any of the other conditions tested (Fig. 3A). In the absence of salt, the unmodified protein also bound to liposomes in the presence of GDP*AlFx, indicating an electrostatic contribution to membrane binding, while farnesylated hGBP1 sedimented with liposomes in buffers containing 0, 130, or 300 mM potassium chloride (Fig. 3B). Thus, under physiological salt conditions, membrane binding of hGBP1 is dependent on the lipid modification of the C terminus and a specific conformation, which occurs during GTP hydrolysis.

GTPase activity

The effect of farnesylation and liposome binding on the GTPase activity of hGBP1 was measured by HPLC (Fig. 4). At 37°C, both farnesylated and unmodified hGBP1 showed

a cooperative behavior, explained by self-association and self-activation of hGBP1. The fit of a quadratic equation to the data for the unmodified form displayed a maximum specific activity of 85 min⁻¹ and a dimer dissociation constant of 4 μM. For the farnesylated hGBP1, the dimer dissociation constant was 2-fold higher, while the maximum activity of farnesylated hGBP1 was identical to the unmodified form. The major product of GTP hydrolysis by the unmodified form at 37°C was GMP (85% of total) but the attachment of the farnesyl moiety resulted in a change of the product ratio to 30% GMP. The presence of folch liposomes had no significant impact on the GTPase activity of any modified form of hGBP1 (Fig. 4, filled circles).

DISCUSSION

Isoprenylation is essential for the membrane interaction and biological function of CaaX box-containing proteins. The potentially different biochemical properties of lipid-modified hGBP1 led us to pursue the purification of farnesylated hGBP1. We demonstrate the utility of our expression systems for the production of farnesylated protein. The availability of milligram quantities of modified hGBP1 now allows for a detailed biochemical and mechanistic analysis. Coexpression of the two subunits of FTase is long established (27, 28) and has been used to purify the enzyme for the in vitro modification of various CaaX box-containing proteins (14, 29–31). Whereas N-terminal myristoylation of proteins by coexpression in *E. coli* has been described (32), this is not the case for farnesylation. We use a set of bicistronic expression vectors for the coexpression of hGBP1 with the two subunits of the human FTase in *E. coli* cells. Because *E. coli* cells produce farnesylpyrophosphate as a natural metabolite, our method is both simple and efficient. Thus, it offers a great advantage compared with the in vitro farnesylation of proteins, especially if large amounts are required without the need for further modifications. Geranylgeranylpyrophosphate is only present in *E. coli* in low amounts, but a recent report has shown that geranylgeranylpyrophosphate synthase can be actively expressed in *E. coli* to increase them (33). So in principle, our coexpression system could even be adapted to allow geranylgeranylation in *E. coli*.

In the course of the production of the farnesylated hGBP1 in our *E. coli* system, the complete separation of the modified from the unmodified form of the protein was critical. Due to the small differences of their biophysical parameters, such as charge and molecular weight, a separation of the farnesylated and unmodified forms of hGBP1 was not successful by conventional HIC, ion exchange chromatography, or isoelectric focusing (supplementary Fig. 1 and data not shown). In a recent publication, in vitro modified CaaX proteins have been purified using immobilized cyclodextrin that specifically interacts with the lipid moiety (34). We employed HIC with a novel Butyl Sepharose High Performance resin that dramatically improves the separation in comparison to the previously available HIC resin (compare Fig. 1B and supplementary Fig. 1). Based on the mass spectrometric analysis of the farnesylated and unmodified pools of the protein (Fig. 2), we achieved a complete separation of the two forms.

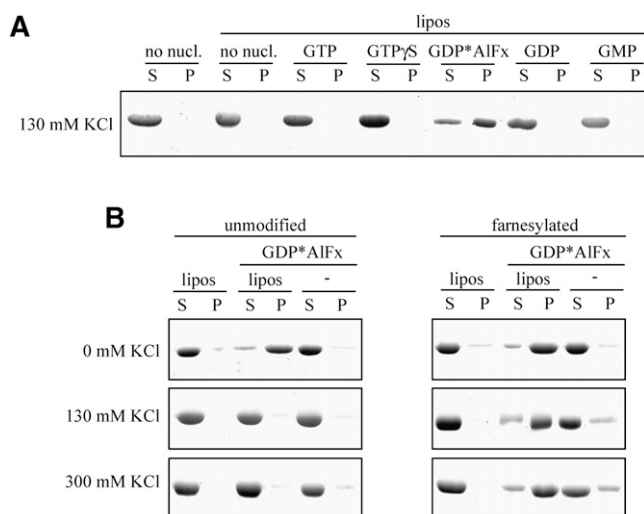


Fig. 3. Nucleotide-dependent liposome binding of hGBP1. Cosedimentation assays of hGBP1 with folch liposomes were analyzed by SDS-PAGE and Coomassie staining. S, supernatant and P, pellet. A: Farnesylated hGBP1 was incubated with or without liposomes in buffer containing 130 mM KCl. Where indicated, guanine nucleotides were added at 200 μM and, additionally, 300 μM AlCl₃ and 10 mM NaF in the case of GDP*AlFx. Association with liposomes was observed only for farnesylated hGBP1 and in the presence of GDP*AlFx but not with GMP, GDP, GTP, or the nonhydrolyzable GTP analog GTPγS. B: Unmodified hGBP1 bound only in low salt conditions and in the presence of GDP*AlFx (left panel). The association of farnesylated hGBP1 with liposomes and in the presence of GDP*AlFx was observed under all salt conditions tested (right panel).

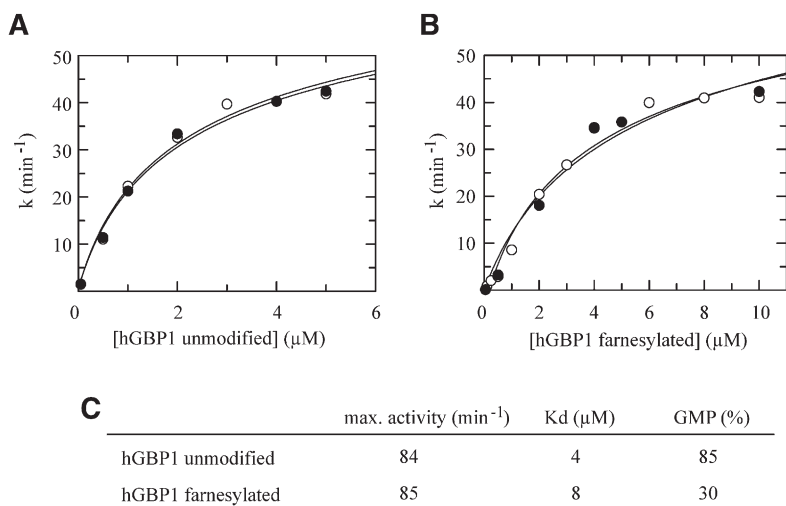


Fig. 4. Concentration-dependent GTPase activity of hGBP1. Different concentrations of (A) unmodified and (B) farnesylated hGBP1 were incubated with 1 mM GTP at 37°C in the presence (closed circles) or in the absence (open circles) of liposomes. The specific activities obtained from linear fits were plotted against the protein concentration. Maximal activity and dissociation equilibrium constant of the hGBP1 dimer were calculated by quadratic fits. C: Comparison of maximal activity, dimer dissociation equilibrium constant (Kd), and produced GMP for unmodified and farnesylated hGBP1.

In the nucleotide-free state, farnesylated hGBP1 displayed no interaction with liposomes. This is in line with data on Ras proteins indicating that farnesylation alone is not sufficient for a stable membrane association of certain proteins (10, 11, 14). However, the isoprenylation site of hGBP1 is preceded by a polybasic region, which should strongly enhance the membrane association similar to K-Ras (35). According to the crystal structures of full-length hGBP1 (23, 36), the C-terminus is supposed to be flexible and solvent exposed. However, farnesylation changes the chromatographic properties of the protein on HIC toward a more hydrophilic behavior (Fig. 1B). Thus, we postulate a conformational change of the C-terminus upon lipid modification. This leads to a covering of the hydrophobic isoprenylated C-terminus inside the structure similar to N-terminally myristoylated Arf proteins (37). In line with this model, modified hGBP1 behaved more hydrophobically than unmodified protein during a denaturing reversed-phase HPLC analysis (data not shown). In the presence of GDP*AlFx, a mimic of the activated state of the GTPase, lipid-modified hGBP1 strongly interacted with lipid-membranes (Fig. 3A) but not in any other nucleotide state. This activation-dependent membrane association can be explained by a reverse conformational change that releases the C-terminus of the protein, which seems to be linked to the tetramerization of the protein in this state (23, 38). A conformational link between the GTPase domain and the C-terminal part had been postulated by the structure of the GTPase domain in the activated state (39) and has recently been experimentally confirmed (40). The tetramerization may also explain why activated unmodified hGBP1 is able to interact with liposomes under low salt conditions, which favor electrostatic interactions, because each tetramer exposes four highly positively charged C-termini.

The maximum GTPase activity of farnesylated hGBP1 is the same as for the unmodified protein (Fig. 4). However, the product ratio of GTP hydrolysis by hGBP1 is influenced by farnesylation. Farnesylated hGBP1 produced only 30% GMP compared with 85% for the unmodified form. This can be explained by a weaker interaction of tetramers of farnesylated hGBP1 that form transiently during GTP hydrolysis. The resulting shorter lifetime of this state could shift the balance from the second hydrolysis step toward the irreversible dissociation of the oligomers (41). This is also indicated by the increase of the apparent dissociation constant of the dimer from 4 to 8 μM. We could not observe any changes in GTP hydrolysis in the presence of liposomes (Fig. 4, filled circles). This is in contrast to other members of the dynamin family, which show an increase in GTPase activity upon membrane binding (17). It is unclear whether for hGBP1 the membrane interaction occurs without effect on the GTPase activity or whether the activated state mimicked by the GDP*AlFx complex is too short-lived for a stable membrane interaction under the conditions of the GTPase assay.

In summary, our data reproduce the in vivo situation where hGBP1 is for the most part cytosolic and only translocates to the Golgi complex upon activation by GDP*AlFx and with an intact isoprenylation site (22). This suggests that in vivo the protein undergoes a cycle of membrane binding and release in which other proteins could be involved to sustain the membrane trafficking. Although the biological significance of these findings remains unclear, recent findings that murine GBPs and immunity-related GTPases are recruited to parasitophorous vacuoles of *Toxoplasma gondii*-infected cells (18, 42) suggest that GTPase activity and membrane binding also regulate the biological function of hGBP1. The ready availability of farnesylated hGBP1 achieved by the coexpression system in *E. coli* described here will greatly facilitate further studies of its subcellular trafficking.

Author contributions

J.M.F. cloned constructs, purified proteins, performed the assays, analyzed the data, contributed conceptually, and wrote the manuscript. S.M. performed the MALDI analysis of modified hGBP1 and wrote the manuscript. G.J.K.P. conceived the study, analyzed the data, and wrote the manuscript.

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