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Murine Guanylate-binding Protein: Incomplete Geranylgeranyl Isoprenoid Modification of an Interferon-γ–inducible Guanosine Triphosphate-binding Protein

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Farnesylation of Ras proteins is necessary for transforming activity. Although farnesyl transferase inhibitors show promise as anticancer agents, prenylation of the most commonly mutated Ras isoform, K-Ras4B, is difficult to prevent because K-Ras4B can be alternatively modified with geranylgeranyl (C20). Little is known of the mechanisms that produce incomplete or inappropriate prenylation. Among non-Ras proteins with C*aa*X motifs, murine guanylate-binding protein (mGBP1) was conspicuous for its unusually low incorporation of [^{3}H]mevalonate. Possible problems in cellular isoprenoid metabolism or prenyl transferase activity were investigated, but none that caused this defect was identified, implying that the poor labeling actually represented incomplete prenylation of mGBP1 itself. Mutagenesis indicated that the last 18 residues of mGBP1 severely limited C20 incorporation but, surprisingly, were compatible with farnesyl modification. Features leading to the expression of mutant GBPs with partial isoprenoid modification were identified. The results demonstrate that it is possible to alter a protein's prenylation state in a living cell so that graded effects of isoprenoid on function can be studied. The C20-selective impairment in prenylation also identifies mGBP1 as an important model for the study of substrate/geranylgeranyl transferase I interactions.

INTRODUCTION

Interest in prenylation has stemmed from the discovery that key proteins in multiple signal transduction cascades contain covalently attached isoprenoids (Casey, 1995). Perhaps the most notable examples are the Ras proteins. Mutated forms of Ras proteins are found in 30% of all human tumors (Lowy and Willumsen, 1993). However, these mutant Ras proteins are not oncogenic if they cannot be prenylated (Lowy and Willumsen, 1993). Prevention of Ras prenylation thus holds promise as a new tactic for cancer chemotherapy (Cox and Der, 1997). To this end, many prenylation inhibitors have been developed, several of which appear to be effective anticancer agents in animal studies and are undergoing clinical trials (Buss and Marsters, 1995; Kohl *et al.*, 1995; Liu *et al.*, 1998; Gelb *et al.*, 1999).

It is currently presumed that, to be effective, these drugs will need to prevent isoprenoid modification of oncogenic Ras entirely. However, forms of Ras that are incompletely modified have received little study (Goalstone and Draznin, 1996; Gadbut *et al.*, 1997), largely because of the assumption, based on direct physical studies, that prenyl proteins are fully and completely modified (Farnsworth *et al.*, 1990; Page *et al.*, 1990; Myung *et al.*, 1999). It is still not known if all functions of oncogenic Ras require prenylation or if some effector pathways may remain active regardless of prenylation state.

The signals that permit isoprenoid attachment are known in some detail. Either a farnesyl (C15) or geranylgeranyl (C20) isoprenoid is attached through a thioether linkage to a cysteine residue at the C terminus of the protein (Zhang and Casey, 1996; Seabra, 1998; Gelb *et al.*, 1999). Enzymes that catalyze isoprenoid modification of proteins are grouped into two major classes: geranylgeranyl transferase II, which recognizes C-terminal X-X-Cys-Cys, Cys-Cys-X-X, and Cys-X-Cys motifs of Rab proteins, and C*aa*X motif prenyl transferases. C*aa*X motifs consist of a cysteine followed by two amino acids that frequently are aliphatic, then the final amino acid of the protein, X. The X residue is currently believed to be the major factor determining which of the two C*aa*X protein prenyl transferases will modify the C*aa*X cysteine (Kinsella *et al.*, 1991; Moores *et al.*, 1991; Yokoyama *et al.*, 1991). Farnesyl transferase (FTase) modifies proteins

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with X residues such as Met, Ser, Ala, Gln, or Asn. Geranylgeranyl transferase I (GGTase I) preferentially modifies C*aa*X proteins with X residues of Leu or Phe. However, in cells treated with an FTase inhibitor, the K-Ras4B protein, whose C*aa*X motif (CVIM) is usually modified by FTase, can become C20 modified (James *et al.*, 1996; Rowell *et al.*, 1997; Zhang *et al.*, 1997; Sun *et al.*, 1998). This ability of GGTase I to modify particular FTase substrates is a difficult problem for the design of drugs to prevent Ras protein farnesylation. Current inhibitors are designed to specifically inhibit FTase and can do little to prevent K-Ras4B cross-prenylation by GGTase I.

Despite the details identified regarding how isoprenoids are attached to proteins, little is known of the mechanisms that GGTase I or FTase use to exclude inappropriate proteins. FTase and GGTase I are heterodimers with a shared α subunit but distinct β subunits (Zhang and Casey, 1996). The β subunit binds the isoprenoid and is therefore responsible for the lipid specificity of each prenyl transferase. Recent crystallographic studies of FTase indicate that amino acids from both α and β subunits contribute to the site at which the C*aa*X motif of the protein substrate binds (Park *et al.*, 1997; Strickland *et al.*, 1998). This combination presents a further challenge for the design of C*aa*X-based inhibitors for FTase, which must bind FTase tightly yet avoid interactions with the GGTase I enzyme to preserve the function of critical proteins that are C20 modified.

Although Ras and other small GTPases are the most well-known prenyl proteins, several other classes of C*aa*X-containing proteins exist. One such group is the family of guanylate-binding proteins (GBPs). GBPs are 65-kDa proteins of still unknown function that are highly induced by interferons and that were originally characterized based on their ability to bind to guanine nucleotide affinity columns (Cheng *et al.*, 1991; Wynn *et al.*, 1991). Six of the eight GBPs identified thus far possess C*aa*X motifs (Cheng *et al.*, 1991; Wynn *et al.*, 1991; Asundi *et al.*, 1994; Schwemmle *et al.*, 1996; Han *et al.*, 1998; Vestal *et al.*, 1998). Only one of the GBPs (human hGBP1) has a C15-type C*aa*X (CTIS) and appears to be farnesylated, as predicted (Schwemmle and Staeheli, 1994; Nantais *et al.*, 1996). Other GBP family members have C20-type C*aa*X boxes. The C*aa*X sequence (CTIL) of murine mGBP1 was predicted to be a good motif for isoprenoid attachment, based on the successful prenylation of five proteins with the same CTIL sequence (rat p67 GBP, murine GBP2, Gγ4, murine mRpgr protein, and a plant calmodulin [Kalman *et al.*, 1995; Vestal *et al.*, 1996, 1998; Yan *et al.*, 1998; Rodriguez-Concepcion *et al.*, 1999]). However, despite having the hallmarks thought to be necessary, even favorable, for prenylation, isoprenoid attachment to mGBP1 proved difficult to detect. Detailed examination of possible reasons for the meager incorporation of [^{3}H]mevalonate ([^{3}H]MVA) indicated that the defect was not the result of problems in cellular isoprenoid metabolism or prenyl transferase activity but arose from mGBP1 itself.

MATERIALS AND METHODS

Construction of DNAs

The 1.8-kilobase mGBP1 coding region in pRC/RSV (Wynn *et al.*, 1991) was amplified by PCR and cloned directionally into the pcDNA3 vector (Invitrogen, Carlsbad, CA) with the use of *Not*I and *Xba*I sites included on the PCR primers. Additional mutants were generated from this mGBP1 gene with the use of reverse primers that harbored the desired mutations. The GBP:Ras chimera, *Chim*, was constructed with the use of a primer complementary to the coding strand of the vector, with 24 mGBP1 nucleotides plus 57 nucleotides corresponding to the final 18 codons of K-Ras4B, plus a stop codon and an *Xba*I site. Full-length hGBP1 was cloned into pcDNA3 after PCR amplification from the pHMG-SVpolyA vector (Cheng *et al.*, 1991) with the use of 5′ and 3′ primers containing *Bam*HI or *Not*I restriction sequences, respectively. Sequences of the mutated regions in all DNAs were verified before use in transfections.

Cell Culture, Transfection, and Interferon Treatment

COS-1 cells were maintained in DMEM (Life Technologies, Grand Island, NY) containing 10% calf serum (Hyclone, Logan, UT) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin/streptomycin. Purified plasmid DNA (1–2 μg) was introduced into COS-1 cells with the use of Lipofectamine (Life Technologies), and cells were harvested 48 h later. RAW264.7 cells were maintained in RPMI medium (Life Technologies) with similar additions but with 10% FBS (Hyclone). Endogenous mGBP1 was induced by treatment of RAW264.7 cells with interferon-γ (IFNγ; 300 U/ml) and lipopolysaccharide (LPS; 10 μg/ml) for 20 h.

Metabolic Labeling, Electrophoresis, and Fluorography

Transfected COS-1 cells were labeled overnight in medium containing 10% serum and 100 μCi/ml [^{3}H]MVA (American Radiolabeled Chemicals, St. Louis, MO) in the presence of compactin (a generous gift from D. Graves, Iowa State University, Ames, IA) at 50 μM or as indicated in the figure legends. RAW264.7 cells were simultaneously exposed to IFNγ/LPS and labeled with 100 μCi/ml [^{3}H]MVA or 50 μCi/ml [^{3}H]geranylgeraniol (American Radiolabeled Chemicals) in the presence of 50 μM compactin. Cells were lysed directly in electrophoresis sample buffer, and samples were separated by SDS-PAGE. Proteins were transferred by electroblotting onto a polyvinylidene difluoride membrane (New England Nuclear, Boston, MA), the membrane was sprayed with En^{3}Hance (DuPont/New England Nuclear), and the ^{3}H-labeled proteins were detected by fluorographic exposure. After fluorography, blots were stripped of fluorographic enhancer by rinsing with methanol and Tris-buffered saline containing 0.5% Tween 20. GBPs were then detected directly by immunoblotting the same membrane used for fluorography.

Immunoblotting, Immunoprecipitation, and Subcellular Fractionation

GBPs were detected with the use of a rabbit polyclonal antibody to recombinant mGBP1 (provided by D. Paulnock, University of Wisconsin, Madison, WI) with a biotinylated secondary antibody and alkaline phosphatase (Vector Laboratories, Burlingame, CA). Endogenous H-Ras in COS-1 cells was detected similarly with the use of the H-Ras–specific mouse mAb 146-03E4 (Quality Biotech, Camden, NJ). Immunoprecipitates were isolated by solubilizing cells as described (Vestal *et al.*, 1998) and incubating the clarified supernatant with anti-GBP–coated Pansorbin (Calbiochem, La Jolla, CA). Samples were washed and resuspended in electrophoresis sample buffer for analysis by SDS-PAGE. Membranes (P100) were separated from cytosol (S100) by centrifugation at 100,000 $\times$ *g* as described (Nantais *et al.*, 1996). Proteins in both fractions were precipitated by the addition of 4 volumes of cold acetone, collected by centrifugation, and resuspended in electrophoresis sample buffer.

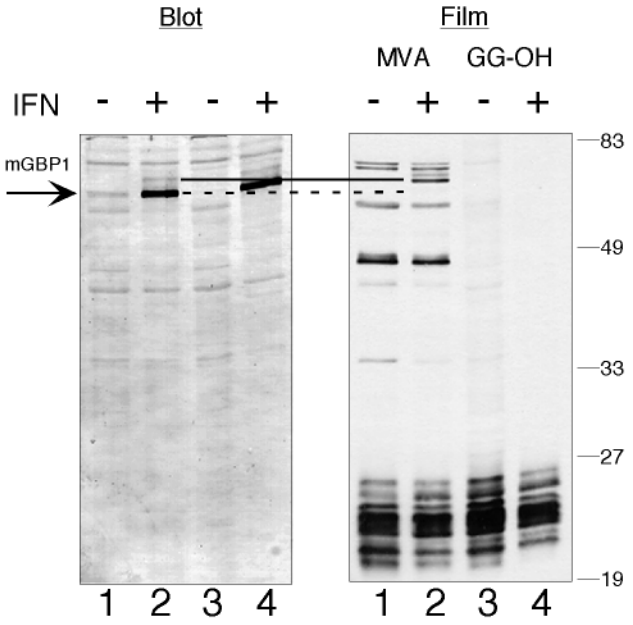


Figure 1. Incorporation of [^{3}H]isoprenoid into mGBP1 cannot be detected in IFNγ-treated RAW264.7 cells. RAW264.7 cells were labeled for 18 h with either [^{3}H]MVA (lanes 1 and 2) or [^{3}H]GG-OH (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of IFNγ and LPS. Total cell lysates were displayed by SDS-PAGE, and mGBP1 was detected by immunoblotting (Blot). "Film" indicates the fluorogram from the immunoblot shown, after 14 d of exposure. With either labeled precursor, no radiolabeled band of the correct size for the induced mGBP1 protein (dotted line) was detected after IFNγ/LPS. However, two new [^{3}H]MVA-labeled proteins (solid line) of slightly slower mobility appeared after IFNγ/LPS treatment. Positions of molecular weight markers are shown on the right.

RESULTS

Endogenous mGBP1 Is Poorly Labeled by [^{3}H]MVA in IFNγ-treated RAW264.7 Cells

Our early studies on the effects of IFNγ/LPS on protein prenylation in murine bone marrow–derived macrophages had identified a 65-kDa prenyl protein (p65) that was strongly induced by this cytokine (Vestal *et al.*, 1995). Incorporation of [^{3}H]MVA into p65 was sensitive to the farnesyl transferase inhibitor BZA-5B, implying that p65 was farnesylated. Clear potential candidates for p65 were the 65-kDa IFN-inducible murine GBPs. However, both identified murine GBPs had C20-type C*aa*X motifs, and the mGBP2 protein appeared to be successfully C20 modified (Vestal *et al.*, 1998). Therefore, mGBP1 was examined to determine if it would also be C20 modified or might instead be farnesylated. Unexpectedly, when the endogenous mGBP1 protein was induced by treatment of RAW264.7 cells with IFNγ (Figure 1), no prenyl protein was visible on the film at the correct molecular weight. Just above the position of mGBP1, two IFNγ-inducible proteins were clearly labeled with [^{3}H]MVA. These minor proteins were also recognized by the polyclonal anti-GBP serum. This indicated that RAW264.7 cells were capable of attaching isoprenoids to IFNγ-induced proteins and suggested that some of these were potential GBP family members. Surprisingly, although the immunoblot showed that mGBP1 was abundantly expressed, the amount of isoprenoid it contained appeared to be below our level of detection.

Because [^{3}H]MVA is converted into both [^{3}H]farnesyl pyrophosphate and [^{3}H]geranylgeranyl pyrophosphate ([^{3}H]GGPP), the possibility was tested that the poor labeling of mGBP1 resulted from difficulties in the transport of [^{3}H]MVA or the production of [^{3}H]GGPP in RAW264.7 cells. Examination of the other proteins present in the radiolabeled cell lysates did not support this theory, because many 21- to 28-kDa small GTPases of the Rho and Rab families, most of which are geranylgeranylated (Reese and Maltese, 1991; Zhang and Casey, 1996), were labeled successfully (Figure 1). To more directly examine geranylgeranyl utilization, cells were incubated with [^{3}H]geranylgeraniol ([^{3}H]GG-OH), an alcoholic isoprenoid that specifically labels C20-modified proteins (Crick *et al.*, 1994). The several proteins between 30 and 80 kDa that had been labeled with [^{3}H]MVA, including the two ~66-kDa IFNγ-induced proteins, did not incorporate label from [^{3}H]geranylgeraniol. This suggested that these larger proteins contained a C15 isoprenoid, as had been reported for other cell lines (James *et al.*, 1994). The group of 21- to 28-kDa proteins was labeled effectively (Figure 1), demonstrating that IFN-treated RAW264.7 cells metabolize [^{3}H]GG-OH to [^{3}H]GGPP properly. However, once again, mGBP1 labeling was below detectable levels. These results indicated that IFN treatment and the presence of mGBP1 did not impair C20 isoprenoid incorporation into the appropriate proteins and thus could not explain mGBP1's poor labeling.

mGBP1 Also Displays Poor Isoprenoid Incorporation in COS-1 Cells

Two other possible explanations for the poor labeling of mGBP1 were that the poorly labeled IFNγ-induced protein was not actually mGBP1 or that the monocytic RAW264.7 cells specifically had difficulty with mGBP1 modification. These possibilities were tested by transiently expressing a molecular clone of authentic mGBP1 in COS-1 cells to produce an amount of mGBP1 similar to that attained naturally through IFNγ treatment. An endogenous prenyl protein that migrated just above mGBP1 (as well as the labeling of other prenyl proteins in the cell lysates) showed no differences in incorporation between mock-transfected cells and cells expressing mGBP1 (Figure 2, top). The positive control hGBP1 protein (Nantais *et al.*, 1996) was clearly labeled (arrowhead). However, isoprenoid incorporation into mGBP1 was still below the level of detection. These results indicated that authentic mGBP1 also encountered difficulties in labeling in another cell type. Thus, the problem in the labeling of mGBP1 was not restricted to monocytic or IFN-treated cells.

To allow us to examine mGBP1 more clearly, immunoprecipitation was used to isolate mGBP1 from the endogenous prenyl protein of COS-1 cells. With this technique, incorporation of small amounts of label into mGBP1 was detected (Figure 2, bottom). The use of immunoprecipitation to isolate larger amounts of mGBP1 from RAW264.7 cells also revealed [^{3}H]MVA incorporation in IFNγ-induced mGBP1 after long film exposure times (our unpublished results). This ability to detect mGBP1 [^{3}H]MVA incorporation in an

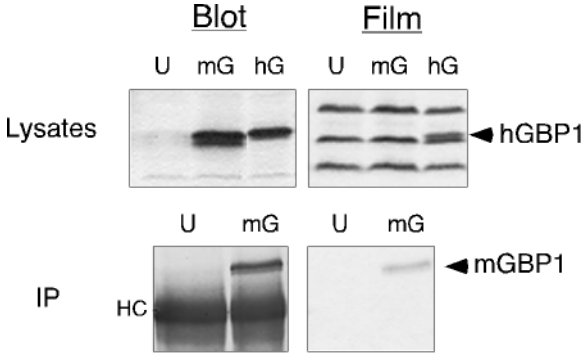


Figure 2. Poor incorporation of [^{3}H]MVA occurs in cloned mGBP1 expressed in COS-1 cells. (Top) COS-1 cells were labeled for 18.5 h with [^{3}H]MVA in the presence of 25 μM compactin, starting at 30 h after transfection with no DNA (U) or DNAs for mGBP1 (mG) or hGBP1 (hG). Cell lysates were resolved by SDS-PAGE, and radiolabeled proteins were detected by fluorographic exposure for 13 d. (Bottom) After transfection and labeling as described above, mGBP1 was isolated by immunoprecipitation (IP) and separated by SDS-PAGE. Incorporation of radiolabel into mGBP1 was detected after fluorographic exposure for 21 d. "HC" denotes the heavy chain of the anti-GBP antibody.

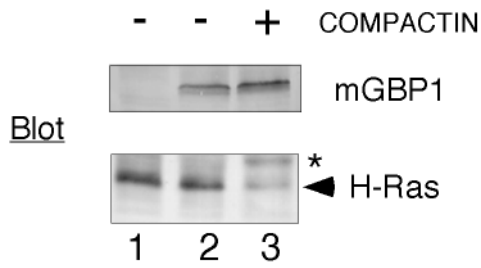


Figure 3. Compactin treatment does not alter mGBP1 mobility. COS-1 cells were transfected with DNA encoding mGBP1 with compactin (50 μM) added to the medium 5 h after transfection and remaining until samples were prepared 42 h after transfection. Cell lysates were separated by SDS-PAGE, and endogenous H-Ras or the transfected mGBP1 was detected by immunoblotting. Lane 1 is from cells transfected with empty vector, and lanes 2 and 3 are from mGBP1-transfected cells treated either in the absence or the presence of compactin. The asterisk (*) denotes unprocessed H-Ras; the arrowhead shows fully processed, farnesylated H-Ras.

immunoprecipitate did not derive from a more favored interaction of the GBP antiserum with prenylated forms of the protein, because the serum could capture uniform amounts of proteins from lysates even when the proteins showed up to eightfold differences in labeling (see below). Reciprocally, the poor labeling of mGBP1 seen directly in cell lysates (Figure 1) also indicated that immunoprecipitates had not selectively lost a prenylated form of mGBP1. Thus, the antiserum could recognize equally both prenylated and nonprenylated forms of mGBP1. Therefore, neither native, cytokine-induced, nor artificially expressed mGBP1 was totally devoid of isoprenoid, but each appeared to contain so little lipid that the small amounts present were difficult to detect unless the protein was concentrated and purified by immunoprecipitation.

Finally, to determine if the problem with mGBP1 labeling might be kinetic, and might arise from a slow equilibration of C20 pools or inefficient modification by prenyl transferase, the amount of compactin used to inhibit cellular hydroxymethylglutaryl-CoA reductase was decreased (see Figure 4, top) to avoid unintentional depletion of C20 isoprenoids (Rilling *et al.*, 1993), and extended labeling periods (up to 52 h; our unpublished results) were used. Both approaches failed to improve mGBP1 [^{3}H]MVA incorporation. These data indicated that mGBP1 underwent neither rapid but short-lived prenylation, nor slow but eventual prenylation, but was, at all times, poorly labeled.

Poor Labeling of mGBP1 Is Not Due to Removal of a Prenylated C Terminus

All of the experiments described above indicated that, despite the expectation that the mGBP1 C*aa*X motif should make the protein a good target, the difficulties underlying mGBP1's poor labeling did not involve isoprenoid metabolism or changes in prenyl transferase activity. Therefore, reasons for limited incorporation that might arise from special properties of the protein itself were considered. An initial hypothesis was that mGBP1 processing might resemble that of prelamin A. The prelamin A protein undergoes farnesylation but subsequently loses the isoprenoid when the C-terminal 18 amino acids are removed (Kilic *et al.*, 1997). It was estimated that if a C-terminal domain of similar size were removed from mGBP1, the change in length should be detectable, because the 589-amino acid mGBP1 and the 592-amino acid hGBP1 proteins can be distinguished on our gel system (see Figure 2). When compactin was used to limit isoprenoid synthesis and force accumulation of precursors of prenylated proteins, the endogenous H-Ras protein in the COS-1 cells collected in its unprocessed form, but the mobility of the transfected mGBP1 protein did not change (Figure 3). The two forms of mGBP1 with slightly different mobilities also persisted after exposure to compactin and both incorporated [^{3}H]isoprenoid (Figures 4 and 5) at the same low levels, indicating that neither protein was a nonprenylated precursor of the other. Presumably, some other modification or internal initiation of translation generates these two forms. Thus, compactin treatment failed to cause accumulation of any specific precursor form of mGBP1 and also failed to detect any potential isoprenoid modification that might have been difficult to observe with radiolabeling techniques.

mGBP1 with a C15-type CaaX Motif Is Modified Well

A second possibility was that the bulk of newly synthesized mGBP1 might be concealed in some location that was inaccessible to GGTase I and (because they share an α subunit) FTase. This possibility was tested by creating a chimeric mGBP1 (designated *Chim*) with a signal for farnesylation derived from K-Ras4B. [^{3}H]MVA incorporation into this chimeric GBP:Ras protein showed a prominent increase above the native, wild-type mGBP1 (mGBP1wt) (Figure 4), showing that FTase had no difficulty gaining access to the *Chim* protein. Therefore, mGBP1 was not sequestered.

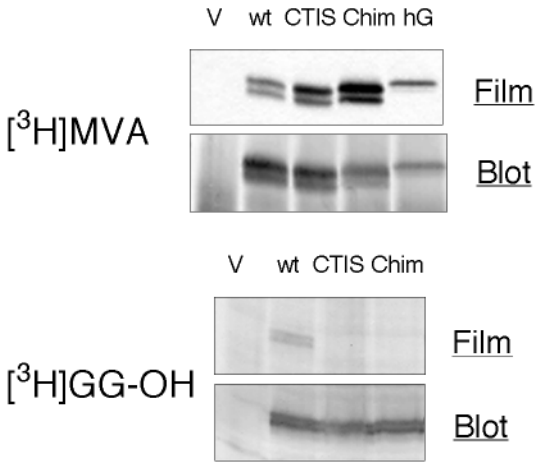


Figure 4. [3H]MVA labeling improves in mGBP1 proteins with C15-type C*aa*X boxes. COS-1 cells were transfected with empty vector (V) or DNAs encoding wild-type mGBP1 (wt), hGBP1 (hG), or mGBP1 variants with C15-type C*aa*X motifs (CTIS and Chim). Cells were labeled with either 100 μCi/ml [3H]MVA plus 10 μM compactin or 50 μCi/ml [3H]GG-OH and no compactin, and immunoprecipitates (top panels) or cell lysates (bottom panels) were prepared and analyzed by SDS-PAGE. Incorporation of radiolabel was detected by fluorographic exposure for 22 d ([3H]MVA) or 28w ([3H]GG-OH), and then GBPs on the membranes were visualized by immunoblotting.

However, the loss of 18 residues and their replacement with a lysine-rich domain was a rather significant alteration of the mGBP1 C terminus. To address more precisely how well FTase could modify mGBP1, a less drastic mutant of mGBP1 was made with only the final X amino acid changed from leucine to serine. This mutant, designated CTIS, also incorporated more label than mGBP1wt (Figure 4). To determine if the increased labeling of the CTIS protein resulted simply from the change to FTase or also might result from an unintended improvement in presentation of the serine at the C terminus to a prenyl (farnesyl) transferase, an additional mutant was constructed. An alanine substitution in the X position of C*aa*X (CTIA) was chosen to more closely mimic the leucine present in the natural C*aa*X motif. The mGBP-CTIA protein also showed improved labeling, to an extent similar to that of the mGBP-CTIS protein (our unpublished results). Because this conservative change was unlikely to alter the C-terminal structure, it appeared that increased [3H]MVA incorporation resulted from the predicted change in the modifying prenyl transferase from GGTase I to FTase.

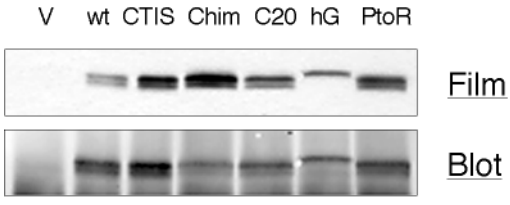


Figure 5. The C terminus of mGBP1 selectively hinders C20 modification. COS-1 cells were transfected with vector DNA or DNAs for various GBPs and labeled with [3H]MVA. Immunoprecipitates were formed and separated by SDS-PAGE. After fluorographic exposure for 22 d, GBPs present in the immunoprecipitates were visualized by immunoblotting.

The size of isoprenoid attached to these proteins was verified with the use of [3H]GG-OH labeling. The mGBP1 protein incorporated small amounts of [3H]GG-OH (Figure 4, bottom), providing evidence that what little isoprenoid was incorporated into mGBP1 was of the expected, C20 type. The *Chim* and the CTIS proteins were not labeled by the [3H]C20 isoprenoid (Figure 4, bottom), indicating that these proteins were no longer substrates for GGTase I. Thus, the lipid detected on CTIS and *Chim* after [3H]MVA labeling was likely a C15 farnesyl moiety, as intended by their C*aa*X motifs. These results implied that mGBP1 was acceptable as a substrate for FTase as long as it had the appropriate C*aa*X motif. In addition, mGBP1 was not the still-elusive farnesylated p65 protein of bone marrow–derived macrophages.

The C Terminus of mGBP1 Interferes with C20 Modification

At this point, all of the data suggested the unexpected possibility that the problem with mGBP1 prenylation was C20-selective. To determine if it was the C terminus or a more distant part of mGBP1 that caused this difficulty, a new C20 version of *Chim* was constructed by remodeling the C15-type K-Ras4B CVIM motif of *Chim* back to CTIL. This C20-*Chim* would thus be modified by [3H]C20 isoprenoid of the same specific activity as mGBP1wt. The only differences between mGBP1wt and C20-*Chim* were in the C-terminal 14 amino acids that mimicked those of K-Ras4B, adjacent to the C20-C*aa*X motif. As shown in Figure 5, [3H]MVA labeling of C20-*Chim* was twofold to threefold higher than mGBP1wt. Therefore, mGBP1 modification by C20 isoprenoid could be improved by inserting 14 K-Ras4B amino acids directly upstream of the C*aa*X motif. This result identified this region of the C terminus as part of the mGBP1 structure that impaired C20 modification. Significantly, this replacement did not improve C20 prenylation to the same level as the C15-*Chim*, indicating that additional internal structures of mGBP1 contribute to and continue to impede C20 modification.

Among these C-terminal residues of mGBP1wt were three consecutive prolines (Figure 6). To determine if these prolines hindered C20 modification of mGBP1, a new mutant was constructed in which the CTIL motif was retained but the prolines were changed to arginines, the residues found in these positions in hGBP1. This P-to-R mutant was labeled approximately threefold better than mGBP1wt, to a level similar to that of the more extensively altered C20-*Chim*. Therefore, among the C-terminal residues of mGBP1wt, these prolines were responsible for at least a portion of the difficulty in C20 modification. It should be noted that these prolines may also impair FTase interaction, because the CTIS mutant, which retains the prolines, was also not prenylated as well as the C15-*Chim* protein. GGTase interaction appears to be hindered by both these prolines and more N-terminal regions and thus is more severely affected.

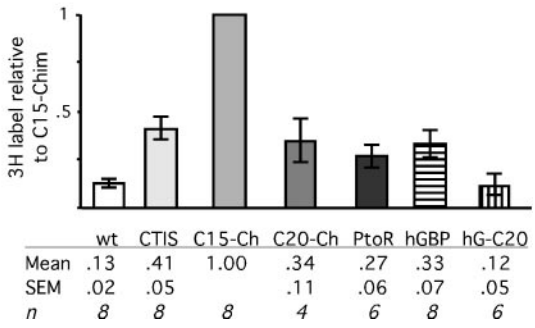
Isoprenoid Modification of mGBP1 Is Incomplete

The consistent inequalities in labeling observed among these various GBPs suggested that these proteins might not be

A

mGBP-1	C-terminal Residues	Predicted Isoprenoid
wt	LQLRQEIEKIKNMPPPRSCTIL	C20
CTIS	LQLRQEIEKIKNMPPPRSCTI**S**	C15
C15-Chim	LQLR**SKDGKKKKKKSKTKCVIM**	C15
C20-Chim	LQLR**SKDGKKKKKKSKTK**CTIL	C20
P to R	LQLRQEIEKIKNM**RRR**RSCTIL	C20
huGBP-1		
hu-wt	IMKNEIQDLQTKMRRRKACTIS	C15
hu-CTIL	IMKNEIQDLQTKMRRRKACTI**L**	C20

B



	wt	CTIS	C15-Ch	C20-Ch	PtoR	hGBP	hG-C20
Mean	.13	.41	1.00	.34	.27	.33	.12
SEM	.02	.05		.11	.06	.07	.05
n	*8*	*8*	*8*	*4*	*6*	*8*	*6*

Figure 6. C-terminal residues of GBPs and [^{3}H]MVA incorporation relative to C15-*Chim*. (A) C-terminal amino acids of various GBP constructs are aligned. Changes from wild-type mGBP1 or hGBP1 are indicated in boldface type. (B) With the use of a fluorogram and immunoblot from the same membrane, [^{3}H]MVA incorporation and protein amounts of each immunoprecipitated GBP were quantified by scanning, and values of [^{3}H]MVA-derived label were corrected for variations in protein recovery. The amount of ^{3}H for each GBP was then expressed relative to C15-*Chim*. Values shown are averages of these relative amounts ± SEM. The number of independent experiments is indicated by *n*. One-way analysis of variance indicated that all GBP proteins incorporated significantly greater amounts of ^{3}H than mGBP1 ($p < 0.05$), with the exception of the C20-modified hGBP-CTIL (hG-C20), which was not significantly different from mGBP1.

fully prenylated in mammalian cells (Figure 6A). Direct measurement of mGBP1's isoprenoid content by mass spectrometry was not practicable, because methods have not yet been developed for purification of sufficient amounts of mGBP1 from mammalian cells (where the impairment occurs), especially given the small portion of native protein that appeared to be prenylated. Therefore, the fraction of each protein that contained isoprenoid was determined by dividing the amounts of [^{3}H]isoprenoid incorporated into the various proteins by the amount of each protein that was expressed. To increase the accuracy of this analysis, both ^{3}H and protein measurements for each protein were drawn from a single immunoprecipitate blotted onto a single membrane. The membrane was first exposed to film for detection of ^{3}H, then developed with GBP antiserum for quantification of protein. As an additional control, comparisons of immunoblots from total cell lysates and immunoprecipitates of cells expressing the various GBPs showed that all forms, including those well or poorly labeled, were immunoprecipitated with equal efficiency by the antibody. To determine the reliability of the calculations, the amounts of the C15-*Chim* proteins on the gel were varied to verify that the immunoblot measurements were in the linear range of detection. These experiments gave identical results.

These calculations indicated that the C15-*Chim* protein contained the most [^{3}H]isoprenoid (Figure 6B). The ^{3}H:protein ratio of C15-*Chim*, therefore, was set to 1 (because each protein molecule could contain no more than one isoprenoid, although it conceivably might contain less), and the ratios of the other GBP variants were expressed relative to this value. This analysis quantified the previous visual results and indicated that mGBP1wt contained only 13 ± 2% as much [^{3}H]isoprenoid as C15-*Chim* (Figure 6B), implying that >85% of mGBP1 was not prenylated. Although the C20-*Chim* and P-to-R proteins were labeled more strongly than mGBP1wt, modification of these proteins also appeared to be incomplete (~30% of C15-*Chim*). Thus, all of the mGBP variants with C20-type C*aa*X motifs appeared to be poorly prenylated (Figure 6B). Even the C15-type CTIS protein was modified only ~40% as well as the C15-*Chim*. The fact that the CTIS and C15-*Chim* proteins had different levels of [^{3}H]C15 labeling further confirmed that differences detected in the incorporation of ^{3}H radioactivity reflected differences in the amount of isoprenoid attached. Thus, the level of isoprenoid modification of mGBP1 that occurred within an intact cell could be manipulated over an eightfold range by altering the last 18 residues. A more precise replacement of the three prolines with arginines could double the amount of C20-modified mGBP1, whereas simply switching to a C15-modified form could triple levels of prenylated mGBP1.

Interestingly, hGBP1 was also labeled less well than the C15-*Chim*. These results suggested that modification of hGBP1, although efficient enough to allow detection, was also incomplete. Finally, hGBP1 with the C20 motif CTIL was labeled as poorly as mGBP1 (12 ± 5% of C15-*Chim*). The hGBP-C20 protein provided a second example in which prenylation was far less than stoichiometric amounts. The threefold difference in labeling of native hGBP1 and hGBP-CTIL illustrated again that in a pair of proteins in which all non-C*aa*X structures were identical, C15 and C20 modification could be different.

Prenylation of GBPs Does Not Affect Membrane Association

Because for some proteins isoprenoid modification enhances membrane binding, mutant GBPs with wide variations in prenylation were examined to determine if this might lead to variable extents of membrane binding. The mGBP1wt, CTIS, P-to-R, and hGBP1 proteins were largely soluble (~70%; Figure 7A), even though their extents of isoprenoid modification differed threefold, from 15 to 45%. Earlier work had already found 75% of [^{3}H]MVA-labeled hGBP1 in cytosolic fractions (Nantais *et al.*, 1996), showing that the prenylated form of hGBP1 was not restricted to membranes. Prenylation thus had little impact on the membrane association of these GBPs.

However, C15-*Chim* exhibited a distinct distribution, with significantly greater association with the membrane fraction (~60%). When compactin was used to deplete the amount of farnesyl available for prenylation of C15-*Chim* (Figure 7B), the portion of C15-*Chim* present in the membrane fraction

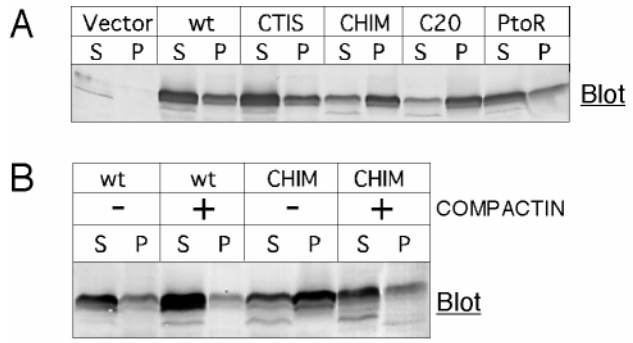


Figure 7. Membrane association of GBPs that differ in prenylation state. (A) COS-1 cells were transfected with DNAs for the indicated proteins and 48 h later separated into cytosolic (S) and membrane (P) fractions. Samples were separated by SDS-PAGE, and GBPs were detected by immunoblotting. (B) COS-1 cells were transfected with DNAs encoding mGBP1wt or C15-*Chim*. Treatment with 50 μM compactin was started 5 h later. After 48 h, lysates were separated into cytosolic (S) and membrane (P) fractions and separated by SDS-PAGE, and GBPs were detected by immunoblotting.

decreased. The compactin sensitivity of C15-*Chim* membrane interaction provided additional evidence, independent of radioactive labeling, that indicated the C15-*Chim* contained significant amounts of isoprenoid.

DISCUSSION

mGBP1 is the first example of a protein in which a seemingly adequate C*aa*X motif almost completely escapes modification. The RhoB GTPase has been shown to exist naturally as a mixture of C15- and C20-modified forms (Adamson *et al.*, 1992), but the stoichiometry of isoprenoid attachment has been presumed to be nearly complete. The mGBP1 protein, rather than existing as a fully prenylated protein modified by one or the other isoprenoid, represents one of the few documented cases of incomplete prenylation. The ability of mGBP1 to evade prenylation is particularly unusual because it applies selectively to modification of the protein with C20 isoprenoid. Because the CTIL C*aa*X sequence can be C20 modified in other proteins, it is clear that this negative control of isoprenoid modification not only arises from regions of mGBP1 outside of its C*aa*X box but must be predominant over the otherwise acceptable C*aa*X motif's role.

Prenylation of mGBP1 Is Incomplete

Identification of mGBP1 as a protein with a severe deficit in [^{3}H]MVA labeling but with an outwardly acceptable C*aa*X motif was unexpected. Careful examination of cellular isoprenoid utilization determined that difficulty with production of [^{3}H]isoprenoid or IFN-induced alteration of prenyl transferase activity was not the cause of the poor incorporation.

Our results provide an important foundation for the notion that difficulty in detecting the incorporation of [^{3}H]isoprenoid into a protein should not be ignored as an experimental or cellular flaw but may actually result from incomplete prenylation of the substrate protein. From comparison of labeling of mGBP1 and the C15-*Chim* protein, we calculate that only 15% of mGBP1wt molecules contain isoprenoid. This estimate is based on the assumption that the specific activity of the [^{3}H]farnesyl pyrophosphate and [^{3}H]geranylgeranyl pyrophosphate pools used to modify these proteins will be equal after >18 h of labeling in minimal compactin. Even if C20 and C15 isoprenoids were to continue to harbor differences in specific activities, the much better labeling of mGBP1's C20 counterpart, C20-*Chim*, indicates that, at best, only one-third of mGBP1wt is likely to be modified. Therefore, the bulk of mGBP1 within an intact cell genuinely appears to lack isoprenoid. Even this simple estimate gives mGBP1 the distinction of being the most poorly prenylated protein identified to date (Farnsworth *et al.*, 1990; Page *et al.*, 1990). There are reports that there may be some nonfarnesylated Ras proteins in cholesterol-deprived cardiac cells (Gadbut *et al.*, 1997) and insulin-starved 3T3-L1 adipocytes (Goalstone and Draznin, 1996); additionally, the Rab24 protein also appears to be prenylated inefficiently by the Rab GGTase II (Erdman *et al.*, 2000). Our work with mGBP1 and hGBP1 now suggests that serious deficits in prenylation can occur in other proteins and cell types and with all three classes of prenyl transferase.

Native mGBP1 thus appears to exist persistently in the cell as a mixture of C20-modified and (more predominantly) nonmodified forms. However, the amount of isoprenoid-modified mGBP1 could be increased up to eightfold by replacing mGBP1's 18 C-terminal residues. Furthermore, mutant proteins with varying degrees of C20 or C15 modification could be produced either by altering more specific residues of the C-terminal domain or by changing the prenyl transferase responsible for modification to FTase. Thus, the extent of mGBP1 prenylation can be manipulated through relatively modest changes in the C-terminal domain. Similarly, mutation of C-terminal residues of other proteins may allow mixed populations of prenyl and nonmodified proteins to be produced within a living cell and isoprenoid-sensitive functions studied. Experiments are under way to determine if replacing the C terminus of K-Ras4B with that of mGBP1 can produce a mixture of lipidated and nonlipidated forms of an oncogenic Ras. This would allow the study of responses that might be encountered if treatment with prenyl transferase inhibitors were only partially effective.

Mechanism Limiting Prenylation of mGBP1

The incomplete prenylation of mGBP1 does not appear to result from prelamin A–like proteolysis or from sequestration of the protein from cellular prenyl transferases. Simply changing the C*aa*X motif of mGBP1 to a form recognized by FTase significantly improved mGBP1 modification. This result also indicates that the C*aa*X motif of mGBP1 is not likely to be buried within the structure of the protein, because such masking would presumably impede interaction with either FTase or GGTase I.

Occupation of the mGBP1 C*aa*X cysteine by another modifying group remains a possibility, although we have not been able to detect incorporation of [^{3}H]palmitate into mGBP1 (our unpublished results). ADP ribosylation of the mGBP1 C*aa*X is a theoretical possibility, because pertussis toxin modifies the α subunits of heterotrimeric G_i and G_o proteins at a cysteine in the position analogous to the C*aa*X (West *et al.*, 1985). However, in normal circumstances, these

α subunit cysteines are unmodified (Jones and Spiegel, 1990) and available to interact with heptahelical receptors (Blahos *et al.*, 1998; Yang *et al.*, 1999). Additional information on the physiological function of mGBP1 will be needed to form a clearer picture of whether the C*aa*X cysteine of mGBP1 might undergo an alternative modification.

Notably, the mechanism that interferes with isoprenoid attachment to mGBP1 appears to differ from that of the G protein α subunits. For the pertussis toxin–sensitive G_i α proteins, the glycine in the second position of the "pseudo-C*aa*X" sequence (CGLF) interferes with prenyl transferase interaction, making this sequence a very poor substrate (Jones and Spiegel, 1990). In contrast, the CTIL C*aa*X motif of mGBP1 has been shown to be a good substrate for prenylation in four other proteins (Kalman *et al.*, 1995; Vestal *et al.*, 1998; Yan *et al.*, 1998; Rodriguez-Concepcion *et al.*, 1999). Thus, the mechanism that negatively regulates mGBP1 prenylation is sufficiently strong to limit prenylation of an acceptable C*aa*X motif.

The Defect in mGBP1 Prenylation Is Selective for C20 Modification

The negative influence of C-terminal non-C*aa*X regions on mGBP1 prenylation is intriguing because it appears to selectively impair the ability of GGTase I to modify the protein. This is seen most clearly with the CTIS mutant, which retains all residues of the wild type except for the final residue that confers FTase recognition and that is modified three times as well as native mGBP1. One major structural impediment to GGTase I interaction appears to be in the C-terminal region of mGBP1, because replacement of 14 C-terminal residues (C20-*Chim*) or the somewhat unusual triplet of prolines (P-to-R) increases mGBP1's C20 modification approximately threefold. However, neither of these mutants of mGBP1 show the eightfold improvement in MVA incorporation seen with C15-*Chim*. Thus, N-terminal structures of mGBP1, in addition to its C terminus, appear to contribute to hindering GGTase I–mediated prenylation. The C20-selective impairment in prenylation identifies mGBP1 as an important model for the study of substrate/GGTase I interactions. With the use of mGBP1 as a platform, additional model structures that can produce this selective interference can be tested and help guide the design of compounds to better prevent C20 modification of K-Ras4B. Including non-C*aa*X elements modeled on mGBP1 may decrease a prenyl transferase inhibitor's affinity for GGTase I while retaining good FTase interaction.

It will be important to determine which distant residues outside of the C-terminal region also contribute to the difficulty in C20 modification. Work with chimeric Gγs of heterotrimeric G proteins has suggested that GGTase I recognizes protein sequences outside of the C*aa*X box (Kalman *et al.*, 1995). In vitro prenylation assays and further testing of deletion mutants of mGBP1 in living cells will be necessary to clarify the location of these residues and whether they interfere directly with GGTase I interaction or bind another protein that impairs enzyme access.

An explanation of why mGBP1 might evade prenylation is not yet clear. Very little is known about the cellular function of any of the GBP proteins. One study indicates that hGBP1, like the more well-studied IFN-inducible MxA GTPase protein, may produce antiviral effects (Anderson *et al.*, 1999). The recent solution of the three-dimensional structure of hGBP1 led to speculation that this protein might be structurally related to the dynamin family of GTPases (Prakash *et al.*, 2000). Whether mGBP1 will show any natural variation in its prenylation or stay largely unmodified remains to be studied. Two somewhat divergent forms of murine GBPs (mag-2 and mGBP3) lack C*aa*X motifs and therefore will never be prenylated (Wynn *et al.*, 1991; Han *et al.*, 1998). The variety of mGBP1 proteins constructed here, especially the C15-*Chim* protein with its gain in prenylation and membrane association, may be useful for studies on GBP function.

The exceptionally poor isoprenoid modification of mGBP1 indicates that our understanding of protein prenylation in the intact cell is far from complete. Our results clearly show that full prenylation of a protein, even one with an excellent C*aa*X motif, is not automatic, suggesting that mechanisms that regulate isoprenoid attachment do exist but appear to function via the structure of the protein substrate rather than through changes in transferase activity. Such information is particularly needed for the newly described prenyl transferases of protozoa (Yokoyama *et al.*, 1998) and fungi (Omer and Gibbs, 1994) and for isoprenoid-modified viral proteins such as hepatitis delta antigen (Glenn *et al.*, 1998), in which it may be possible to exploit differences between pathogen and mammalian enzymes and their recognition of protein substrates in the treatment of infectious diseases.

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