The Interferon (IFN)-induced GTPase, mGBP-2

ROLE IN IFN-γ-INDUCED MURINE FIBROBLAST PROLIFERATION*

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To investigate the function of mGBP-2, a member of the interferon (IFN)-induced guanylate-binding protein family of GTPases, NIH 3T3 fibroblasts were generated that constitutively expressed mGBP-2. mGBP-2 induced a faster growth rate, with the highest expressing clones showing approximately a 50% reduction in doubling time. mGBP-2-expressing cells also grew to higher density and exhibited partial loss of contact growth inhibition, as evidenced by the formation of foci in post-confluent cultures. In addition, mGBP-2-expressing cells showed decreased dependence on serum-derived growth factors. However, they did not lose the requirement for anchorage-dependent growth. Finally, NIH 3T3 cells expressing mGBP-2 formed tumors in athymic mice. An mGBP-2 protein carrying a point mutation (S52N) that reduced GTP binding failed to produce these phenotypes when expressed at the same levels as wild type. The additional finding that IFN-γ treatment of NIH 3T3 cells resulted in an increase in proliferation similar to that observed for mGBP-2 in the absence of other IFN-induced proteins suggests that mGBP-2 may indeed be important for these growth changes.

Cellular responses to interferons (IFNs)1 involve the transcriptional induction of hundreds of genes (reviewed in Refs. 1–4), with some of the most robustly IFN-induced genes belonging to three families of GTPases, NIH 3T3 fibroblasts were generated that constitutively expressed mGBP-2. mGBP-2 induced a faster growth rate, with the highest expressing clones showing approximately a 50% reduction in doubling time. mGBP-2-expressing cells also grew to higher density and exhibited partial loss of contact growth inhibition, as evidenced by the formation of foci in post-confluent cultures. In addition, mGBP-2-expressing cells showed decreased dependence on serum-derived growth factors. However, they did not lose the requirement for anchorage-dependent growth. Finally, NIH 3T3 cells expressing mGBP-2 formed tumors in athymic mice. An mGBP-2 protein carrying a point mutation (S52N) that reduced GTP binding failed to produce these phenotypes when expressed at the same levels as wild type. The additional finding that IFN-γ treatment of NIH 3T3 cells resulted in an increase in proliferation similar to that observed for mGBP-2 in the absence of other IFN-induced proteins suggests that mGBP-2 may indeed be important for these growth changes.

* This work was supported by American Cancer Society Grant RPG-98-034-01-CIM (to D. J. V.), National Institutes of Health Grant CA-6220 (to G. C. S.), and a Cleveland Clinic Bridge grant (to D. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: IFN, interferon; GBP, guanylate-binding protein.
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concludes mGBP-2 alters the growth characteristics of murine fibroblasts. Cells constitutively expressing mGBP-2 form foci when allowed to grow to post-confluence. The robustness of this phenotype correlates with the expression level of mGBP-2 and is not observed in cells expressing mGBP-2 with a mutant GTP binding domain. In this mutant mGBP-2, a single amino acid in the P loop has been changed from Ser at position 52 to Asn, a mutation equivalent to the traditional S17N mutation in Ras-like proteins that reduces their relative affinities for GTP (36). Cells expressing mGBP-2 also grow at a faster rate and to a higher density. In addition, they have a reduced need for serum-derived growth factors for proliferation. However, these cells fail to grow as colonies in soft agar, suggesting that they have retained anchorage-dependent growth. In addition, they grow as tumors in athymic mice. Whereas IFN-γ treatment of NIH 3T3 cells has little effect on cell growth, IFN-γ enhances NIH 3T3 cell proliferation. The magnitude of the IFN-γ-induced reduction in doubling time is comparable with that observed for cells constitutively expressing mGBP-2 in the absence of other IFN-induced proteins. Taken together these data indicate that mGBP-2 functions by contributing to a signaling pathway that controls growth regulation and supports a role for mGBP-2 in IFN-γ-mediated growth regulation. These studies provide information that the highly abundant GBPs can function in regulation of some aspects of growth control by IFNs.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The rabbit polyclonal antiserum against mGBP-2 was described previously (16). Anti-FLAG monoclonals M2, M5, and rabbit anti-actin were purchased from Sigma. Recombinant murine IFN-γ and IFN-β were purchased from Calbiochem.

Plasmid Constructions—Construction of the amino-terminal FLAG epitope-tagged version of mGBP-2 was described (16). To make the equivalent of the Ras17Ser to Asn mutant in mGBP-2, a two-step PCR mutagenesis was performed. For the first amplification the template for both products was the plasmid G1 (18). To amplify the 5’ region of mGBP-2, the forward primer was 5’-ATATGGCGGCCGCTCTAGAGATC-GATGTTTGGTCGCTC-3’. To amplify the 3’ region, the forward primer was 5’-GCCAGGGCAAAACTACTGTAAGACC-3’, and the reverse primer was 5’-GTGTGAGATCTGGCCTTGAGTAGATGTGAC-TCC-3’. The products were isolated and purified and added together as templates for the second PCR that used 5’-ATATGGCGGCCGCTCTAGAGATC-GATGTTTGGTCGCTC-3’ as forward primer and 5’-GTGTGAGATCTGGCCTTGAGTAGATGTGAC-TCC-3’ as reverse primer. The amplified full-length product was digested with NolI and BglII and inserted into NotI/BglII cut pCMV2FLAG (NH) (gift of Lawrence Quilliam). Sequencing of the S52N mutant revealed a base change in the 3’ region generated by PCR. This was corrected by digestion of both NB17 and NB4 with HindIII and BglII. The ~1100-bp fragment was isolated and ligated into NB17.

To generate the constructs to be used for in vitro transcription and translation of mGBP-2 and S52N mGBP-2, a Myc epitope tag was inserted into pCDNA3.1hygro(-) (CLONTECH) by annealing the following oligonucleotides and insertion into XhoI/NolI cut vector to generate c-myc-pcDNA2.1hygro(-). 5’-CCTCTGCTAGATTGAAAGCCACCAGCATACTCC-3’ and 5’-AAAGGGCGGCGGGTTTCTTGTCTTTAACCT-GTCC-3’. To generate c-myc-pcDNA3.1hygro(-)vanGBP-2, the construct containing mGBP-2 described above was digested with NotI/BglII, and the insert was ligated into NotIBamHI cut c-myc-pcDNA3.1hygro(-). To generate c-myc-pcDNA3.1hygro(-)S52N mGBP-2, the construct containing S52N mGBP-2 (above) was digested with NotI/EcoRV, and the insert was ligated into NotIEcoRV cut c-myc-pcDNA3.1hygro(-).

Cell Culture and Transfections—NIH 3T3 cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Cells were transfected with FuGENE 6 (Roche Molecular Biochemicals) per the manufacturer’s instructions. mGBP-2 constructs were transfected with pSV2Neo into NIH 3T3 cells at a ratio of 10 mGBP plasmids per 1 pSVNeo plasmid. Stable transfectants were selected in 400 μg/ml G418 (Invitrogen), and clones were screened for GBP expression by Western blot.

Western Blot—Cell lysates were size-fractionated by SDS-PAGE and transferred to Immobilon membranes, and Western blot analysis was performed as described (16, 18). Detection was performed using the ECL system (Amersham Biosciences).

Immunofluorescence—Cells on cover slips in 6-well dishes were processed for indirect immunofluorescence as described (16).

Focus Formation Assays—Focus formation was performed as described (37), with modification. Cells were plated at 5 × 10^4 cells per 6-cm dish. After 7 days the cells were washed gently, fixed in 10% methanol, 10% acetic acid, and stained in 1% crystal violet. Each assay was performed in triplicate and at least 3 times.

Growth In Low Serum—Analysis of growth in low serum was performed as described (37). Cells were plated at 1000 cells per dish in 6-cm dishes (in triplicate) and allowed to adhere overnight in 10% serum. The next day the media was changed to Dulbecco’s modified Eagle’s medium containing 0, 1, 2, 5, or 10% serum. After 14 days cells were washed gently with phosphate-buffered saline and stained with crystal violet. For several experiments the cells were counted using a hemocytometer at the end of the 14 days rather than stained.

Growth Rate and Saturation Density—Cells were plated at 1 × 10^5 cells per dish in Dulbecco’s modified Eagle’s medium containing 10% serum. Cells were trypsinized and viable cells counted by hemocytometer daily. For growth rates of IFN-treated cells, NIH 3T3 cells were allowed to adhere for 2 h before the addition of 500 units/ml murine IFN-γ or 1000 units/ml murine IFN-β. Media and IFNs were replaced after 24 h and every other day after that. All assays were performed in triplicate. Two different lots of murine IFN-γ (Calbiochem) were used and both elicited enhanced growth. To calculate doubling times, a region of the growth curve that most closely approached linear was chosen. By using values of x and y within the linear portion, the time it took to double the number of cells was calculated.

Binding to Nucleotide Agaroses—GTP-garose (Sigma) was equilibrated and made into a 50% slurry in 20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 5 mM MgCl2, 0.01% Triton X-100 (Binding buffer). These experiments are a modification of previous analyses by Cheng et al. (13). Labeled mGBP-2 and S52N mGBP-2 were generated using the Promega TNT kit and [35S]methionine. The two constructs generated equal amounts of labeled proteins. Equal amounts of TNT lysate (2 μl) were added to 70 μl of packed binding beads. Beads were incubated in vitro for 5 h at 4 °C. The beads were washed three times with 1 ml of cold binding buffer and eluted with SDS-PAGE sample buffer and incubation at 100 °C for 5 min. Samples were run into SDS-PAGE gels, dried, and analyzed by PhosphorImager. Quantification was performed using the ImageQuant for Macintosh program version 1.2.

Cell Cycle Analysis—Actively growing cells were trypsinized and analyzed for cell cycle by staining cells with propidium iodide and flow cytometry analysis using ModFit (Becton Dickinson, San Jose, CA).

Tumor Formation in Athymic Mice—Three- to four-week-old NCR male athymic nude homozygous (nu/nu) mice (Tacomic Farma) were irradiated with 20 rads followed by intradermal injection of 2 million control 20 or clone 3 cells into flanks in the mid-axillary line. Tumor sizes were measured using the formula for prolate spheroid as follows: volume = (4/3)πr^2h, where r = mean minor axis and h = major axis. Tumor control and eight clone 3 injection sites were followed for tumor formation, and the results are presented as mean tumor volume ± S.E.

RESULTS

Generation of Stable Transfectants Expressing mGBP-2 in NIH 3T3 Cells—To begin to understand the function of the robustly IFN-induced GTPase mGBP-2 (16), and stable cell lines were established that expressed mGBP-2 at a variety of steady state levels. To be confident that the phenotype we observed was not the consequence of variations in the properties of one particular clone of NIH 3T3 cells, two separate transfections and clonal isolations were performed using two different batches of NIH 3T3 cells. The highest expressing cell lines were clones 2 and 4 from the first isolation. The control clone chosen for further analysis from this screen was control 20. A second isolation of clones was performed using a new batch of NIH 3T3 cells at lower passage levels. The two highest expressing clones from this screen were clone 1 and clone 11. The control clone chosen from the second screen was control 16. The levels of expression of mGBP-2 in these transfectants were compared with those of NIH 3T3 cells treated with IFN-γ or IFN-β for 24 h (Fig. 1A). As observed previously,
IFN-γ is a more robust inducer of mGBP-2 than IFN-β (16), with IFN-γ promoting expression of only about 37 ± 3% as much mGBP-2. Clones 1 and 3 have levels of mGBP-2 expression that more closely approximate that of IFN-γ-treated NIH 3T3 cells (115 ± 22 and 105 ± 22% of the mGBP-2 in IFN-γ-treated cells, respectively), whereas clones 4 and 11 express mGBP-2 at levels more similar to those subsequent to IFN-β treatment (31 ± 9 and 26 ± 3% of the IFN-γ amounts, respectively). These data indicate that, compared with GBP levels during IFN exposure, none of the clones grossly overexpress mGBP-2.

To examine the role of a wild type GTP binding domain in mGBP-2 function, a mutant mGBP-2 was generated that contained a single point mutation in the first conserved region of the tripartite GTP binding domain (P loop). This mutation (S52N) is directly comparable with the serine to asparagine point mutation at position 17 of Ras (S17N), a mutation that has been made in a wide variety of GTPases and has often generated GAPases with reduced affinity for GTP (36). This serine is involved in binding to magnesium ions in the nucleotide-binding pocket in members of the Ras family and in hGBP-1 (28, 29). The FLAG epitope-tagged S52N GTPase mutant was used to generate stable cell lines. As shown in Fig. 1A, the clone expressing S52N mGBP-2 that was used for these studies expresses the mutant mGBP-2 at levels comparable with the wild type mGBP-2 expression of clone 3 (91 ± 18%).

To verify that this point mutation did indeed generate a mGBP-2 protein with altered nucleotide binding, the ability of GBPs to bind to GTP-immobilized agarose was exploited (13). Equal amounts of labeled in vitro transcribed and translated mGBP-2 and S52N mGBP-2 were incubated with GTP-agarose, and the relative levels of bound protein were analyzed by PhosphorImager analysis (Fig. 1B). Wild type mGBP-2 bound to GTP-agarose more robustly than did the S52N mGBP-2. The amount of S52N mGBP-2 bound to GTP-agarose was 44.3 ± 8.4% (n = 4) that of wild type mGBP-2, supporting the concept that this mutation reduces the protein’s affinity for GTP.

Intracellular Localization of mGBP-2 and S52N mGBP-2—We have demonstrated that endogenous mGBP-2 expressed in IFN-γ-treated NIH 3T3 cells is found in a punctate distribution throughout the cell cytoplasm but is also localized to conspicuous vesicle-like structures of heterogeneous size, number, and distribution (16) (Fig. 2A). As a prelude to functional assays using the stable cell lines, we first asked whether mGBP-2 targeted correctly when constitutively expressed in the absence of other IFN-induced proteins or whether its localization required the presence of other IFN-induced proteins. Although immunofluorescence localization is not expected to detect subtle differences in protein localization, it can be used to look for gross protein mistargeting or aggresome formation. Indirect immunofluorescence of NIH 3T3 cells stably expressing FLAG epitope-tagged mGBP-2 showed a staining pattern indistinguishable from that of the endogenous mGBP-2 protein in IFN-γ-treated cells (Fig. 2C). Having determined that lipid modification was necessary for vesicle targeting of mGBP-2 containing a wild type GTPase domain (16), it remained to be determined whether the GTP-binding mutant had an intracellular distribution that differed from that of wild type mGBP-2. For small GTPases, such as members of the Rab family, isoprenoid modification is necessary for vesicle targeting, but it is not sufficient. A relatively large proportion of the protein remains in the cytoplasm as a consequence of an isoprenoid-
mediated protein-protein interaction. The fraction of Rab proteins associated with vesicles is the GTP-bound or active portion of the protein (38, 39). Indirect immunolocalization of the S52N mutant of mGBP-2 was indistinguishable from the wild type mGBP-2, suggesting that mGBP-2 targeting to membranes is independent of GTP binding (Fig. 2E). To date the importance of membrane localization by mGBP-2 has not been correlated with any known function. These observations that mGBP-2 and the GTP-binding mutant show intracellular distributions indistinguishable from the endogenous protein suggest that any differences in the phenotype observed for these cells are not the consequence of gross mistargeting of the respective proteins.

**The Expression of mGBP-2 in NIH 3T3 Cells Accelerates Cell Growth**—Whereas interferons induce an antiproliferative response in many cells, one notable exception is fibroblasts, where IFN-γ treatment can promote primary fibroblast proliferation *in vitro* (40–46). Consequently, the NIH 3T3 cells expressing mGBP-2 and the GTP-binding mutant were examined for changes in their growth properties. To determine whether there were changes in the rate of cell growth, cell counts were performed subsequent to plating equal numbers of each clone. These cells were followed for 3–4 days after plating. Beyond that time the curves began to plateau and doubling times slowed for some clones, presumably due to cell density. Representative growth curves generated for mGBP-2-expressing cells from both clonal isolations are shown (Fig. 3, A and B).

![Graph A](image1.png)

**Graph A**. NIH 3T3 cells expressing mGBP-2 have accelerated growth rates. Control and mGBP-2 expressing NIH 3T3 cell were plated at $1 \times 10^6$ cells per dish and counted by hemocytometer daily. All assays were set up in triplicate. A, a representative growth curve from the first set of clones isolated is presented as follows: control 20 (diamond), clone 4 (circle), S52N (square), and clone 3 (triangle). B, a representative growth curve from the second set of clones is presented as follows: control 16 (diamond), clone 11 (circle), and clone 1 (triangle). C, the doubling times for each clone were calculated as described under “Experimental Procedures” and expressed as mean doubling time in hours ± S.D. D, the doubling times for each clone are expressed as percent of the control doubling time on a per experiment basis. * represents $p < 0.05$. 

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**Graphs B and C**. NIH 3T3 cells expressing mGBP-2 have accelerated growth rates. Control and mGBP-2 expressing NIH 3T3 cell were plated at $1 \times 10^6$ cells per dish and counted by hemocytometer daily. All assays were set up in triplicate. A, a representative growth curve from the first set of clones isolated is presented as follows: control 20 (diamond), clone 4 (circle), S52N (square), and clone 3 (triangle). B, a representative growth curve from the second set of clones is presented as follows: control 16 (diamond), clone 11 (circle), and clone 1 (triangle). C, the doubling times for each clone were calculated as described under “Experimental Procedures” and expressed as mean doubling time in hours ± S.D. D, the doubling times for each clone are expressed as percent of the control doubling time on a per experiment basis. * represents $p < 0.05$. 

**Graph D**. NIH 3T3 cells expressing mGBP-2 have accelerated growth rates. Control and mGBP-2 expressing NIH 3T3 cell were plated at $1 \times 10^6$ cells per dish and counted by hemocytometer daily. All assays were set up in triplicate. A, a representative growth curve from the first set of clones isolated is presented as follows: control 20 (diamond), clone 4 (circle), S52N (square), and clone 3 (triangle). B, a representative growth curve from the second set of clones is presented as follows: control 16 (diamond), clone 11 (circle), and clone 1 (triangle). C, the doubling times for each clone were calculated as described under “Experimental Procedures” and expressed as mean doubling time in hours ± S.D. D, the doubling times for each clone are expressed as percent of the control doubling time on a per experiment basis. * represents $p < 0.05$. 

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of 16.6 ± 2.6 (clone 3; n = 5) and 16.0 ± 2.6 h (clone 1; n = 4). The two clones expressing mGBP-2 at lower levels had doubling times of 26.8 ± 3.8 (clone 4; n = 5) and 27.3 ± 8.3 h (clone 11; n = 4). These data show that mGBP-2 expression at the level observed for IFN-γ treatment of NIH 3T3 cells (clones 3 and 1) shortens the doubling time of NIH 3T3 cells by almost 50% (Fig. 3D). The failure of cells expressing the GTP-binding site mutant of mGBP-2 to grow at these rapid rates (30.38 ± 10.7; n = 5) shows the requirement of GTP binding and/or hydrolysis for modulating cell growth rates (Fig. 3C).

Flow cytometry was used to evaluate the proportion of mGBP-2-expressing cells in each stage of the cell cycle. Comparison of actively growing control 20 and clone 3 cells consistently showed a slight increase in the proportion of mGBP-2 expressing cells in S phase with a concomitant decrease in the proportion of cells in G0/G0 phase compared with control cells (Fig. 4). However consistent these differences were, the magnitude was small and not statistically significant. That mGBP-2 did not result in differences in the proportion of cells in each stage of the cell cycle was confirmed by flow cytometry of control 16 and clone 1. These data suggest that the length of each of the different stages of the cell cycle is proportionally equal to the number of independent experiments.

of 60.8 ± 0.3 (clone 3; n = 5) 50% (Fig. 3D) and 16.0 ± 2.6 h (clone 1; n = 4). The two clones expressing mGBP-2 at lower levels had doubling times of 26.8 ± 3.8 (clone 4; n = 5) and 27.3 ± 8.3 h (clone 11; n = 4). These data show that mGBP-2 expression at the level observed for IFN-γ treatment of NIH 3T3 cells (clones 3 and 1) shortens the doubling time of NIH 3T3 cells by almost 50% (Fig. 3D). The failure of cells expressing the GTP-binding site mutant of mGBP-2 to grow at these rapid rates (30.38 ± 10.7; n = 5) shows the requirement of GTP binding and/or hydrolysis for modulating cell growth rates (Fig. 3C).

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NIH 3T3 Cells Treated with IFN-γ Have Accelerated Growth Rates—Although the literature contained several examples of IFN-γ-induced proliferation of fibroblasts, these studies were performed with primary fibroblasts. Therefore, the growth of NIH 3T3 cells was examined in the presence or absence of IFN-γ or IFN-β (Fig. 5A). IFN-β had no significant antiprolif-erative effect on these cells (time for doubling of 32.5 ± 6.5 h compared with 31.2 ± 0.1 h for untreated controls; n = 3). However, IFN-γ treatment was growth promoting for NIH 3T3 fibroblasts. The IFN-γ-treated cells grew faster than either untreated or IFN-β-treated cells (21.5 ± 3.4 h; n = 4) (Fig. 5A). Because the NIH 3T3 cells showed greater variability in growth rates, we also presented the doubling times as percent of control (Fig. 5B). When the doubling times of IFN-γ-treated cells are compared with the untreated cells on a per experiment basis, IFN-γ treatment reduces the doubling time to 60 ± 4% of untreated cells.

NIH 3T3 Cells Expressing mGBP-2 Grow to Higher Density—We continued evaluation of the growth changes mediated by mGBP-2 by determining whether NIH 3T3 cells expressing mGBP-2 showed density-arrested growth. For these experiments cells were allowed to grow for 5 days after having reached confluence and were examined both by microscopy and subsequent to crystal violet staining of the cell monolayer. All of the control clones grew until they formed a single cell monolayer and then stopped growing (Fig. 6A). However, all of the transfectants expressing mGBP-2 continued to grow and formed foci (Fig. 6A). The number and size of these foci correlated with the level of mGBP-2 expression. The morphology of these foci are shown in Fig. 6B. The cells containing the S52N mutant failed to form foci, demonstrating that a wild type GTP binding domain is required for the formation of foci.

To examine more directly the differences in saturation density, growth curves of the cells expressing mGBP-2 were continued until cell numbers plateaued (Fig. 7). Similar to the correlation observed between expression level and doubling times, the clone expressing mGBP-2 at levels comparable with IFN-β treatment (clone 4) reached saturation at cell numbers comparable with control cells (clone 20) (about 3 × 10⁶ cells per
6 cm dish; Fig. 7A), whereas the highest expressing cells (clone 3) did not stop expanding until the cells had reached a density of about $4 \times 10^6$ cells per 6-cm dish. S52N mGBP-2 grew at a similar rate to control cells (Fig. 3) but did not growth-arrest until a cell density intermediate between control and high expressing cells. There were, however, exceptions to this correlation. Clone 11 cells that express at low levels grew erratically and on occasion would grow to the same density as the highest expressing clone of that isolate (not shown). NIH 3T3 cells when treated with IFNs grew in a similar manner. The cells treated with IFN-α and the untreated cells stopped growing at about $3 \times 10^4$ cells/6-cm dish, whereas treatment with IFN-γ resulted in density arrest at closer to $4 \times 10^5$ cells/dish (Fig. 7B).

The observation that mGBP-2 can induce the formation of foci and partial loss of contact growth inhibition suggested that mGBP-2 may mediate oncogenic transformation. Other hallmarks of such transformation are the reduced dependence on exogenous growth factors manifested by the ability to grow in reduced serum, the loss of anchorage-dependent growth as assessed by growth in soft agar, and the ability to form tumors in athymic mice. NIH 3T3 cells expressing mGBP-2 are unable to grow as colonies in soft agar, suggesting that they have not lost anchorage-dependent growth (data not shown). To determine whether mGBP-2 conferred the ability to grow in lower serum, cells were plated and allowed to attach for 16 h in the presence of 10% fetal calf serum and then were shifted to 2, 5, and 10% fetal calf serum for 14 days. Both clones 1 and 3 grew better than control cells in all serum levels tested (Fig. 8A). To determine whether a wild type GTP binding domain was required for better growth in low serum, control 20, clone 3, and clone S52N were grown in low serum for 2 weeks, and the numbers of cells present were determined by trypsinization and counting. Again, the mGBP-2-expressing cells grew more robustly than the control cells, and wild type GTP binding domain was required for enhanced growth in low serum (Fig. 8B).

To determine whether mGBP-2 expression could promote tumorigenesis of NIH 3T3 cells, clone 3 and control 20 cells were injected intradermally into athymic mice and monitored for tumor formation. The clones had a lag time of about 2 weeks before tumors were large enough for detection, but after that time the tumors generated by mGBP-2-expressing cells grew rapidly (Fig. 9). The tumors generated by mGBP-2 expressing cells were solid, with no grossly detectable necrosis internally. No macroscopic metastatic lesions on other organs were observed.

This report demonstrates the first phenotype for the robustly induced IFN-induced GTPase, mGBP-2. These data show that mGBP-2 is capable of altering a number of growth parameters. mGBP-2 shortens fibroblast doubling time, reduces the need for serum-derived growth factors, induces growth to higher saturation density and focus formation, and induces tumor formation in nude mice.

**DISCUSSION**

We have generated NIH 3T3 cell clones constitutively expressing either the murine IFN-induced GTPase, mGBP-2, or mGBP-2 containing a point mutation in the P loop of the GTP
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binding domain, S52N mGBP-2. Analysis of these cells indicated that mGBP-2 is capable of altering several parameters of cell growth in the absence of other IFN-induced proteins. First, NIH 3T3 fibroblasts expressing mGBP-2 grew faster and to higher saturation density (Figs. 3 and 7). The extent to which the doubling time of NIH 3T3 cells is shortened by mGBP-2 correlates with its level of expression. Expression of mGBP-2 at levels comparable with those of NIH 3T3 cells treated with IFN-γ for 24 h induced cells to grow about twice as fast as cells without mGBP-2 (Fig. 3). Flow cytometric analysis of the DNA content of these cells shows no difference in the proportion of cells in G1, S, or G2/M compared with control transfectants, suggesting that the different phases of the cell cycle are uniformly shortened by mGBP-2 (Fig. 4). Second, the increased density of cell growth observed in mGBP-2-expressing fibroblasts was accompanied by at least a partial loss of contact growth inhibition, as evidenced by the ability to form foci when grown to post-confluence. Foci formation is correlated with mGBP-2 expression level (Fig. 6). Third, the growth advantage of mGBP-2-expressing cells is not abrogated in reduced serum (Fig. 8). Finally, NIH 3T3 cells expressing mGBP-2 show enhanced tumor-forming ability in athymic mice (Fig. 9).

The increased growth rate, partial loss of contact growth inhibition, increased ability to grow in reduced serum, and the ability to form tumors in athymic mice, but retention of substrate-dependent growth, suggest a weak or incomplete form of transformation. This “incomplete” transformation has been observed for other GTPases, most notably members of the Rho family of small GTPases (reviewed in Refs. 47 and 48). The Rho family of GTPases is involved in a wide variety of cellular processes, such as signal transduction, cytokinesis, cell adhesion, migration, and cell proliferation (48–50). In general, transformation by members of the Rho family is much less robust than observed for Ras and requires the expression of proteins containing activating mutations (51–56). Even then, some Rho family members fail to induce all of these parameters of transformed growth. For example, expression of activated RhoA in NIH 3T3 cells does not result in focus formation or growth in soft agar but does promote reduced dependence on serum and tumorigenesis (47). Rho family proteins mediate these phenotypes by interaction with and activation of a wide variety of effector proteins (49, 50). The mechanism(s) by which mGBP-2 mediates similar changes to those observed for activated Rho family members is at this time completely unknown. Certainly, one possibility is that mGBP-2 functions within one of the pathways used to mediate Ras or Rho family-induced cellular changes. Recently, the rat homolog of mGBP-2 was identified as a putative target of Ras transformation, having been identified as a gene robustly induced subsequent to Ras transformation of rat fibroblasts (57).

GTP binding and hydrolysis is crucial for many, but not all, functions of GTPases. We have shown for mGBP-2 that wild type GTP binding is required for its growth promoting activity as well as the ability to form foci and grow in reduced serum. For example, the antiviral activity of the IFN-induced protein MxA does not require GTPase activity (58).

Although interferons are probably best studied for their antiviral and antiproliferative activities, interferons are not antiproliferative for all cell types. For some cells they have no effect on cell proliferation (43–46), and for others they can actually be growth-stimulatory (40–42). IFN-γ has been shown to be growth-promoting or mitogenic for a variety of human fibroblasts, such as human lung, synovial, or dental pulp fibroblasts. The combined observations that IFN-γ is growth-stimulatory for NIH 3T3 fibroblasts and that mGBP-2 is capable of the same growth stimulatory activity in the absence of other IFN-induced proteins support a role for mGBP-2 in IFN-mediated cell growth regulation. However, the mechanism(s) by which mGBP-2 mediates its growth-stimulatory effects remains to be elucidated. As mentioned above, one possibility is that mGBP-2 functions within a pathway used by other small GTPases, such as members of the Rho family. The Rho proteins are important regulators for a number of growth-related processes. Certainly, Rho family proteins are involved in regulation of cytoskeleton organization and cytokinesis. They are also necessary for integrin-mediated signals, and in this capacity...
are actively involved in transducing cell growth signals. Alternatively, mGBP-2 may up-regulate the expression of growth factor receptors or other components involved in mitogenic signaling. How mGBP-2 mediates these growth changes is actively under investigation.

Acknowledgments—We thank Drs. Janice Buss, Dennis Stacey, Alan Wolfman, and Lawrence Quilliam for critical reading of the manuscript; Amy Raber and Cathy Stanko for flow cytometry; and Christoph Carter for assistance in screening and maintenance of NIH 3T3 cell clones.

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