Identification of a Novel GTPase, the Inducibly Expressed GTPase, That Accumulates in Response to Interferon γ*

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Interferon γ is a pleiotropic cytokine that regulates many immune functions. We have identified a novel protein, inducibly expressed GTPase (IGTP), whose expression was regulated by interferon γ in macrophages. In mouse RAW 264.7 macrophages, IGTP mRNA levels were almost undetectable but increased within 1 h of exposure to interferon γ, peaked at very high levels within 3 h, and remained at high levels to at least 48 h; pretreatment of the cells with cycloheximide blocked the majority of mRNA accumulation. In the mouse, the mRNA was highly expressed in thymus, spleen, lung, and small intestine. Using interspecific backcross analysis, the IGTP gene was mapped to mouse chromosome 11. The IGTP cDNA encoded a putative polypeptide of M, 48,507 and pl 7.9 that contained three consensus GTP binding motifs, GXGGXG(S/T), DXXG, and NTKKXD. Both IGTP that had been immunoprecipitated from RAW cells and a glutathione S-transferase IGTP fusion protein were able to convert GTP to GDP in vitro. Subcellular protein fractionation and Western blotting localized IGTP to the cytosol of RAW cells. In addition, the protein was homologous to proteins encoded by three previously cloned cDNAs, IRG-47, TGTP/Mg21, and LRG-47, and thus may be a representative of a new family of interferon γ-regulated GTPases.

Interferon γ (IFN-γ) is a 20-kDa protein that regulates a wide variety of immunological and inflammatory processes (for review see Ref. 1). While production of IFN-γ is limited to CD8+ T cells, some CD4+ T cell subsets (2), and natural killer cells (3), its receptor is found on almost all cell types where it elicits many diverse physiological responses. In macrophages for example, IFN-γ induces major histocompatibility class II expression (4), increases Fc receptor-mediated phagocytosis (5), and mediates removal of neoplastic cells and virally and parasitically infected cells by initiating secretion of cytosolic compounds and tumor necrosis factor α (6–9). In endothelial cells on the other hand, IFN-γ, in combination with tumor necrosis factor α, markedly increases expression of major histocompatibility class I molecules and the cell adhesion molecules, ICAM-1 and ELAM-1, thereby promoting recruitment of immune cells to areas of inflammation (10). These pleiotropic responses are initiated by binding of IFN-γ to its receptor, followed by receptor dimerization and a cascade of primary and secondary events (1). However, these molecular events have not been completely defined, and identification of proteins whose expression is regulated by IFN-γ is the subject of active investigation.

We report here the identification of a 48-kDa protein, designated IGTP, whose expression was rapidly and dramatically increased by IFN-γ in macrophages and fibroblasts. The protein was first recognized as the product of a differentially displayed cDNA in hepatocyte growth factor (HGF)-treated C127 mouse fibroblasts. In these cells, the IGTP mRNA levels showed a slight transient increase following HGF exposure; in addition, mRNA levels were constitutively elevated in C127 and NIH/3T3 cells that were transformed by coexpression of HGF and its receptor Met. These observations and their significance will be discussed elsewhere. In the present work, we characterize the IFN-γ-induced IGTP gene expression, and we demonstrate that the protein product is a GTPase. Because of its expression pattern and its biochemical activity, the protein has been designated inducibly-expressed GTPase (IGTP). The protein had high sequence homology with the proteins encoded by three other cDNAs, IRG-47 (11), TGTP/Mg21 (13), and LRG-47 (14), for which no biochemical or physiological function had been described previously, but which also contained putative GTP binding sequences. IGTP, therefore, may be representative of a new family of GTPases that could potentially mediate the effects of IFN-γ in macrophages and other cells.

EXPERIMENTAL PROCEDURES

Cells and Culture—C127 cells (ATCC CRL-1616, American Type Culture Collection), Met-transfected C127 cells (C127/Met),2 and RAW 264.7 cells (ATCC TIB-71) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin (Life Technologies, Inc.), 100 units/ml streptomycin (Life Technologies, Inc.), and 100 units/ml streptomycin (Life Technologies, Inc.). Hepatocyte growth factor was purified from the supernatant of transformed NIH/3T3 cells that overproduce the factor (15); it was added directly to the growth medium of C127 cells at 200 units/ml. Recombinant mouse interferon γ (Boehringer Mannheim) and lipopolysaccharide (serotype 055:B5, Sigma) were added directly to the culture media of C127 and RAW 264.7 cells at the concentrations indicated in the text.

Northern Blotting—Total RNA was prepared from cultured cells (16) and from mouse tissues (17) using standard acidic phenol/chloroform extraction protocols. 15 μg of RNA samples were separated on 1.2% agarose/formaldehyde gels and used for Northern blot analysis as de-
scribed previously (18). The blots were probed with a \(^{32}\)P-labeled human glyceraldehyde-phosphate dehydrogenase probe isolated as a 1.2-kb PstI fragment of pGIGAP (19), a mouse 1P10 probe isolated as a 0.5-kb EcoRI fragment of the mouse C7-1 (ATCC G3315), a human \(\beta\)-actin cDNA probe (Clontech; supplied with product 7760-1), or an IGTp probe isolated as a 0.28-kb EcoRI fragment of the IGTp CDNA (bases 1645–1927, GenBank accession U53219). The IGTp probe contained entirely 3′-untranslated sequences that were not conserved with the related cDNAs referred to in the text; thus the probe was specific for IGTp.

Differential Display Screening and Library Screening—C127/Met cells were exposed to 200 units/ml hepatocyte growth factor for various times, and total RNA was isolated and used for differential display screening with an RNAimage kit 1 (GenHunter Corp., Brookline, MA) according to the protocols supplied by the manufacturer. One 150-base cDNA fragment, designated A2c, was isolated under primers H-, T-, A- and HAP1 primers of A2c with slightly increased sensitivity from C127/Met cells exposed to HGF for 3–8 h. The DNA fragment was used to screen a mouse spleen cDNA library (Stratagene, La Jolla, CA), from which a 1927-base cDNA clone was isolated and later designated as the IGTp CDNA. The clone was sequenced on both strands using the Applied Biosystems (Foster City, CA) Prism DyeDeoxy Sequencing System. One end of this CDNA corresponded to the 150-base A2c DNA. Based on a partial poly(A) tail present in A2c, the IGTp CDNA was oriented, and a 1419-base open reading frame was identified. The DNA sequence and translated protein were analyzed using Genetics Computer Group (Madison, WI) software.

Plasmid Subcloning and Preparation of GST Fusion Proteins—The plasmid full-length IGTp, pGEX/IGTp, was constructed as described previously (20); this produced an in-frame fusion of the GST gene and the IGTp cDNA, was labeled with an \(\alpha\)-\(^{32}\)P-end labeling kit (Amersham Corp.). The IGTp probe contained as a 0.28-kb EcoRI fragment into the pGEX-KG (20); this produced an in-frame fusion of the GST gene and the IGTp CDNA. The probe, a 0.28-kb EcoRI fragment of the 3′-untranslated region of the IGTp CDNA, was labeled with an \(\alpha\)-\(^{32}\)P-end labeling kit and used as probe.

Interspecific Backcross Mapping—Interspecific backcross progeny were generated by mating (C57BL/6J × C127/Met) F1 females and (C57BL/6J × C127/Met) F1 males as described previously (22). A total of 205 N2 mice were used to map the IGTp locus (see the text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, and Southern blotting were performed as described previously (23). All blots were prepared with Hybond-N nylon membranes (Amersham Corp.). The probe, a 0.28-kb EcoRI fragment of the 3′-untranslated region of the IGTp CDNA, was labeled with an \(\alpha\)-\(^{32}\)P-end labeling kit and used as probe. A description of the probes and restriction fragment length polymorphisms (RFLPs) for I13 and Trp52, two of the loci linked to the IGTp, has been published (24). Recombination distances were calculated using the computer program LINKMAP (25). Genotype was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Immunoprecipitation—Cells were plated on 60-mm plates and grown to confluence. In some experiments, the cells were labeled by washing them three times with Dulbecco's modified Eagle's medium without methionine (Life Technologies, Inc.) and then incubating them in dimethyl sulfoxide as described previously (26). General was added with 10% fetal calf serum (v/v) and 0.5 mM sodium dodecyl sulfate, 0.048 mM EDTA (v/v) sodium dodecyl sulfate, 0.05% (w/v) bromophenol blue) that had been diluted 1:3 with PBS; the suspension was mixed vigorously and boiled for 5 min, and the resin was pelleted by centrifugation for 5 min at 15,000 × g. The proteins in the SDS buffer were separated by 9% SDS-polyacrylamide gel electrophoresis.

GTP Hydrolysis in Cell lysates—Protein was immunoprecipitated from cell lysates as described above, except that the cells were lysed in a lysis buffer of 10 mM CHAPSO, 50 mM Tris, pH 8.0, and 0.15 mM NaCl; immunoprecipitation using this buffer results in slightly higher GTPase activities for some GTP binding proteins (26). \(\alpha\)-\(^{32}\)P] GTP hydrolysis and separation of the nucleotide products by polyacrylamide gel electrophoresis and phosphorimage analysis (27). The values were corrected by subtracting the background activity of the IGTp antibody.

RESULTS

The IGTp CDNA was first identified as a 150-base cDNA fragment that was differentially displayed in hepatocyte growth factor-treated C127 mouse fibroblasts (data not shown). However, it was observed during these experiments that IGTp mRNA levels were dramatically increased by 100 units/ml IFN-\(\gamma\), in both this cell line (data not shown) and in RAW 264.7 mouse macrophages (Fig. 1). While basal IGTp mRNA levels were almost undetectable, they were easily detectable within 1 h of IFN-\(\gamma\) exposure, peaking at very high levels at 3 h, and remaining at high levels to at least 48 h (Fig. 1). Longer exposures of the blot showed that amounts of IGTp mRNA...
mRNA were present before exposure to IFN\(\gamma\) (data not shown). In RAW cells, IGTP mRNA levels were also induced by 1 \(\mu\)g/ml lipopolysaccharide with similar kinetics but to a lesser extent (Fig. 1). With increased lipopolysaccharide exposure times, the mRNA showed a slightly increased mobility which could have resulted from shortening of the poly(A) tail; however, this possibility was not addressed experimentally.

The half-life of the IGTP mRNA was determined by exposing RAW cells to 100 units/ml IFN\(\gamma\) for 3 h and then to the transcriptional inhibitor actinomycin D (4 \(\mu\)M) for various times; the decay of the accumulated mRNA was then followed by Northern blotting. Under these conditions, the mRNA decayed with a half-life of about 4.5 h (data not shown).

Inducibly expressed genes are often classified as primary response genes if induction requires only previously translated transcription factors or as secondary response genes if newly translated factors are required (for review see Ref. 27). To determine if IGTP accumulation required protein synthesis, RAW cells were exposed to 0.1 mM cycloheximide for 30 min and then to 100 units/ml IFN\(\gamma\); mRNA accumulation was followed by Northern blotting (Fig. 2). Inhibition of protein synthesis in this manner blocked the majority of the IGTP mRNA accumulation, although a small amount of mRNA did accumulate (Fig. 2). Conversely, under the same conditions, cycloheximide did not affect the accumulation of IP10 mRNA, which is a primary IFN\(\gamma\) response gene in macrophages (28) (Fig. 2). Therefore, because IGTP mRNA levels did not show detectable increases until 1 h following IFN\(\gamma\) exposure, and because full induction required protein synthesis, IGTP accumulation may be classified as a secondary IFN\(\gamma\) response.

Hybridization of the IGTP cDNA to a total RNA blot containing RNA from several mouse tissues revealed very high expression in the thymus and slightly lower expression in the spleen, lung, and small intestine; expression in brain, heart, kidney, liver, skeletal muscle, and testes was very low or undetectable (Fig. 3). The tissue expression pattern suggested that IGTP may be highly expressed in immune cell populations.

The mouse chromosomal location of Igtp was determined by interspecific backcross analysis using progeny derived from matings of ((C57BL/6 \(\times\) Mus spretus)\(\times\) and C57BL/6\(\times\)) mice.
This page contains a detailed analysis of the Igtp gene, which is involved in the IFN-γ pathway. The text discusses the mapping of the Igtp gene to mouse chromosome 11 and its expression in various cell types. It also describes the cloning of cDNAs and the analysis of their protein expression in response to IFN-γ.

A diagram illustrates the genetic linkage analysis, with each chromosome represented by a bar and the flanking markers shown. The figure shows the distribution of recombinants across different loci, with the percentage of recombinants for each chromosome pair indicated. The Igtp gene is located on chromosome 11, and its expression is upregulated by IFN-γ.

The text further explains the protein expression analysis, where antibodies were used to detect the Igtp protein in various cell types. The protein expression was compared between cells treated with and without IFN-γ.

Overall, the page provides a comprehensive overview of the Igtp gene's mapping, expression, and protein analysis, highlighting its role in the IFN-γ-induced GTPase activity.
tated the LRG-47 protein, and, again, increased GTPase activity was detected in immune pellets from IFN-γ-treated cells, relative to control cells (data not shown). Taken together, these data suggest that IGTP, LRG-47, and possibly the other related proteins are GTPases.

In similar experiments, IGTP was expressed in bacteria as a glutathione S-transferase (GST) fusion protein and, following partial purification by glutathione affinity chromatography (data not shown), was used in a [32P]GTP hydrolysis assay (Fig. 8). The GST-IGTP fusion protein converted about six times more GTP to GDP than was converted by an equivalent amount of GST protein (Fig. 8). This result suggested that the IGTP GTPase activity was inherent to the protein and did not require other mammalian accessory proteins for basal activity.

To determine the subcellular distribution of IGTP, RAW cells were exposed to IFN-γ for 4 h to stimulate synthesis of the protein and then cellular protein was separated into cytosolic and nuclear/particulate fractions; the presence of IGTP and the cytosolic protein β-tubulin (31) was determined by Western blotting (Fig. 9). In some experiments, protein fractions were prepared by briefly incubating the cells in a hypotonic buffer, disrupting the cytosolic membranes by drawing the cells through a 25-gauge needle, and then separating nuclei and particulates from the cytosolic protein fraction by centrifugation. Using this procedure, IGTP was readily detected in the nuclear/particulate protein fraction of IFN-γ-treated cells but not in the cytosolic protein fraction (Fig. 9). Conversely, when the cytosolic membranes were lysed by incubating the cells in an isotonic buffer containing 0.5% (v/v) Nonidet P-40, IGTP localized to the cytosolic protein fraction (Fig. 9). With both procedures, the nuclei appeared intact as determined by light microscopy (data not shown), and β-tubulin was detected only in the cytosolic protein fractions (Fig. 9). These data raised the possibility that IGTP may have been a cytosolic protein that was loosely associated with membranes in the nuclear/particulate fractions and that this association was disrupted by mild treatment with a nonionic detergent such as Nonidet P-40. Cytosolic localization of IGTP was also supported by preliminary results using the IGTP antibody to immunostain IFN-γ-treated RAW cells (data not shown).

**DISCUSSION**

We report here the identification of a 48-kDa GTPase, IGTP, whose expression was rapidly and markedly increased by IFN-γ in macrophages and fibroblasts. Its expression pattern suggested that IGTP could potentially mediate some IFN-γ-induced responses in macrophages and fibroblasts, and its high expression in mouse thymus and spleen suggested that it may also function in other immune cell populations.

We mapped the Igtp gene to a location on mouse chromosome 11 between Ii3 and Trp53. We compared our interspecific map of chromosome 11 with a composite mouse linkage map that reports the location of many uncloned mouse mutations (Fig. 10). The GST-IGTP fusion protein converted about six times more GTP to GDP than was converted by an equivalent amount of GST protein (Fig. 8). This result suggested that the IGTP GTPase activity was inherent to the protein and did not require other mammalian accessory proteins for basal activity.

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The central region of mouse chromosome 11 shares homology to regions of human chromosomes 5q and 17p, and thus, the
human IGTP gene is likely to reside on 5q or 17p. Interestingly, monosomy for chromosome 5 or 5q deletions are frequently associated with myelodysplastic disorders, myeloproliferative syndromes, and acute myeloid leukemia (32, 33). Because Igtp is expressed in myeloid cells, it will be of interest to determine if the human IGTP gene maps to the region on chromosome 5 thought to contain this important myeloid tumor suppressor gene (32).

The IGTP protein contained three GTP-binding motifs, GXXXXGKS/T, DXXG, and NKXD (29), and the protein had inherent GTPase activity. Although many other GTP-binding proteins have been identified, they have diverse cellular functions, and the presence of this biochemical activity in itself does not suggest a cellular function for IGTP. However, the extensive information gained in the biochemical studies of Ras and other GTP-binding proteins may facilitate study of IGTP. For instance, the x-ray crystal structure of Ras (34, 35) has shown that the first and second GTP-binding motifs both contact the phosphates of the GTP molecule and a Mg\(^{2+}\) cofactor, whereas the third motif contacts the purine ring. Mutation of serine 17 in the first GTP-binding motif to asparagine blocks the ability of Ras to hydrolyze GTP, and expression of this Ras mutant in cells blocks the function of wild-type Ras (36). The analogous mutation in dynamin, another GTP-binding protein, also has a dominant-negative effect (37, 38), and this may well be true of IGTP.

**Fig. 7.** GTPase activity of immunoprecipitated IGTP. RAW 264.7 cells were exposed to control conditions or to 100 units/ml interferon γ (IFN-γ) for 4 h, and the cells were then used for immunoprecipitation with preimmune serum or anti-IGTP serum. α-[\(^{32}\)P]IGTP was incubated with the immunoprecipitated proteins or with buffer at 37 °C for the indicated times, and the nucleotide products were separated by polyethyleneimine thin layer chromatography. A, the chromatogram was used for autoradiography, and the positions of GTP and GDP were determined using the fluorescent indicator in the PEI-cellulose plates. B, the amounts of radioactive GTP and GDP were determined with a phosphorimager, and the ratio of GDP to GTP was plotted as a function of time. Other details are described in the text. □, buffer; ◇, control, preimmune; ○, control, anti-IGTP; △, IFN, preimmune; □, IFN, anti-IGTP.

**Fig. 8.** GTPase activity of a GST-IGTP fusion protein. Bacterially expressed GST and GST-IGTP fusion proteins were partially purified using glutathione affinity chromatography. α-[\(^{32}\)P]IGTP was incubated with 10 µg of the purified proteins or bovine serum albumin (BSA) at 37 °C for the indicated times, and the nucleotide products were separated by polyethyleneimine thin layer chromatography. A, the chromatogram was used for autoradiography, and the positions of GTP and GDP were determined using the fluorescent indicator in the PEI-cellulose plates. B, the amounts of radioactive GTP and GDP were determined with a phosphorimager, and the ratio of GDP to GTP was plotted as a function of time. Other details are described in the text. □, BSA; ◇, GST; ○, GST-IGTP.

**Fig. 9.** Subcellular localization of IGTP in RAW 264.7 cells. Cellular protein was fractionated into nuclear/particulate (N/P) and cytosolic (Cy) fractions by lysis of the cells by incubation in a hypotonic buffer followed by passage through a 25-gauge needle (A) or by lysis of the cells by incubation in a buffer containing 0.5% Nonidet P-40 (B). Fractionated protein was used for Western blotting with anti-IGTP and anti-β-tubulin antibodies. The positions of selected molecular mass markers are indicated at the left. Other details are described in the text.
IGTP, an IFNγ-induced GTPase

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