IIGP1, an Interferon-γ-inducible 47-kDa GTPase of the Mouse, Showing Cooperative Enzymatic Activity and GTP-dependent Multimerization*

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*IIGP1 belongs to a well defined family of 47-kDa GTPases whose members are present at low resting levels in mouse cells but are strongly induced transcriptionally by interferons and are implicated in cell-autonomous resistance to intracellular pathogens. Recombinant IIGP1 was expressed in Escherichia coli and purified to homogeneity. Here we present a detailed biochemical characterization of IIGP1 using various biochemical and biophysical methods. IIGP1 binds to GTP and GDP with dissociation constants in the micromolar range with at least 10 times higher affinity for GDP than for GTP. IIGP1 hydrolyzes GTP to GDP, and the GTPase activity is concentration-dependent with a GTP turnover rate of 2 min⁻¹ under saturating protein concentrations. Functional interaction between IIGP1 molecules is shown by nucleotide-dependent oligomerization in vitro. Both cooperative hydrolysis of GTP and GTP-dependent oligomerization are blocked in a mutant form of IIGP1 modified at the C terminus. IIGP1 shares micromolar nucleotide affinities and oligomerization-dependent hydrolytic activity with the 67-kDa GTPase hGBP1 (induced by type I and type II interferons), with the antiviral Mx proteins (interferon type I-induced) and with the paradigm of the self-activating large GTPases, the dynamins, with which Mx proteins show homology. The higher relative affinity for GDP and the relatively low GTPase activity distinguish IIGP1, but this study clearly adds IIGP1 and thus the p47 GTPases to the small group of cooperative GTPase families that appear to characterize the development of intracellular resistance during the interferon response to infection. The present analysis provides essential parameters to understand the molecular mechanism by which IIGP1 participates in this complex resistance program.

Interferons are immunomodulatory cytokines induced either directly by virus infection or by immune and inflammatory stimuli. Type I interferons are secreted by virus-infected tissue cells and by certain activated immune cells such as dendritic cells. Type II or γ-interferon is secreted largely by activated effectors of the innate and adaptive immune systems (natural killer cells, macrophages, and T lymphocytes). Interferons stimulate the transcription of several hundred genes belonging to partially overlapping sets (1). This complex cellular response resolves into a series of programs contributing in different ways to mechanisms of organismal and cellular resistance to pathogens, and this generalization holds for three described families of interferon-inducible GTPases. The antiviral Mx proteins induced exclusively by type I interferons were the first to be characterized (2). More recently, members of two further families, the p65 (guanylate-binding proteins (GBP)1) and the p47 GTPases (3), both induced preferentially by interferon-γ have also been shown to contribute to pathogen resistance (4–9).

Presently, with the possible exception of MxA (2), no explanation is available to connect the biochemical activity of these GTPases with their adaptive function in pathogen resistance. A member of the p65 GTPases from human cells, hGBP1, has been shown to block viral replication and endothelial cell proliferation (4, 10) and has been subjected to a detailed biochemical and structural analysis (11–13). hGBP1 binds to GTP, GDP and GMP with equal affinity and hydrolyzes GTP to GDP and GMP. The GTPase activity of hGBP1 is stimulated at higher protein concentrations, and the presence of GTP or Gpp(NH)p favors the formation of oligomers. The nucleotide-free and the Gpp(NH)p-bound structures reveal many similarities and differences from other GTP-binding proteins. The high intrinsic GTPase activity, the cooperative hydrolytic behavior, and structural properties have suggested that hGBP1, representing the p65 GTPases, may be a functional and possibly also structural model for the family of large GTPases including Mx and dynamin (2, 14). In addition to cooperative GTPase activity, all of these GTPase families share the property of self-assembly into oligomers and higher order multimers, sometimes on specific templates such as lipid membranes in the case of dynamins (15, 16) and viral ribonucleoprotein particles in the case of MxA (2). The binding of GTP to these structures causes conformational changes that have been correlated with function and favors further self-assembly under some conditions (15, 17–19). A critical and still unresolved issue with respect to dynamin is whether they function as conventional regulatory GTPases or as mechanochemical enzymes (20). In view of the proposed structural relationship between the protein families, the resolution of this issue will probably have implications not...

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** Supported by a grant from the Boehringer Ingelheim Fonds.

1 The abbreviations used are: GBP, guanylate-binding protein; mant, 2’3’-O-N-methylanthraniloyl; GTP=S, guanosine 5’-3-O-(thiotriposphate; mGTP=S, mant-GTP=S; Gpp(NH)p, guanosine 5’-β,γ-imido-triphosphate; mGpp(NH)p, mant-Gpp(NH)p; HPLC, high pressure liquid chromatography; mGDP, mant-GDP; GST, glutathione S-transferase.

Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 31, Issue of August 1, pp. 29336–29343, 2003

Received for publication, November 25, 2002, and in revised form, April 16, 2003
Published, JBC Papers in Press, May 5, 2003, DOI 10.1074/jbc.M211973200

29336
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for the production of dynamin but also for the mode of action of its interferon-inducible relatives, Mx and the p65 GTPases. The data that follow now add the p47 GTPases to this list.

IIGP1, with which this study is concerned, is a member of the p47 γ-interferon-inducible GTPase family. The mouse p47 GTPases so far described form a distinct family with no sequence homology to other GTPases outside the GTP-binding region (9). The p47 GTPases have all three GTP-binding motifs GXYXXGKS, P-loop, DXTCG, and (N/T)KXD clearly marked, unlike the p65 GTPase, hGBP1, where the base specific motif (N/T)KKXD motif is functionally replaced by a different structure (11). Four members of the p47 GTPase family have been shown to mediate specific resistance to intracellular pathogens. Fibroblasts stably expressing the p47 GTPase TGGT have reduced susceptibility to cytopathic effects of vesicular stomatitis virus but not of herpes simplex virus (5). Mice with targeted disruptions of other p47 GTPases like IGTP, IRG-47, or LRG-47 are all strikingly susceptible to Toxoplasma gondii infection; in addition, the last also shows susceptibility to Listeria monocytogenes infection (6, 7, 9).

In anticipation of a structural analysis of IIGP1, we here present a quantitative analysis of nucleotide binding and hydrolysis by the purified recombinant protein. IIGP1 is stable in the absence of nucleotides and has a relatively low affinity for guanine nucleotides in the micromolar range. The protein has higher affinity for GDP than for GTP, reflecting a higher dissociation rate for GTP. IIGP1 has low basal GTPase activity that is elevated at higher concentrations of the protein, suggesting cooperative behavior. When nucleotide-free or in the presence of GDP, IIGP1 behaves as a monomer, but it oligomerizes in the presence of GTP or with the nonhydrolyzable GTP analogue Gpp(NH)p. In summary, these properties place IIGP1 in the same functional category as Mx and GBP, the other large interferon-inducible GTPases. However, the distinctive properties of IIGP1 could serve as a key to understand the mechanism of action of this novel GTPase and its function in cell-autonomous pathogen resistance.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**

A 1.2-kilobase restriction fragment of IIGP1 containing the complete coding sequence and artificial SsfI linkers was ligated into SsfI-digested pGEX-4T-2 vector (Amersham Biosciences) and transformed into Escherichia coli BL-21. Cells were grown at 37 °C to an A600 of 0.4, and IIGP1 was expressed as a GST fusion protein upon overnight induction with 1 mM isopropyl-β-D-thiogalactoside at 23 °C. The cells were harvested and resuspended with 1× phosphate-buffered saline, 5 mM EDTA, 200 μM Pefabloc (Roche) and were lysed using a microfluidizer (Microfluidics Corp.) at a pressure of 600 kilopascals. The soluble fraction was purified in a glutathione-Sepharose affinity column (Amersham Biosciences) equilibrated with buffer 1 (1× phosphate-buffered saline, 2 mM dithioerythritol). The GST domain was cleaved by overnight incubation of the protein with thrombin (10 U/ml) at 4 °C. The free IIGP1 was eluted with buffer 1 and was concentrated by ammonium sulfate precipitation.

The protein was further subjected to size exclusion chromatography on a Superdex 75 column (Amersham Biosciences) equilibrated in buffer 2 (50 mM Tris/HCl (pH 7.4), 5 mM MgCl₂, 2 mM dithioerythritol). The fractions were analyzed by SDS-PAGE, and the fractions containing IIGP1 were concentrated to 50 μg/ml by a centrifugal concentrator (Vivaspin) with a 10-kDa cut-off filter. Aliquots were shock-frozen in liquid nitrogen and stored at −80 °C. The concentration of IIGP1 was determined by UV spectrophotometry at 280 nm with the calculated molar absorption coefficient of 35,230 M⁻¹cm⁻¹, based on the number of tyrosine, tryptophan, and cysteine residues (21).

Three versions of the IIGP1 protein are described in this paper, which differ at the C terminus, as shown in Table I. All three proteins were prepared and released from the glutathione affinity matrix by digestion of the GST fusion with thrombin as described above and carried the extension GSPGP/IGSP/ST at the N terminus. The IIGP1-m protein originated from a cloning artifact; it differs from the wild-type by a 2-residue C-terminal truncation followed by the addition of 11 extra C-terminal residues. IIGP1-hs has 6 histidine residues originally introduced as a C-terminal epitope tag.

**Nucleotide Binding Parameters**

Three independent approaches were taken to measure the nucleotide binding properties of IIGP1. Two of these relied on the use of 2′,3′-O-Methylanthramonol (mant)-modified nucleotides. Many nucleotides were used as fluorescent probes, since they exhibit protein-binding properties similar to those of natural nucleotides. The increase in mant fluorescence upon binding of the nucleotides to proteins has been extensively used for the study of nucleotide binding interactions with GTPases. The mant nucleotides used in our study (mGDP, mGTP, and the nonhydrolyzable analogues mGTP[S] and mGpp(NH)p) were synthesized and subsequently purified by ion exchange chromatography as described in Ref. 22. The purity of the nucleotides was analyzed by reverse-phase chromatography. The concentration of the nucleotides was determined by using molar absorption coefficients as described above. Aluminum fluoride solutions were prepared by the addition of 300 μM aluminum chloride and 10 mM sodium fluoride. The formation of AlF₃ and AlF₄ complexes is a pH-dependent process, therefore determinations were done as AlF₃ (23). All experiments were done with buffer 2 at 20°C unless specified.

**Stopped Flow**—In stopped-flow experiments, at least a 5:1 ratio of protein to nucleotide concentrations were mixed together, providing conditions for pseudo-first-order binding kinetics. The time course of the increase in mant fluorescence upon binding of the protein to the mant nucleotides was recorded (SM-17, Applied Photophysics). Mant fluorescence was excited at 360 nm and monitored through a 405-nm cut-off filter. In each case, a single exponential function could be fitted to the data, yielding the observed rate constant kₜ₁. A linear fit of the plot of kₜ₁ versus protein concentration was obtained, the slope of the curve denoting the association rate constant (kₜₒ). The intercept showing the dissociation rate constant (kₜ₋₁). The kₜₒ values are calculated from the ratio of kₜₒ and kₜ₋₁.

**Equilibrium Measurements**—IIGP1 protein was titrated against mant nucleotides. The mant nucleotides (0.5 μM) were excited at 360 nm, and the fluorescence was monitored at 430 nm (Fluoromax 2; Spex Industries). The increase in fluorescence upon the stepwise addition of the protein was measured, and at each step the values were averaged over 1 min. The equilibrium dissociation constants, Kₜₒ, were obtained by fitting a quadratic function to the data as described in Ref. 24.

**Isothermal Titration Calorimetry**—Isothermal titration calorimetry (Microcal Inc.) was used to measure the binding of unlabeled nucleotides to IIGP1. The heat of binding was detected upon the stepwise addition of nucleotide into a cell containing the protein. The data were fitted using the manufacturer’s software, yielding the enthalpy of the reaction, the dissociation constant (Kₜₒ), and the stoichiometry factor (N) as described in Ref. 25.

**GTP Hydrolysis Assay**

Different concentrations of GTP and protein were mixed and incubated at 37 °C in buffer 2. At different time points, aliquots were removed and subjected to reverse-phase HPLC analysis for GTP and GDP. Samples were run under isocratic conditions on a C₁₈ column (0.4 × 25 cm filled with 5-μm ODS Hypersil) with buffer 3 (10 mM H₂PO₄/H₂PO₄(na) 100 mM KCl, pH 7.4). Nucleotides were detected at 252 nm in a UV absorption detector (Beckman System Gold 166). The concentration-dependent GTPase activity of the three forms of IIGP1 protein (Table I) was measured at a GTP concentration of 700 μM and at a range of protein concentrations.

**Dynamic Light Scattering**

Dynamic light scattering was performed using a DynaPro molecular sizing instrument (Protein Solutions) equipped with a temperature control unit. The sample was filtered through a microsample syringe, where 50 μl of the sample was passed through a filtering device using a 0.22-μm filter into a 12-μl Quartz cuvette. The sample (either protein alone or protein with different nucleotides) in buffer 2 was illuminated by a 25-milliwatt, 750-nm wavelength solid state laser. The time scale and scattered light intensity were recorded for each measurement. All measurement was evaluated by autocorrelation, from which the translational diffusion coefficient (Dₛ) was calculated. The Mₛ was estimated from the hydrodynamic radius Rₛ, which was derived from Dₛ using the Stokes-Einstein equation and the sample temperature (T = 2°C) using the standard
RESULTS

Recombinant IIGP1 Expression and Purification

IIGP1-wt, IIGP1-m, and IIGP1-His (see Table I) were expressed as N-terminal GST fusion proteins in E. coli BL-21 cells. Fig. 1 shows the purification of IIGP1-m. The incubation conditions were adjusted to maximize the yield of soluble proteins by varying the concentration of IPTG, temperature, time of induction, and cell density. A large fraction of the expressed protein was insoluble even under the best conditions, as shown in Fig. 1 (lane 2). However, a significant portion was soluble (lane 1) and was purified as described (see “Experimental Procedures”), and the GST domain of the fusion protein was cleaved by incubation with thrombin on the resin (lane 4). The eluted protein was further purified by size exclusion chromatography (lane 6). The purified protein was analyzed by reverse-phase HPLC and found to be nucleotide-free.

Nucleotide Binding

Equilibrium Titrations—The binding parameters of IIGP1-m for guanine nucleotides were determined at equilibrium in titrations of protein against constant mant nucleotides. Fig. 2a shows the titration curve for the increase in fluorescence upon the binding of mant-GDP (mGDP) to IIGP1-m. IIGP1-m was added from a 600 μM stock solution, and at each step the values for the increase in fluorescence were averaged over a period of 1 min. Dissociation constants were calculated from curves fitted to the data following Ref. 24. The $K_d$ value for nucleotide binding is in the micromolar range, and mGDP shows higher affinity than mGTP/S (Table II). mGpp(NH)p, another nonhydrolyzable analogue of GTP, shows weak binding in comparison with the other nucleotides ($K_d$ of ~130 μM). Interestingly, we could not detect any increase in fluorescence upon the addition of aluminum fluoride (AlF₃) to IIGP1-mGDP solution (data not shown), and in confirmation, no decrease in the dissociation rate of mGDP could be detected by stopped flow in the presence of AlF₃. Thus, monomeric IIGP1 in free solution in the presence of GDP appears not to be able to stabilize the transition state of GTP hydrolysis through binding of AlF₃.

The interactions of unlabeled GDP and GTP/S with IIGP1-m were additionally measured by isothermal titration calorimetry in order to investigate possible interference of the mant group in the fluorescence-based binding assay. Fig. 2b (upper lane) shows the raw data for binding upon the stepwise addition of GDP to IIGP1-m. The exothermic process is reflected in a negative power pulse from which the heat of the reaction is calculated by integration. The enthalpy of the reaction normalized to the concentration of injected GDP was plotted against the molar ratio of GDP and IIGP1-m as shown in the lower panel. The parameters defining the theoretical curve yielded a $K_d$ for this interaction of 2.5 μM and an enthalpy of association of ~15 kcal/mol. In addition, 1:1 stoichiometry is evident from the data. The results from Table II suggest that the mant modification does not significantly influence the binding affinities of GDP and GTP/S for IIGP1.

Dynamics of Nucleotide Binding—The dynamics of mant nucleotide binding to IIGP1 were determined by stopped flow. mGTP/S was used to control for potential complications in the measurement of GTP binding caused by hydrolysis. No significant difference was seen (Tables II and III), no doubt due to the slow turnover of GTP by IIGP1 (see below). The IIGP1 protein was used in a large molar excess over the nucleotide in order to obtain pseudo-first-order reaction kinetics (shown for IIGP1-m in Fig. 3). A single exponential function was fitted to the data, where the resulting time constant corresponds to the inverse observed rate constant ($k_{obs}$). The observed rate constants for the binding of mGDP were plotted against IIGP1-m concentration. The slope of the straight line denotes the association rate constant ($k_{on}$), and the intercept shows the dissociation rate constant ($k_{off}$). $k_{off}$ was also measured in a displacement experiment by the addition of excess unlabeled nucleotide to IIGP1-mGDP preformed complex. This leads to a quasi-irreversible dissociation of mGDP and therefore to a single exponential decrease of fluorescence as shown in the lower inset in Fig. 3.

The $K_d$ values of different nucleotides to IIGP1-m using equilibrium methods are compared with $K_d$ from stopped-flow experiments in Table II. The three different methods gave comparable results. The nucleotide binding affinities determined by stopped flow for the native IIGP1 protein and for the two forms varying at the C terminus (Table I) are shown in Table III. It is apparent that the $K_d$ values and the rate constants are similar for all three proteins, suggesting that the C terminus is not involved in nucleotide interaction with IIGP1 (see below). The association rate constants are similar for the different nucleotides, but the dissociation rate constants vary significantly. The smaller $k_{off}$ for mGDP accounts for the higher affinity compared with mGTP and mGTP/S, as observed in the equilibrium titrations described above (Table II). To summarize the results from three different methods and from the three C-terminal versions of IIGP1, the dissociation constant for GDP is ~1 μM, whereas for GTP it is ~15 μM.

**IIGP1 as a GTPase**

In all GTPases, the identity of the nucleotide bound to the protein and the rate of conversion of GTP to GDP by hydrolysis are crucial factors determining their functional regulation (26). We therefore document a detailed analysis of the kinetics of GTP hydrolysis by IIGP1. The rate of hydrolysis and product formation was analyzed by reverse-phase HPLC. The activity of the three forms of IIGP1 (IIGP1-wt, IIGP1-m, and IIGP1-his)
at 2–100 μM was analyzed over GTP concentrations from 0.05 to 2 mM. ATP was not hydrolyzed, confirming nucleotide specificity and indicating that our preparations were free of phosphatases (data not shown).

IIGP1 hydrolyzes GTP to GDP and not to GMP. A representative curve for the time course of GTP hydrolysis by IIGP1-wt is shown in Fig. 4a. The complete hydrolysis of 700 μM GTP to GDP with 50 μM IIGP1 at 37 °C is documented. The rate constant for GTP hydrolysis was obtained from the initial rates, since later values are affected by product inhibition.

Magnesium ions were found to be essential for the GTPase reaction; severely impaired GTPase activity was observed in the presence of 10 mM EDTA (Fig. 4b). Surprisingly, in view of the results reported above, where GDP binding to IIGP1 could not be directly stabilized by AlF₃, the hydrolysis of GTP by IIGP1 was greatly inhibited by the addition of AlF₃, suggesting that there are conditions under which AlF₃ can stabilize the GDP-bound state and inhibit nucleotide exchange (Fig. 4b).

The catalytic activity of IIGP1 at different protein and nucleotide concentrations was determined, as shown in Fig. 4c. The Michaelis-Menten equation was applied to a plot of specific activity (at a range of protein concentration) versus substrate concentration. Intriguingly, a small increase in $K_m$ was observed with increased protein concentration, suggesting functional cooperativity between IIGP1 molecules.

In order to investigate the cooperativity effect more precisely, the dependence of the specific GTPase activity on IIGP1 concentration was measured at a fixed GTP concentration of 700 μM as shown in Fig. 4d (closed circles, IIGP1-m; open circles, IIGP1-wt). The maximum specific rate of GTP hydrolysis by IIGP1 was close to 2 min⁻¹ (see Fig. 4c, where 50 μM IIGP1-m and 2 mM GTP was used). The increase in activity of

**Fig. 3.** Stopped flow. The increase in fluorescence upon mixing 0.2 μM mGDP with 4 μM IIGP1 is monitored through a 405-nm cut-off filter after excitation at 360 nm. A single exponential curve is fitted to the data (top inset) to get the observed rate constant $k_{\text{obs}}$, and a linear fit to the plot of the observed rate constant versus protein concentration is shown, deriving the $k_{\text{on}}$ and $k_{\text{off}}$. The exponential decrease of fluorescence upon the addition of excess GDP (400 μM) to the IIGP1 (4.8 μM)-mGDP (3.9 μM) preformed complex is shown in the bottom inset, yielding the $k_{\text{off}}$.

**TABLE III**

| Nucleotides | $k_{\text{on}}$ | $k_{\text{off}}$ (displacement) | $K_d$ (μM) |
|-------------|----------------|-------------------------------|-------------|
| mGDP        | 1.30           | 14.28                         | 4.20        |
| IIGP1-wt    | 1.55           | 0.99                          | 0.64        |
| IIGP1-m     | 1.65           | 1.6                           | 0.97        |
| IIGP1-his   | 1.31           | 0.70                          | 0.53        |
| mGTP        | 0.98           | 21.6                          | 22          |
| IIGP1-wt    | 1.30           | 21                            | 16.1        |
| IIGP1-m     | 14.28          | 12.9                          | 15.7        |
| mGTP₅S      | 4.20           | 66                            |             |
| IIGP1-m     |                |                               |             |
the protein with an increase in protein concentration observed in the case of IIGP1-wt and IIGP1-m demonstrates a cooperative mechanism of GTP hydrolysis. Intriguingly, the GTP hydrolysis rate of IIGP1-his C-terminally modified protein showed marginal if any protein concentration dependence over the tested range, remaining at or close to the basal rate of 0.1 min⁻¹ (Fig. 4d, closed triangles).

GTP-dependent Formation of IIGP1 Oligomers

Other GTPases that show cooperative activity have also been shown to form enzymatically active oligomers in the presence of nucleotides (reviewed in Ref. 2). The oligomerization of IIGP1-m was investigated using dynamic light scattering at 4 °C at protein concentrations of 50–100 μM and nucleotide (GDP, GTP, and Gpp(NH)p) concentrations of 1 mM. The change in the Rh of IIGP1 (derived from the unimodal fit analysis by Dynamics 4.0 software) versus time was plotted for each of the data sets with and without nucleotides. Fig. 5a shows the time-dependent oligomerization of IIGP1-wt with GTP and Gpp(NH)p. A striking increase in hydrodynamic radius was detectable after the addition of GTP and also, but to a lesser degree, after the addition of Gpp(NH)p. No detectable higher molecular forms were found with the apoprotein (nucleotide-free) or in the presence of GDP, since the Rh is 3.1 nm, which corresponds to the expected molecular mass of 47 kDa. The difference in oligomer formation between GTP- and Gpp(NH)p-bound forms could be due to their difference in nucleotide binding affinities.

As shown in Fig. 4d, IIGP1 modified by the addition of a C-terminal His₆ tag showed essentially no cooperative GTPase activity. It was therefore of interest to know whether the C-terminal modification also blocked the formation of nucleotide triphosphate binding oligomers. Using the dynamic light scattering assay, IIGP1-his showed no oligomerization in the presence of GTP or Gpp(NH)p (Fig. 5a) where the curve for IIGP1-his in the presence of GTP overlays with nucleotide-free IIGP1-wt, IIGP1-wt GDP. The hydrodynamic radius of IIGP1-his in the presence of GTP persists at around 3 nm, thus a monomer.

Since oligomerization was possible only in the presence of GTP or Gpp(NH)p, and not in the presence of GDP, the reversibility of these oligomers was tested with respect to GTP hydrolysis. In order to show directly the relationship between IIGP1-catalyzed GTP hydrolysis and oligomer formation, we used light scattering in a fluorescence cuvette from which aliquots for nucleotide analysis were taken simultaneously. The time course of the oligomerization in a solution of GTP (1 mM) and IIGP1-wt (50 μM) is shown in Fig. 5b. The initial rise in light scattering was followed by a slow decrease associated with progressive hydrolysis of GTP. This time-dependent oligomerization was analyzed by HPLC as described under “Experimental Procedures.” IIGP1 hydrolyzes GTP to GDP. The percentage breakdown of 700 μM GTP (open circles) with 50 μM IIGP1 to GDP (closed circles) with time is shown. b, effect of Mg²⁺ and AlF₃ on the GTPase activity of IIGP1. The time course of GTP hydrolysis with 25 μM IIGP1 and 700 μM GTP was also analyzed as shown by the closed circles. c, Michaelis-Menten plot. The specific activity of IIGP1 at a range of protein concentrations (2–50 μM) was plotted against the substrate concentration. The Michaelis-Menten equation was fit to the data to derive the parameters. d, cooperativity. IIGP1 shows a cooperative GTPase activity, with a 7-fold increase in the specific activity of the protein at a concentration of 700 μM GTP in the case of IIGP1-wt (open circles) and IIGP1-m (closed circles) shown. The IIGP1-his protein (closed triangles) fails to show cooperativity.

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**Fig. 4. Kinetics of GTP hydrolysis.** a, time course of GTP hydrolysis by IIGP1. The rate of GTP hydrolysis and GDP formation was analyzed by HPLC as described under “Experimental Procedures.” IIGP1 hydrolyzes GTP to GDP. The percentage breakdown of 700 μM GTP (open circles) with 50 μM IIGP1 to GDP (closed circles) with time is shown. b, effect of Mg²⁺ and AlF₃ on the GTPase activity of IIGP1. The time course of GTP hydrolysis with 25 μM IIGP1 and 700 μM GTP was also analyzed as shown by the closed circles. c, Michaelis-Menten plot. The specific activity of IIGP1 at a range of protein concentrations (2–50 μM) was plotted against the substrate concentration. The Michaelis-Menten equation was fit to the data to derive the parameters. d, cooperativity. IIGP1 shows a cooperative GTPase activity, with a 7-fold increase in the specific activity of the protein at a concentration of 700 μM GTP in the case of IIGP1-wt (open circles) and IIGP1-m (closed circles) shown. The IIGP1-his protein (closed triangles) fails to show cooperativity.
GTP-dependent Oligomerization and Activity of IIGP1

Comparative nucleotide binding affinities in GTPases (28–35)

| Similar means less than 5-fold difference, and higher means more than 10-fold difference. |
|-----------------------------------|-----------------|-----------------|
|                                  | Very high affinity | High affinity  |
|                                  | Low affinity     |                 |
| Ras, Ran                         | EF-Tu, Go        | hGBP1, Mx, Dynamins, IIGP1 |
| Similar GTP/GDP affinity         | Higher GTP affinity | Higher GDP affinity |

| Ras, Ran | GP/GDP affinity | Dynamins | EF-Tu, IIGP1 |
|----------|-----------------|----------|--------------|
|          |                 |          |              |
|          |                 |          |              |
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**DISCUSSION**

GTPases regulate diverse cellular processes in invertebrates and vertebrates modulated by their ability to bind guanine nucleotides and hydrolyze GTP (27). Nucleotide binding affinities are described for a variety of GTPases, a few of which are represented and grouped in Table IV (28–35). IIGP1 has a micromolar \( K_d \) value and is therefore grouped with the large GTPases but, like EF-Tu, has a higher affinity (15-fold) for GDP than for GTP.

Due to slow dissociation rates, Ras and EF-Tu bind GDP very tightly (picomolar and nanomolar \( K_d \) values), and GDP release is accelerated by the exchange factors Sos and EF-Ts, respectively (30, 36, 37). For IIGP1, the nucleotide dissociation rate constants are large, 21 s\(^{-1}\) for GTP and 3.6 s\(^{-1}\) for GDP. This indicates very short lifetimes for the complexes as compared with Ras-like GTPases, Go or EF-Tu. Given the cellular concentrations of GTP and GDP (38), the molar ratio of the GDP and GTP states of IIGP1 should be roughly 3:1 in favor of GDP, but switching states might well not require a putative guanine nucleotide exchange factor because of the high dissociation rates. In contrast to these expectations derived from consideration of IIGP1, a direct investigation of bound nucleotide from the ex vivo purified p47 GTPase, IGTP, for which unfortunately no quantitative nucleotide binding parameters are available, showed only detectable bound GTP (39). It may be, however, that the ratio of GTP-bound and GDP-bound forms is altered in vivo by interaction partners that change the relative affinities for nucleotides. Until now, no interaction partners have been described for any of the p47 GTPases.

GTPase activity has been shown to be crucial for the regulation of almost all GTPases. With respect to the switch GTPases, the time from the binding of GTP to its hydrolysis is critical for downstream cellular functions and is therefore targeted for precise modulation. For example, Ras has a very low intrinsic hydrolytic rate (0.03 min\(^{-1}\)). Here, functional efficiency is achieved by accelerating the turnover rate by a factor...
GTPase activity may rather be similar to Ras-like GTPases or from the large self-associating GTPases. The presence of nucleotide, a property that distinguishes Rac1, however, the formation of oligomers is independent of this case, the accelerated GTPase activity upon oligomerization has also been described for the wild-type despite the C-terminal extension.

form of IIGP1 studied here, IIGP1-m, appears to be essentially acceleration of GTPase activity. The other C-terminal modified form of IIGP1 are likely to be essential for regulating its function in vivo, although there is no direct evidence for IIGP1 forming stable oligomers under in vivo conditions. Increased GTPase activity upon oligomerization has also been described for the small GTPase Rac1, and a functional significance for the activation of the dimeric effector Pak has been documented (43). In this case, however, the formation of oligomers is independent of the presence of nucleotide, a property that distinguishes Rac1 from the large self-associating GTPases.

The IIGP1-his protein modified at the C terminus shows neither GTP-dependent oligomerization nor concentration-dependent GTPase activity, thus laying emphasis on the significance of the C terminus in the formation of oligomers. We do not see any influence of the histidine tag in IIGP1-his on nucleotide binding, suggesting that the tag interferes with oligomerization rather than shielding or delaying the GTP binding/hydrolysis. The properties of IIGP1-his strengthen the notion that the native environment in the interferon-g-induced cell will help us further in analyzing the significance of oligomerization and regulated GTPase activity as well as membrane association for the function of the protein in vivo.

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J. Biol. Chem. 2003, 278:29336-29343.
doi: 10.1074/jbc.M211973200 originally published online May 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211973200

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