GTPase properties of the interferon-induced human guanylate-binding protein 2

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Abstract Guanylate-binding proteins (GBPs) were originally described as proteins that are strongly induced by interferons and are capable of binding to agarose-immobilized guanine nucleotides. hGBP1, the first of two members of this protein family in humans, was recently shown to represent a novel type of GTPase that hydrolyzes GTP predominantly to GMP. We now report that purified recombinant hGBP2 also hydrolyzes GTP very efficiently, although GDP rather than GMP was the major reaction product. The biochemical parameters of this reaction were as follows: $K_m = 313$ μM, turnover number = 22 min^{-1}. Both hGBP1 and hGBP2 failed to hydrolyze GDP, however, GDP was an effective inhibitor of the hGBP2- but not the hGBP1-catalyzed GTP hydrolysis reaction. Thus, hGBP1 and hGBP2 have similar biochemical properties, but show pronounced differences in product specificity.

Key words: Interferon; Human; GTPase; GTP hydrolysis; Guanylate binding

1. Introduction

The interferon-induced guanylate-binding proteins (GBPs) were originally discovered as a result of their ability to bind to agarose-immobilized guanine nucleotides [1,2]. A characteristic feature of GBPs is that they lack a tripartite GTP-binding consensus motif: the classical third element of this motif (N/TXPG) [3] is missing in human [4], mouse [4], rat [5] and chicken [6] GBPs. The physiological role of the GBPs in the interferon response is unknown: mouse strains which fail to express GBP1, but which express other GBP family members, are healthy and do not show enhanced susceptibility to viral and other pathogens [7–9].

Two co-expressed GBPs were identified in humans, designated hGBP1 and hGBP2 [4], both of which have molecular masses of about 67 000 Da. Due to a C-terminal CaaX motif, recombinant hGBP1 can be isoprenylated in vitro [10], suggesting that it is a membrane-associated protein. We have recently shown that hGBP1 is a novel type of GTPase that converts GTP mainly to GMP, presumably via two consecutive cleavages of a single phosphate group [10]. The K_m for this reaction is high, and purified hGBP1 can perform multiple cycles of GTP hydroloysis in the absence of accessory factors [10]. Here, we report that hGBP2 is a GTPase that resembles hGBP1 in many respects. However, GDP is the major reaction product of the hGBP2-catalysed hydrolysis reaction.

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2. Materials and methods

2.1. Determination of the 5′-proximal sequence of the hGBP-2 mRNA

The nucleotide sequence near the 5′ end of the hGBP2 mRNA was determined by the 5′ RACE procedure according to the manufacturer's protocol (Gibco-BRL). For cDNA synthesis we used the primer 5′TTTGCCTTGATTCGATC3′ which corresponds to positions 449–433 of the previously isolated hGBP-2 cDNA clone [4] and poly-$(A)^+$ RNA from HaCat cells treated with 1000 units per ml each of interferon-γ and interferon-α2a. After C-tailing with terminal deoxynucleotide transferase, the cDNA was amplified by PCR using the RACE anchor primer and the primer 5′GTTGGTCCATGGCCTGCTGGTTGATGGTTCCC3′ which corresponds to positions 410–379 of hGBP2 cDNA [4]. The PCR products were cloned into the TA cloning vector pCR (InVitrogen, San Diego) and the inserts of individual clones were sequenced.

2.2. Expression constructs

The plasmid pHis-hGbp1, which permits high-level expression of histidine-tagged hGBP1 in *E. coli*, has previously been described [10]. pHis-hGbp2, which codes for a histidine-tagged variant of hGBP2, was constructed by replacing the coding sequence of hGBP1 for that of hGBP2. The appropriate hGBP2 cDNA fragment was generated by PCR from a cDNA library derived from interferon-induced human fibroblasts [11] using the primer 5′ACTTAGGATCCGCTCCAGAGATCAACTTG3′ which carries a *Bam*HI restriction site and which corresponds to positions 160–177 of the RACE hGBP2 cDNA clone (Fig. 1A) and the primer 5′ATTGAACGTCGACGGCAAATTTTGCTCCTTGGACT3′ which carries a *Sal*I restriction site and which corresponds to positions 1784–1763 of the previously characterized hGBP2 cDNA [4]. The PCR product was digested with *Bam*HI and *Sal*I and cloned into the corresponding sites of pQE9. The resulting plasmid codes for a protein that consists of the N-terminal polypeptide Met-Arg-Glu-Ser-$(His)_6$-Gly-Ser fused to the alanine at position 2 of the full-length hGBP2 sequence (Fig. 1B).

2.3. Purification of recombinant GBPs from E. coli

Histidine-tagged hGBP2 protein was enriched from the bacterial lysate by Ni-chelate affinity chromatography [10]. This material was then applied to a MonoQ FPLC chromatography column (Pharmacia, Freiburg, Germany) equilibrated with buffer A (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM DTT, 0.1% NP40), and bound proteins were eluted with a linear gradient of 100–1000 mM NaCl. hGBP2 eluted at about 300 mM NaCl. A second round of MonoQ chromatography was performed with the peak fraction of the first column. The purity of hGBP2 was more than 95%. Histidine-tagged hGBP1 was purified as described [10].

2.4. HPLC analysis

Analysis of nucleotides by HPLC was performed as described [12] with slight modifications. Briefly, a C-18 reversed-phase column (0.4×25 cm filled with ODS Hypersil, 5 μm, Bischoff, Leonberg, Germany) was run at ambient temperature at a flow rate of 1 ml/min in 50 mM Na-phosphate (pH 6.5) containing 0.2 mM *t*-butylammonium bromide, 3% (v/v) acetonitrile, and 0.2 mM NaN_3. In this system, GMP eluted at 4.8 min, GDP at 7.5 min and GTP at 10.6 min. The absorption was measured at 252 nm with a VWM-2141 UV detector (Pharmacia) and the signals were quantified with a C-R5A integrator (Shimadzu, Kyoto, Japan).

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2.5. Nucleotides

Radiolabeled [α-^{32}P]GTP (3000 Ci/mmol) was purchased from Amersham. The non-hydrolyzable nucleotide analogs guanosine 5′-[β,γ-thio]triphosphate (GTPγS), guanosine 5′-[β,γ-imido]triphosphate (GMP-PNP) and adenosine 5′-[β,γ-imido]triphosphate (AMP-PNP) were obtained from Boehringer Mannheim. GTP, GDP, GMP, ATP, UTP and CTP were purchased from Pharmacia.

2.6. Assay for GTPase activity

GTPase assays were performed with 0.02 mg/ml of either purified His-hGBP2 or purified His-hGBP1 [10] in buffer B (13 nM [α-^{32}P]GTP (3000 Ci/mmol), 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 10% glycerol, 0.1 mM DTT and 100 nM adenosine 5′-[β,γ-imido]triphosphate) at 37°C as described [10]. The concentration of unlabeled GTP was 100 μM, except where the indicated concentrations were used. At various times, the reaction was stopped by adding an equal volume of a stop solution containing 2 mM EDTA and 0.5% SDS. Samples were spotted onto polyethyleneimine-cellulose (PEI) thin-layer chromatography plates (MN300, Macherey und Nagel, Düren, Germany) and resolved in buffer C (1 M acetic acid, 1 M LiCl). The plates were exposed to X-ray film. The signals were quantified with a digital autoradiograph LB286 (Berthold, Wildbad, Germany). To calculate specific GTPase activities, time points within the linear range of the hydrolysis reaction were used. To determine the K_m and turnover number, GTPase reactions were performed under optimized conditions. The GTP concentrations varied from 0.1 to 2.0 mM.

3. Results

3.1. hGBP2 mRNA sequence

Since the previously cloned hGBP2 cDNA was an incomplete copy of the corresponding mRNA [4], we set out to isolate the missing part by the 5′ RACE technique using poly(A)$^+$ RNA of IFN-γ-treated HaCat cells. Five RACE cDNA clones were sequenced. The sequence of one of these clones is shown in Fig. 1A (GeneBank accession no. M 55543). A second clone had an identical sequence, except that it carried some additional nucleotides at the 5′ end.

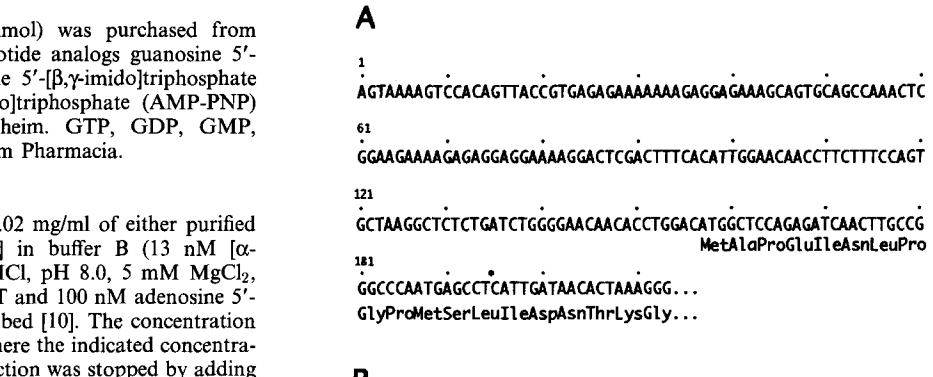

Fig. 1. Structure of hGBP2 at the N-terminus. (A) Nucleotide sequence of the first 213 residues of a 5′ RACE cDNA clone and deduced N-terminal amino acid sequence of hGBP2. The C-residue at position 195 which was not found in the previously characterized incomplete hGBP2 cDNA clone [4] is marked by an asterisk. (B) Sequence comparison of the N-terminal 19 amino acids of hGBP1 and hGBP2, respectively.

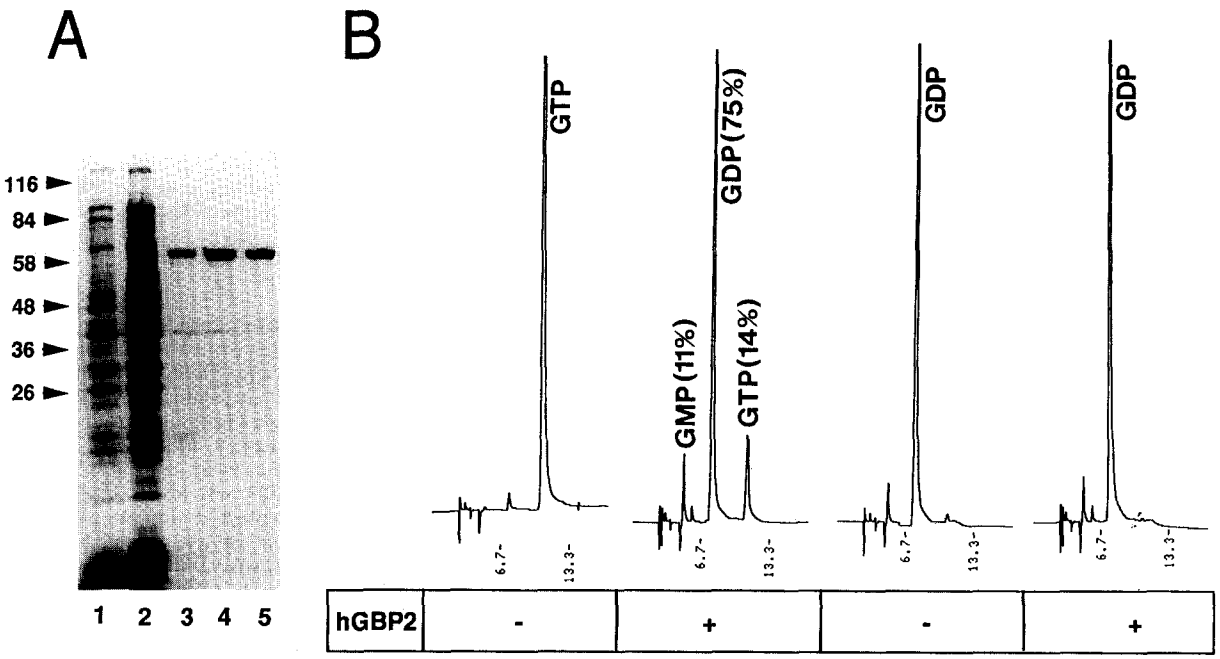

Fig. 2. Purification and GTPase activity of His-hGBP2. (A) Purification of histidine-tagged hGBP2 from *E. coli*. Samples were analysed on 10% SDS-PAGE before (lane 1) and after (lane 2) induction of the culture with IPTG. His-hGBP2 was affinity-purified from the soluble cell fraction by Ni-agarose chromatography (lane 3), and subsequently loaded onto a MonoQ column. Bound material was eluted at 300 mM NaCl (lane 4). Fractions with the highest purity were reapplied to MonoQ chromatography and eluted with 300 mM NaCl (lane 5). (B) GTPase activity of His-hGBP2. GTPase assays were carried with either 100 μM GTP or 100 μM GDP in the presence or absence of MonoQ-purified His-hGBP2 (0.02 μg/μl) for 30 min at 37°C. The reaction products were analysed by HPLC. The chromatogram was monitored at 252 nm. The identity of the nucleotide peaks and their retention times are indicated. The peaks were integrated, and the relative concentrations of the various nucleotides (in percent) are given.

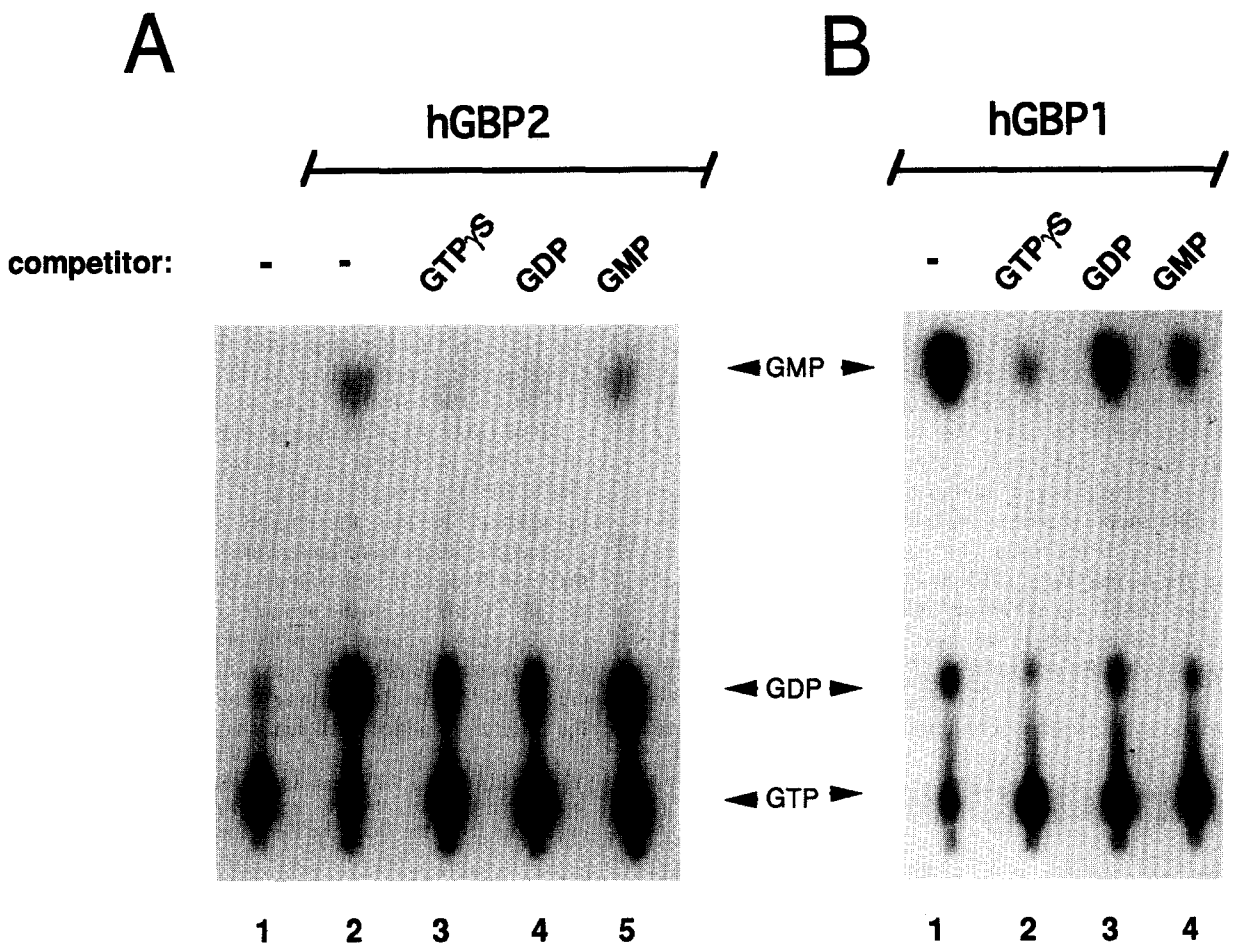

Fig. 3. Inhibition of hGBP2 and hGBP1 by various nucleotides. GTPase assays performed in the presence of (A) MonoQ-purifed His-hGBP2 (0.02 μg/μl) and (B) MonoQ-purified hGBP1 (0.02 μg/μl) and a 15-fold molar excess of the indicated nucleotides over radiolabeled GTP. Samples were analyzed by PEI thin-layer chromatography and autoradiography. The positions of GTP, GDP and GMP are indicated.

The other three clones had 5′ truncations, but were otherwise identical. All RACE clones differed from the published hGBP2 sequence [4] by an extra C residue at position 195 (Fig. 1A), indicating that the previously deduced sequence of the N-terminus of hGBP2 was incorrect. The first initiation codon of the new sequence at position 157 (Fig. 1A) marks the beginning of the hGBP2 coding region. It is located in a favorable context for translation initiation in eukaryotes [13] and is preceded by an in-frame stop codon at position 22. Comparison of the predicted N-termini of hGBP2 and hGBP1 revealed a high degree of sequence identity (Fig. 1B). Thus, hGBP2 is composed of 591 amino acids and has a calculated molecular mass of 67 189 Da.

3.2. hGBP2 is an enzyme that converts GTP to GDP and GMP

For biochemical studies we cloned the complete coding region of hGBP2 into the *E. coli* expression vector pQE9. The resulting construct codes for N-terminally histidine-tagged hGBP2, designated His-hGBP2. Histidine tagging allows for rapid and efficient purification of recombinant proteins from bacterial lysates [10]. His-hGBP2 was strongly induced in *E. coli* after induction with IPTG (Fig. 2A, lanes 1,2) and could be isolated from the soluble fraction of bacterial lysates by Ni-agarose chromatography (Fig. 2A, lane 3). Partially purified His-hGBP2 readily bound to MonoQ and eluted at about 300 mM NaCl (Fig. 2A, lane 4). After a second round of MonoQ chromatography, His-hGBP2 had a purity of about 95% as revealed by Coomassie blue staining of the gel (Fig. 2A, lane 5).

The enzymatic activity of His-hGBP2 was assayed by incubation of purified protein with 100 μM GTP at 37°C and analysis of the reaction products by HPLC. GTP was rapidly hydrolysed to GDP and GMP (Fig. 2B). Quantitation of the signals revealed that 75% of the substrate was converted to GDP and 11% was converted to GMP. No hydrolysis of ATP, CTP or UTP was observed under these conditions (data not shown), indicating that the observed activity of His-hGBP2 was specific for GTP. This latter result further demonstrated that our hGBP2 preparation was essentially free of contaminating phosphatases. His-hGBP2 produced by expressing a second independent hGBP2 cDNA clone exhibited identical biochemical properties (data not shown). The K_m of the His-hGBP2-catalysed hydrolysis of GTP was 313 μM. The specific GTPase activity of our best preparation of His-hGBP2 was 325 nmol/min per mg, corresponding to a turnover number of 22 min^{-1}.

Since GMP was a reaction product, we wished to determine whether GDP could serve as a substrate for His-hGBP2. To

test this, hGBP2 was incubated with 100 μM GDP at 37°C and the products were analysed by HPLC. His-hGBP2 failed to hydrolyse GDP under these conditions (Fig. 2B).

3.3. *The apparent affinities of hGBP2 for GDP and GMP differ from those of hGBP1*

His-hGBP2 and the previously characterized His-hGBP1 [10] are both GTPases that convert GTP to GMP (Fig. 3). Since the product ratios differed markedly, we investigated whether the two enzymes have different affinities for GDP and GMP. We therefore performed competition experiments with the GTP analog GTPγS. When a 15-fold molar excess of GTPγS was used, hydrolysis of GTP by either His-hGBP2 or His-hGBP1 was strongly inhibited (Fig. 3). GTPγS was not hydrolysed to GMP by either His-hGBP1 [10] or His-hGBP2 (data not shown). The non-hydrolysable GTP analog GMP-PNP was a similarly potent inhibitor of His-hGBP2 (data not shown). Differences between hGBP1 and hGBP2 were observed when GDP was used as a competitor: GDP strongly inhibited the hydrolysis of GTP by His-hGBP2 (Fig. 3A, lane 4), but had almost no inhibitory effect on His-hGBP1 (Fig. 3B, lane 3). When GMP was used as a competitor, GTP hydrolysis by hGBP1 was inhibited (Fig. 3B, lane 4), whereas that of His-hGBP2 was not (Fig. 3A, lane 5). Thus, for both enzymes, the most prominent reaction products also proved to be the most efficient competitors.

4. Discussion

We have recently shown that hGBP1 is an unusual GTPase that hydrolyzes GTP predominantly to GMP [10]. We now demonstrate that hGBP2 has similar GTPase activity but that in contrast to hGBP1, the major reaction product of the hGBP2-catalysed hydrolysis of GTP is GDP rather than GMP.

The previously cloned cDNA for hGBP2 is an incomplete copy of the corresponding mRNA. Using the 5′ RACE procedure, we obtained the sequence information necessary for reconstruction of the hGBP2 open reading frame. Our analysis showed that the formerly characterized GBP2 cDNA clone carried a mutation and that the predicted N-terminal sequence of hGBP2 was incorrect. A cDNA encoding full-length hGBP2 was expressed in *E. coli* as a N-terminally histidine-tagged protein to facilitate its purification. Highly purified His-hGBP2 hydrolysed GTP mainly to GDP (85–90%) and to a lesser extent to GMP (10–15%). Formation of GMP was indeed due to an intrinsic activity of hGBP2, since this activity co-purified with His-hGBP2 and since unrelated proteins purified under identical conditions lacked such activity ([12] and unpublished data). hGBP2 thus resembles the previously characterized hGBP1 [10] by being an unconventional GTPase that converts GTP to GDP and GMP. However, a remarkable biochemical difference between hGBP1 and hGBP2 is that the former enzyme hydrolyzes GTP predominantly to GMP, whereas the latter enzyme hydrolyzes this substrate predominantly to GDP. Accordingly, the two enzymes also differ in their sensitivities to inhibition by the reaction products: GDP potently inhibited hGBP2 but not hGBP1, while GMP strongly inhibited hGBP1 but not hGBP2. Taken together, the biochemical properties of hGBP2 strikingly resemble those of the recently cloned GBP homolog of the chicken [6].

Our experiments with the second member of the human GBP family confirm previous results with hGBP1, which showed that these GTPases have unusual biochemical properties [10], and further emphasize the unique position of the GBPs in the superfamily of GTPases. Unlike conventional GTPases, GBPs strongly bind to agarose-immobilized GTP, GDP or GMP [4]. Nonetheless, they bind guanine nucleotides only poorly in solution, as indicated by the fact that all purified GBPs have empty nucleotide binding pockets ([10] and unpublished results). Presumably as a consequence of these properties, GBPs have characteristically high K_m values for the GTP hydrolysis reaction and can perform multiple cycles of GTP hydrolysis in the absence of nucleotide exchange factors. The most unusual property of GBPs is that they are able to synthesize GMP. The physiological significance of increased GMP levels resulting from the action of GBPs in the interferon-induced cell remains obscure.

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