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Supporting Material

: Dimerization And Its Role In GMP Formation By Human Guanylate Binding Proteins

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Materials and methods

Cloning and constructs: The cloning of the wild type, mutant and truncated proteins containing the globular domain of hGBP-1 has been described earlier (17). The truncated proteins containing the helical domains were prepared using appropriate forward and reverse primers by standard PCR techniques with the full-length hGBP-1 as a template. The amplified genes were cloned into pET22b(+) by using NdeI/NotI restriction sites. The constructs were confirmed by DNA sequencing. These were used for bacterial expression and purification as a C-terminal 6xHis tag proteins.

Full-length hGBP-2 gene was PCR amplified from IFN-γ treated HeLa cells using specific primers and standard RT PCR techniques. The amplified gene was cloned into pET28b (+) by using BamHI and XhoI restriction sites. The construct was confirmed by DNA sequencing. The construct generated was used for bacterial expression and purification as an N-terminal 6x His tag protein.

Mutagenesis: Mutagenesis was done using specific primers and Quick change site directed mutagenesis kit from Stratagene. Mutations were verified by sequencing.

Expression and purification: The wild type, mutant and truncated proteins of hGBP-1 were over-expressed and purified as described earlier (17). For the truncated proteins containing helical domains, the protein was expressed at 37°C. But for hGBP-2, the expression temperature was shifted to 25°C. These proteins were similarly purified as described earlier (17). The concentration of the proteins was determined using a Bio-Rad protein assay dye.

Assays for GTPase activity: GTPase assays were carried out with a buffer of 50mM Tris, pH 8.0, 100mM KCl, 5mM $MgCl_2$, 0.1mM DTT and 6.6 nM radiolabeled [α-^{32}P] GTP (3000 Ci/mmol, Perkin Elmer). The concentration of unlabeled GTP (Sigma, USA) was used as indicated in the text. The reaction was stopped by adding 250 mM EDTA (final concentration). The assay was carried out at 37°C unless specified. The nucleotides were separated and quantified using autoradiography as described earlier (17).

The specific activity was measured for various concentrations of proteins with GTPase assay buffer containing trace amount of radiolabeled [α-^{32}P] GTP and 50 μM unlabelled GTP at 37°C. Aliquots were taken at different time intervals and the progress of GTP hydrolysis was analyzed as described earlier (17). The specific activity was determined by dividing the initial rate derived from a linear fit with the protein concentration.

Gel filtration chromatography: The analytical gel filtration chromatography was performed using a Perkin Elmer HPLC system, USA. A Biosep-SEC-S 4000 column from phenomenex was used. Mobile phase contained 50mM Tris at pH 8.0, 100mM KCl and 5mM $MgCl_2$. Additionally, 200 μM GppNHp analogue was used in the mobile phase wherever the experiment was carried out in the presence of analogue. A flow rate of 1 ml/min was used. A standard curve was generated from the elution volumes of proteins with known molecular weight such as amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa) and the molecular weight of the protein was determined based on the retention volume plotted as V_e/V_o (elution volume/void volume).

Protein immobilizations: To prevent dimerization, the proteins were immobilized in two different ways as described below.

i) Using microtiter (ELISA) plates. Proteins were incubated for overnight at 4°C in coating buffer (CO_3^{2-}/HCO_3^-, pH 9.6) on ELISA plates. 20 µg of proteins were used in a volume of 50µl/well. After overnight incubation, the coating buffer was removed followed by washing three times with phosphate buffered saline containing 0.05% v/v tween-20. Appropriate negative and positive controls were used. The binding of proteins was detected using sandwich ELISA method with anti His-tag antibody and a secondary antibody incorporating proper blocking with bovine serum albumin.

ii) Using Cyanogen bromide activated sepharose beads: CNBr activated sepharose was obtained from GE healthcare and was used according to manufacturer's protocol. Briefly, 0.1M $NaHCO_3$ at pH of 8.3 was used for binding. The protein was used as 5mg per ml of bead medium. The beads were stored in 50mM Tris pH 8.0, 100mM KCl and 5mM $MgCl_2$ at 4°C.

Fluorescence experiments:

i) ANS binding assay: 1 µM of protein was taken in a buffer containing 50 mM Tris, 5 mM $MgCl_2$, and 100 mM KCl, pH 7.5. The experiment was carried out with and without substrate analogue GppNHp. The concentration of the analogue was used 200µM and the mixture was kept for 2hrs. The reaction mixture was then titrated with ANS which was prepared in 20mM Tris-HCl, pH 7.5. The data were collected at room temperature (25ºC). All fluorescence measurements were carried out on a FluoroMax 4 (Horiba Jobin Yvon) spectrofluorimeter. An excitation wavelength of 350 nm was used. The emission spectra were recorded from 400 to 630 nm. The monochromator slit width was kept at 5 nm for both excitation and emission measurements.

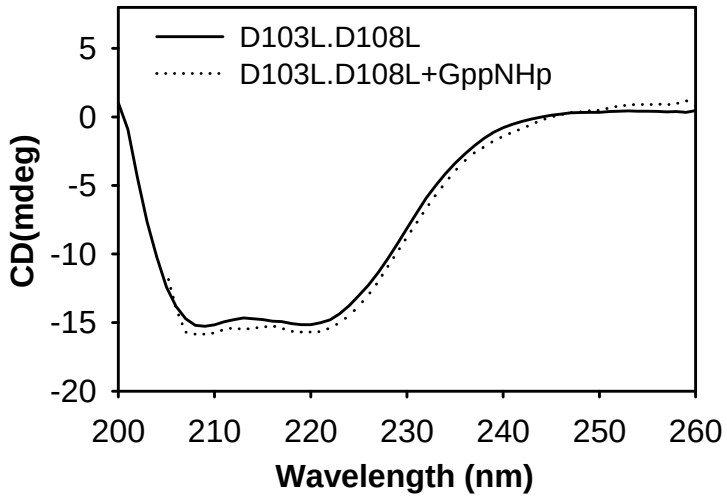
ii) Intrinsic tryptophan: The fluorescence measurements were carried out on a Fluorolog 3 (HORIBA Jobin Yvon) spectrofluorimeter. The fluorescence excitation wavelength of 280 nm was used. The emission spectra were recorded from 300 to 400 nm and clearly showed that the fluorescence was dominated by tryptophans only. The monochromator slit width was kept at 2 nm for both excitation and emission measurements. All measurements were done using 50 mM Tris, 5 mM $MgCl_2$, 100 mM KCl, pH 7.5, and at room temperature. The fluorescence measurements of the protein samples were carried out with an optical density of below 0.1 at 280 nm to avoid inner filter effect.

Circular dichroism measurements: The CD measurements of the wild type and mutant proteins were carried out both in the absence and presence of substrate analogue GppNHp using a Chirascan (Applied Photophysics) spectropolarimeter. The concentration of the protein and the analogue was used 1 and 200 µM respectively. All measurements were carried out at room temperature 25ºC using a buffer containing 20 mM Tris, 5 mM $MgCl_2$ and 100 mM KCl, pH 7.5. The optical cell of 1mm path length was used for these studies. For experiments with the analogue, the mixture was kept for at least an hour before collecting the data. The data were collected before the saturation of high voltage.

Protein cross-linking: The cross-linking experiments were carried out by mixing 20µM of protein with 10 mM zero length cross-linker *EDC* (1-Ethyl-3-[3-dimethylaminopropyl]carbo-

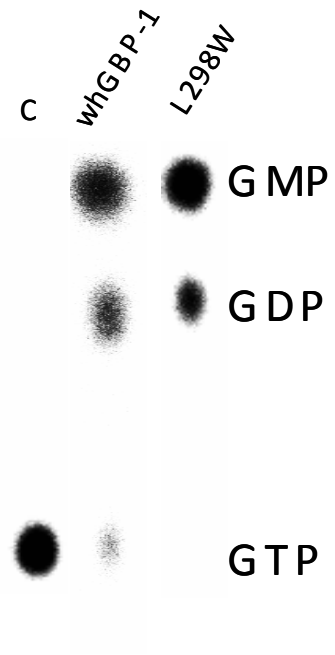
diimide Hydrochloride), Thermo Scientific, and 20 mM NHS (N-Hydroxy Succinimide) in 100 mM phosphate buffer at pH 7.0. The mixture was kept for 3 hrs.

Fig. S1



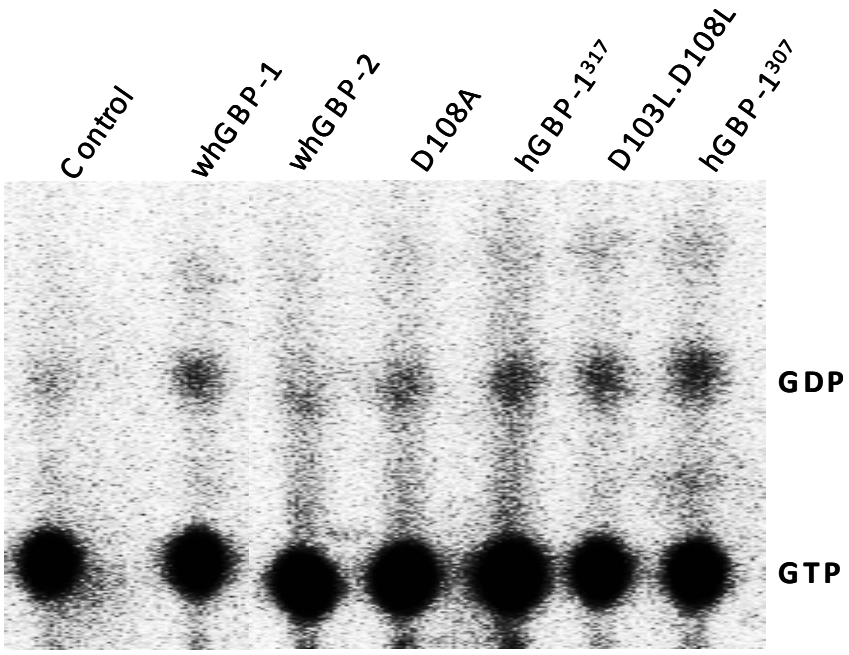
Circular dichroism measurement of the double mutant D103L.D108L with and without the substrate analogue at 25 ^{0}C. 1 μM of the double mutant was mixed with 200 μM GppNHp containing the assay buffer as described in the methods. The data was collected before the saturation of high voltage.

Fig. S2



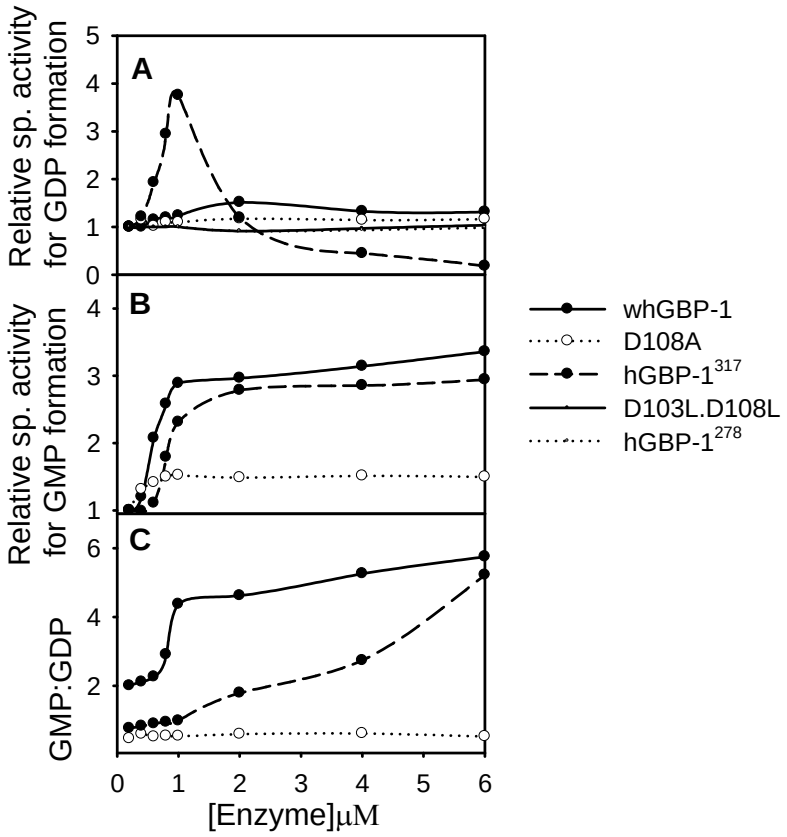
Activity assay of L298W using radiolabeled [α-^{32}P] GTP as described in the methods. The wild type hGBP-1 was used as a positive control. The concentration of the proteins was 0.4 μM.

Fig. S3



Activity assay of the dimeric immobilization proteins using radiolabeled [α-^{32}P] GTP. The assays were carried out as described in the text. After immobilization, the dimeric proteins were extensively washed in order to remove the bound analogue.

Fig. S4



A) The concentration dependence specific GTPase activity measurement for GDP formation. Relative specific activity was plotted against the concentrations of the enzymes. The experiment was carried out for various proteins as shown above. The activity was measured as described in the methods. The lines drawn through the experimental data points show the trend of the curve. The specific activity for hGBP-1^{317} initially increased with increasing concentrations of the protein but after ~ 1 μM concentration, it decreased with increasing concentrations of the protein. This may due to the external GDP which is released from the active site after the first catalysis rebinds to the protein for subsequent catalysis as this truncated protein is known to exhibit GDPase activity. B) The same experiment was carried out for GMP formation. C) The ratio of GMP to GDP was determined for various concentrations of the protein. The assay was described in the methods.