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Human Guanylate Binding Protein-1 Is a Secreted GTPase Present in Increased Concentrations in the Cerebrospinal Fluid of Patients with Bacterial Meningitis

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Interferon-γ-induced GTPases are key to the protective immunity against microbial and viral pathogens. As yet, the cell interior has been regarded as the exclusive residence of these proteins. Here we show that a member of this group, human guanylate binding protein-1 (hGBP-1), is secreted from cells. Secretion occurred in the absence of a leader peptide via a nonclassical, likely ABC transporter-dependent, pathway, was independent of hGBP-1 GTPase activity and isoprenylation, and did not require additional interferon-γ-induced factors. Interestingly, hGBP-1 was only secreted from endothelial cells but not from any of the nine different cell types tested. Clinically most important was the detection of significantly ($P < 0.001$, Mann-Whitney *U*-test) increased hGBP-1 concentrations in the cerebrospinal fluid of patients with bacterial meningitis ($n = 32$) as compared to control patients ($n = 74$). In this first report of a secreted GTPase, we demonstrate that secreted hGBP-1 may be a useful surrogate marker for diagnosis of bacterial meningitis. ***(Am J Pathol 2006, 169:1088–1099; DOI: 10.2353/ajpath.2006.060244)***

GTPases induced by interferon (IFN)-γ are key to the protective immunity against microbial and viral pathogens. IFN-γ-induced GTPases are classified into three groups: the small 47-kd GTPases, the Mx proteins, and the large 65- to 67-kd GTPases [also known as guanylate binding proteins (GBPs)].[1] The human 65- to 67-kd GTPase family consists of five members (hGBP-1 to hGBP-5).[2] All of these bind guanine nucleotides with relatively low affinity,[3] and the third motif [(N/T)KXD] of the classical tripartite GTPase motif is substituted by a conserved arginine-aspartic acid (RD)-motif.[4,5]

hGBP-1 is the best characterized member of the 65- to 67-kd GTPases. It hydrolyzes GTP with a high intrinsinc turnover rate; however, in contrast to other GTPases, it mainly yields GMP and inorganic phosphate.[6] The crystal structure of hGBP-1 has been resolved both in the GTP-bound and in the nucleotide-free state and was found to be composed of two domains: an N-terminal, compact globular domain harboring the GTPase function and a C-terminal, index finger-like, purely α-helical domain.[3,5]

hGBP-1 has been shown to exhibit antiviral activity against vesicular stomatitis virus and encephalomyocarditis virus.[7] In addition, it regulates the inhibition of proliferation and invasion of endothelial cells in response to IFN-γ.[8,9] Of note, hGBP-1 expression can be induced in many different cell types *in vitro*, but is almost exclusively associated with endothelial cells *in vivo*.[10]

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Three of the five hGBPs (hGBP-1, -2, and-5) carry an isoprenylation motif at their C-terminal end. Isoprenylation is involved in membrane association of proteins[11] and has been shown to cause association of murine GBP-2 with vesicular cytoplasmic membranes.[12] Recently, it has been demonstrated in HeLa cells that hGBP-1 can translocate to the Golgi membranes.[13] The translocation process required the GTPase activity of hGBP-1, stable induction of a protein structure resembling the GTP-bound form [induced by aluminum fluoride (AlF)], a functional isoprenylation signal, and IFN-γ stimulation of the cells.[13]

Presence in vesicles and/or Golgi localization are hallmarks of secreted proteins.[14] Two secretion pathways have been described. The classical secretion pathway requires a leader/signal peptide at the N-terminal end of the protein[15] and is sensitive to Golgi-disturbing agents such as brefeldin A and monensin.[16,17] In addition, a growing list of proteins including IL-1β, fibroblast growth factor, and galectin-1 can be exported from cells in the absence of a functional endoplasmic reticulum (ER)-Golgi system by unconventional/nonclassical secretion pathways.[18–20] Characteristics of nonclassically secreted proteins are 1) the lack of a leader peptide; 2) localization outside of classical secretory compartments such as ER and Golgi, often with granular appearance; and 3) secretion in the presence of brefeldin-A and/or monensin.[20] Golgi association of hGBP-1 under specific conditions and vesicular localization of GBPs directed us to investigate whether hGBP-1 may be secreted and which secretion pathways and signaling motifs may be involved. In addition, we analyzed the cell-type specificity of hGBP-1 secretion and the relevance of secreted hGBP-1 in human diseases.

Materials and Methods

Clinical Samples

Lumbar punctures were performed for diagnostic purposes after informed consent of patients and in agreement with the recommendations of the local ethics committee of the University of Erlangen. After centrifugation, cerebrospinal fluid (CSF) samples were stored at −70°C until analysis. We examined patients with acute bacterial meningitis (n = 32) and control individuals (n = 74). Control patients suffered from noninflammatory diseases of the nervous system. Their CSF findings (cell number and protein concentration) were normal. Patients with acute bacterial meningitis had typical signs and symptoms of meningitis (fever, headache, meningism), a neutrophil CSF pleocytosis (3919 ± 3786 leukocytes/μl), evidence of severe blood-CSF barrier disruption (417 ± 356 mg/dl protein), and a positive CSF culture or polymerase chain reaction (PCR)-based detection for bacteria (*Streptococcus pneumoniae*, n = 16; *Staphylococcus aureus*, n = 7; *Escherichia coli*, n = 3; *Neisseria meningitides*, n = 2; *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, group A streptococci, group B streptococci, all n = 1). Sex distribution and age were not statistically different between meningitis patients and controls.

Cell Culture and Harvesting of CM

Primary human umbilical vein endothelial cells (HUVECs) and smooth muscle cells were purchased from Cambrex Bio Science (Verviers, Belgium) and cultivated in EBM-2-MV (Cambrex) and SmGM-3 medium (Cambrex), respectively. Primary human dermal fibroblasts were isolated from healthy donors and cultivated in Dulbecco's modified Eagle's medium (DMEM)-10% fetal bovine serum (FBS) as described.[21] All primary cultures were used between passages 5 and 7 (split ratio, 1:4). The human keratinocyte line (HaCaT) and HeLa cells were cultivated in DMEM-10% FBS (DMEM: PAA, Cölbe, Germany; FBS: Biochrom, Berlin, Germany).

For IFN-γ (Roche, Mannheim, Germany) stimulation ~5×10^5 cells were starved overnight in 3.5 ml of the respective low medium (EBM-2–0.5% FBS; DMEM–0.5% FBS) and subsequently were treated with the cytokine in the same medium. In the following, the conditioned medium (CM) was harvested by centrifugation at 1000 × g for 10 minutes and stored at −80°C until analysis. Unless otherwise indicated all stimulations were performed in triplicate. One representative experiment of at least three is shown.

Cell Viability and Permeability

Cell viability and permeability were analyzed by determination of lactate dehydrogenase (LDH) activity in CM using a commercially available assay (CytoTox 96 nonradioactive cytotoxicity assay; Promega, Mannheim, Germany) according to the manufacturer's protocol and propidium iodide (Molecular Probes, Karlsruhe, Germany). For propidium iodide staining cell monolayers were washed with phosphate-buffered saline (PBS) once and incubated with propidium iodide solution (0.3 μg/ml, in PBS) for 3 minutes. Cells that incorporated propidium iodide as well as total cell numbers were determined in 10 high-power optical fields of an Axiovert 25 fluorescence microscope (Zeiss, Jena, Germany). To determine cells with compromised permeability in the CM, cells were harvested by centrifugation (1000 × g, 10 minutes) and treated as described above.

Retroviral Transduction and Transfection of HUVECs

HUVECs were stably transduced with the retroviral vectors pBABE-GFP-hGBP-1, pBABE-hGBP-1, pBABE-control, and pBABE-GFP as described.[8] Transient transfection was performed with SuperFect (Qiagen, Hilden, Germany) as described.[22] For transfection studies plasmids were constructed by insertion of cDNA of hGBP-1 or well-characterized mutants of hGBP-1 (hGBP-1-ΔCAAX, hGBP-1-D184N, helical domain)[8] fused to an N-terminal

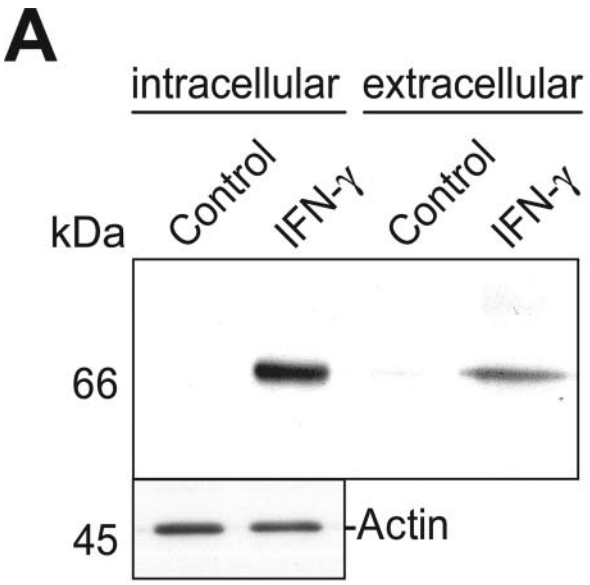
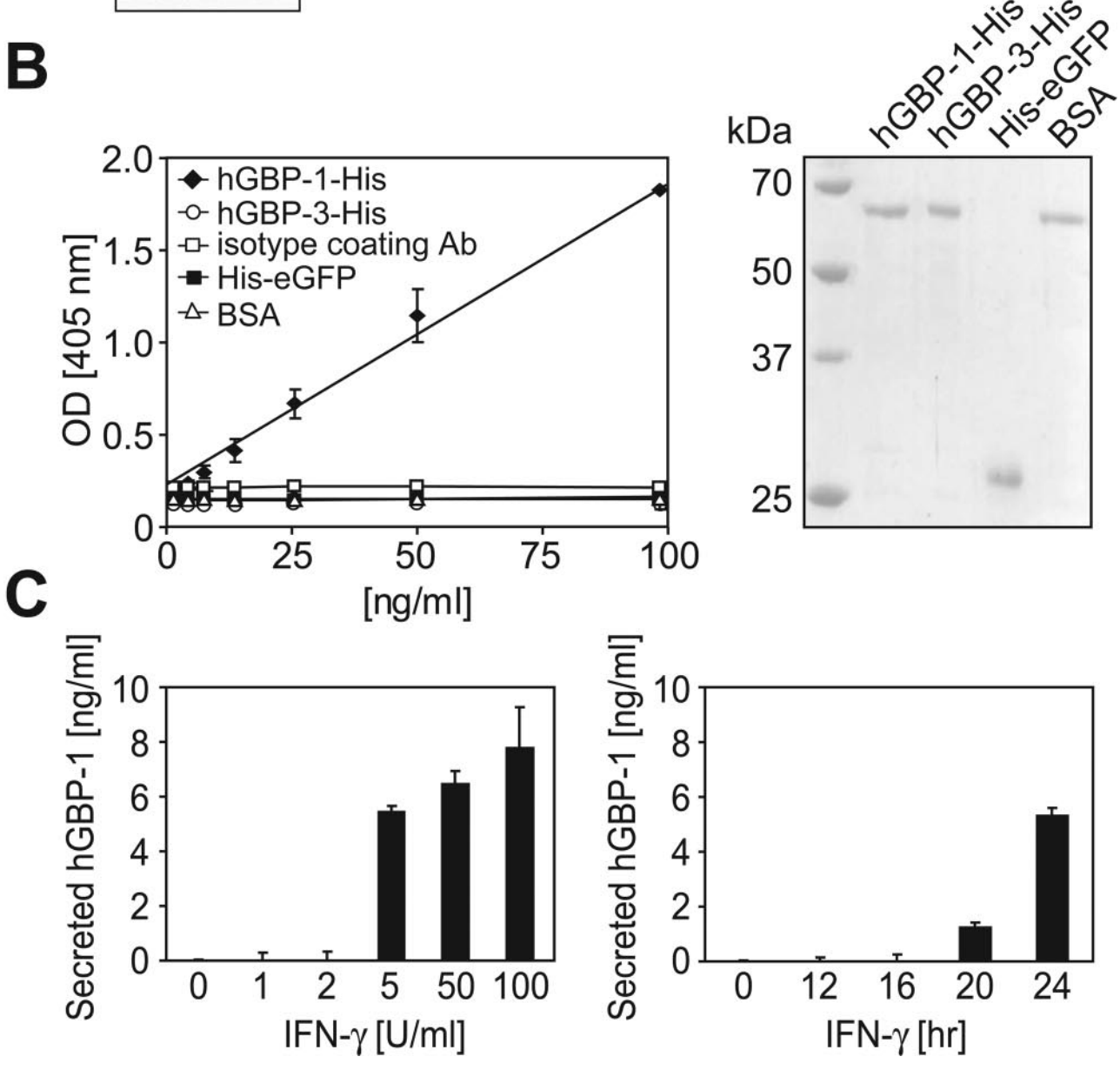


Figure 1. hGBP-1 is present in the CM of HUVECs. **A:** HUVECs were either untreated (control) or stimulated with IFN-γ (100 U/ml) for 24 hours. Intracellular hGBP-1 expression was analyzed by Western blot analysis of cell lysates. Actin was used as a loading control. Extracellular hGBP-1 was assayed by immunoprecipitation of CM and subsequent Western blot analysis. **B:** Fresh culture medium was supplemented with increasing concentrations of purified recombinant hGBP-1-His (black rhombus, C-terminal His-tag), hGBP-3-His (white circles), His-eGFP (black squares, N-terminal His-tag), or BSA (white triangles) and subjected to the hGBP-1 ELISA (**left**). In one experiment an isotypic (IgG_1) monoclonal rat control antibody was used for coating (white squares). Coomassie staining of the different control proteins used for the hGBP-1 ELISA (**right**). **C:** HUVECs were stimulated for a constant period of time (24 hours) with increasing concentrations of IFN-γ (**left**) and with a constant amount of IFN-γ (100 U/ml) for increasing periods of time (**right**). hGBP-1 secreted into the CM was detected by ELISA. All experiments were performed in triplicates.



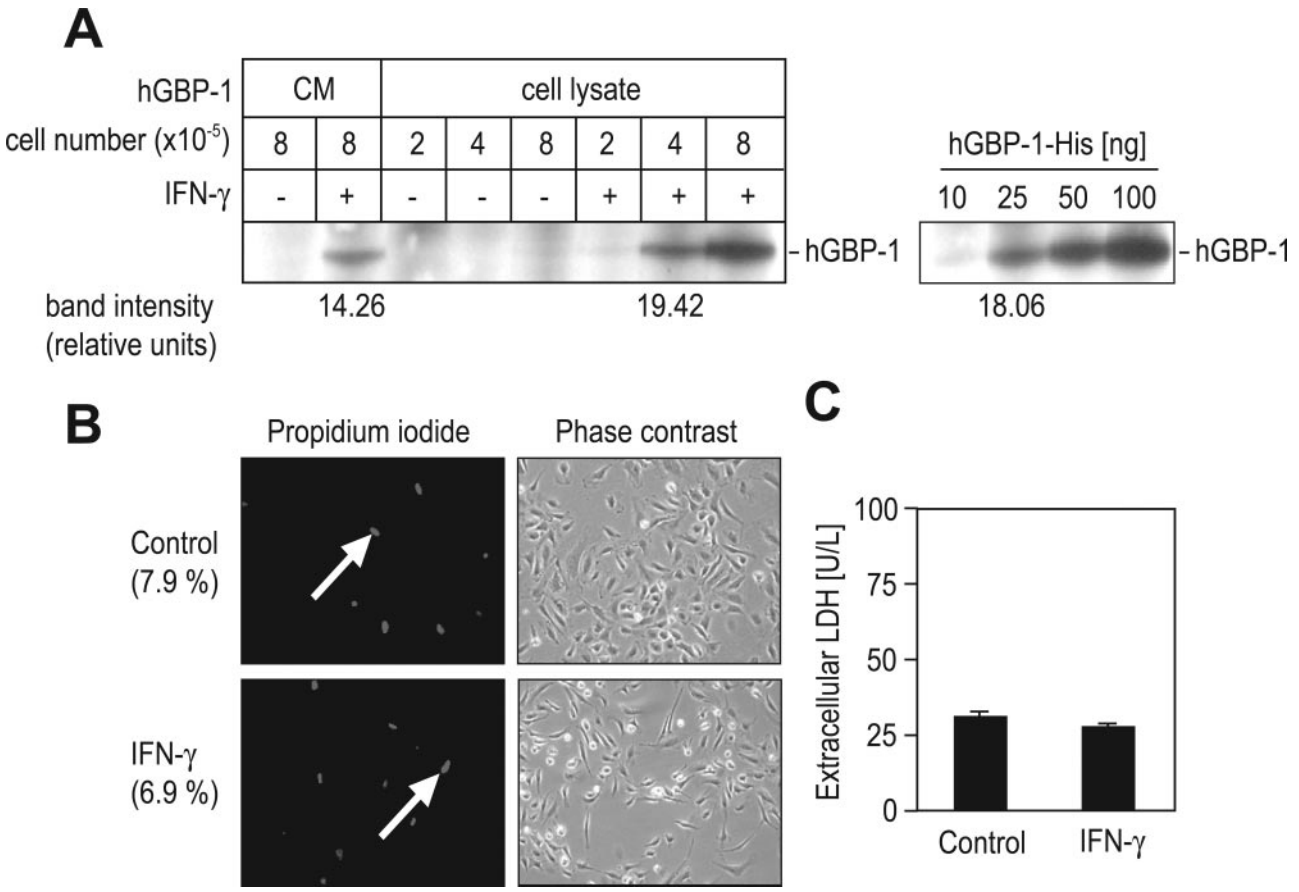


Figure 2. hGBP-1 is secreted from HUVECs. **A:** HUVECs were either untreated (−) or stimulated with IFN-γ (100 U/ml) for 24 hours (+). CM, cell lysates (**left**), and defined amounts of recombinant hGBP-1-His (**right**) were subjected to immunoprecipitation using equal experimental conditions. Optical densities of bands in nonsaturated levels were determined and are given in relative units. **B:** Compromised cell permeability was analyzed in untreated (control) and IFN-γ-treated (100 U/ml, 24 hours) HUVECs using propidium iodide staining. Cells with compromised cell permeability (propidium iodide, **arrows**) and total cell numbers (phase contrast) were estimated in 10 optical fields. The relative percentage of cells with compromised permeability, both in the monolayer and harvested from the cell culture supernatant, is given in **brackets**. All experiments were performed in triplicate. **C:** LDH activity was determined in the CM of untreated (control) and IFN-γ-treated (100 U/ml, 24 hours) HUVECs. Each value was determined in triplicate, means and SDs are shown.

Flag-tag encoding sequence into the pMCV1.4 expression vector (Mologen, Berlin, Germany).

Enzyme-Linked Immunosorbent Assay (ELISA)

hGBP-1

MaxiSorp immunoplates (Nunc, Wiesbaden, Germany) were coated overnight with 1 μg/ml of purified rat anti-hGBP-1 monoclonal antibody (clone 1B1,[10] capture antibody) or an isotypic control antibody (IgG_1, directed against a mutant form of E-cadherin) in PBS. Plates were rinsed with PBS-0.1% Tween 20 (PBS-T), blocked with PBS-1% skim milk for 30 minutes and incubated with the samples in triplicates for 2 hours. CM was used undiluted, CSF was diluted 1:8 in PBS-1% skim milk. Subsequently, the plates were washed four times with PBS-T, incubated (2 hours) with rabbit anti-hGBP-1 polyclonal antibody[8] [1:2500 in PBS-1% skim milk-0.1% Tween 20 (PBS-ST), detection antibody], rinsed four times with PBS-T and incubated (1 hour) with alkaline phosphatase-conjugated anti-rabbit antibody (1:2000 in PBS-ST; Zymed, Berlin, Germany). The wells were rinsed four times with PBS-T, *p*-nitrophenyl phosphate staining solution (Zymed) was added for 1 hour, and the color reaction was quantified at 405 nm in a microplate reader (model 680; Bio-Rad, Munich, Germany). Standard curves were obtained with recombinant purified hGBP-1-His protein (0 to 100 ng/ml), either diluted in fresh culture medium or in pooled standardized normal serum (diluted 1:8 in PBS-1% skim milk; Sigma-Aldrich, Munich, Germany). The ELISA was linear up to 100 ng/ml of hGBP-1 and had a detection limit of 4 ng/ml.

IP-10

IP-10 was measured using the human IP-10 Quantikine ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer's protocol.

Inhibition of Secretion

Monensin A sodium salt and glyburide [5-chloro-*N*-(4-(cyclohexylureidosulfonyl)phenethyl)-2-methoxybenzamide] were purchased from Sigma-Aldrich and dissolved in methanol and dimethyl sulfoxide, respectively. Final concentrations of organic solvents in CM were al-

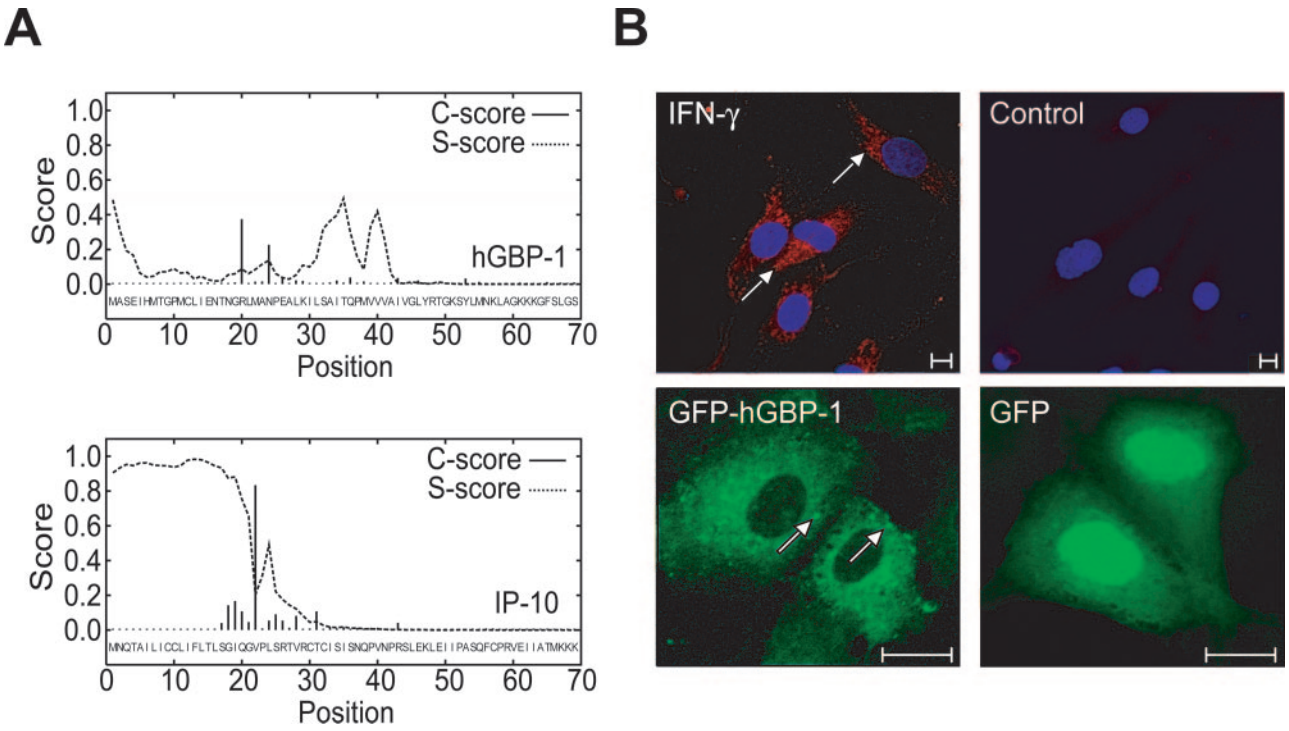


Figure 3. hGBP-1 does not have a leader peptide and is granularly distributed in IFN-γ-treated HUVECs. **A:** The presence of a leader peptide in hGBP-1 (**top**) and in the classically secreted IP-10 (**bottom**) was analyzed using SignalP 3.0 software. The S-score (dotted line) indicates the probability of an amino acid to be part of a leader sequence, and the C-sore (solid line) indicates the probability of a potential cleavage site. Positions with a high C-score and a drop in the S-score indicate the presence of a potential signal peptide. **B:** HUVECs untreated (control) or stimulated with IFN-γ (100 U/ml, 24 hours, IFN-γ) were subjected to immunofluorescence analysis with a monoclonal anti-hGBP-1 antibody. Nuclei were counterstained with DAPI (blue). GFP-hGBP-1 (**bottom left**) and GFP (**bottom right**) were constitutively expressed in HUVECs after retroviral transduction with pBABE-GFP-hGBP-1 and pBABE-GFP. hGBP-1 staining (red) and GFP-hGBP-1 fluorescence (green) are indicated by **arrows**. Scale bars = 10 μm.

ways less than 1.9% for dimethyl sulfoxide and 0.6% for methanol to avoid toxicity. Monensin and glyburide were added to the cultures 1 hour before IFN-γ.

Western Blotting

Western blotting was performed as described,[8,10] using the following antibodies: anti-ABCA-1 (dilution, 1:100; Acris, Hiddenhausen, Germany), anti-actin (1:1000, Sigma-Aldrich), anti-Flag (1:1000; ABR, Golden, CO), anti-GAPDH (1:60,000; Chemicon, Hampshire, UK), anti-hGBP-1 (clone 1B1, hybridoma supernatant 1:500), all horseradish peroxidase-conjugated secondary antibodies were diluted 1:5000 and were purchased from Amersham Biosciences, Freiburg, Germany.

Coomassie Staining

Different recombinant proteins (300 ng each) and 10 μl of unstained Precision Plus protein standard (Bio-Rad) were separated in a 10% sodium dodecyl sulfate polyacrylamide gel. The gel was washed with water three times and stained with SimplyBlue SafeStain (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol.

Immunoprecipitation

From CM

Cell culture supernatants were harvested, residual cells were removed by centrifugation (1000 × *g*, 10 minutes) and a protease inhibitor cocktail (0.02 mg/ml pancreas extract, 0.005 mg/ml pronase, 0.5 μg/ml thermolysin, 3 μg/ml chymotrypsin, and 0.33 mg/ml papain; Roche) was added. For hGBP-1 immunoprecipitation (IP), 5 ml of supernatant were supplemented with 12 μl of rabbit preimmune serum and 80 μl of protein A/G agarose beads (Oncogene, San Diego, CA) and incubated for 3 hours at 4°C. The beads were removed by centrifugation, 80 μl of protein A/G agarose beads and 12 μl of rabbit polyclonal anti-hGBP-1 serum[8] were added and incubated overnight at 4°C. For Flag IP 250 μl of Sepharose CL-6B (Sigma-Aldrich) was added to 3 ml of supernatant and incubated for 1 hour at 4°C. After removal of the beads 10 μl of Flag-agarose M2 (Sigma-Aldrich) was added (2 hours). In both cases beads were washed four times in PBS-T (4°C) after incubation, resuspended in 2× Laemmli sample buffer (Bio-Rad), boiled for 5 minutes, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis.

Table 1. Leader Peptide Analysis of hGBP-1

Protein	D-score	Leader sequence	Mechanism of secretion
hGBP-1	0.12		
Annexin 1	0.253		Probenicide-sensitive ABC-transporter[44,45]
bFGF	0.03		ABC transporter[46–48]
Galectin 1	0.13		Membrane blebbing[19]
MIF	0.112		ABC transporter[49]
IL-1β	0.056		ABCA1 transporter, endocytosis[29,50]
IP-10	0.861	MNQTAILICCLIFLTLSGIQG	ER-Golgi[25]
MMP-2	0.812	MEALMARGALTGPLRALCLLGCLLSHAAA	ER-Golgi[28]
VEGF	0.887	MNFLLSWVHWSLALLLYLHHAKWSQA	ER-Golgi[51]
IL-6	0.953	MNSFSTSAFGPVAFSLGLLLVLPAAFP	ER-Golgi[52]
IgG_1-Fc	0.894	MWFLTTLLLWVPVDG	ER-Golgi

The D-score of hGBP-1 and different classically [IFN-γ-inducible protein-10 (IP-10), matrix metalloproteinase-2 (MMP-2), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), immunoglobulin G_1- constant region (IgG_1-Fc)] and nonclassically [basic fibroblast growth factor (bFGF), macrophage migration inhibitory factor (MIF), interleukin-1β (IL-1β)] secreted proteins was determined with SignalP 3.0 software. A D-score above 0.43 indicates the presence of a leader peptide.

From Cell Lysates

Cellular proteins were extracted using 40 μl of RIPA buffer,[8,10] diluted in 5 ml of fresh culture medium cell CM supplemented with the protease inhibitor cocktail (see above) and subjected to the same IP procedure as above (CM samples). Using recombinant hGBP-1-His, we showed that 40 μl of RIPA buffer in 5 ml of fresh culture medium did not affect IP.

Immunofluorescence Analysis

Immunofluorescence analysis was performed as described.[10]

Signal Peptide Prediction

Signal peptide prediction was performed using the SignalP version 3.0 server (*http://www.cbs.dtu.dk/services/SignalP*).[23,24]

Statistical Analysis

Statistical analysis was performed using SPSS version 13.0 software. Analysis was based on Mann-Whitney *U*-test performed as a nonparametric test for independent samples with unknown distribution. hGBP-1 levels were compared with meningitis status (0 = negative/1 = positive) of the patients.

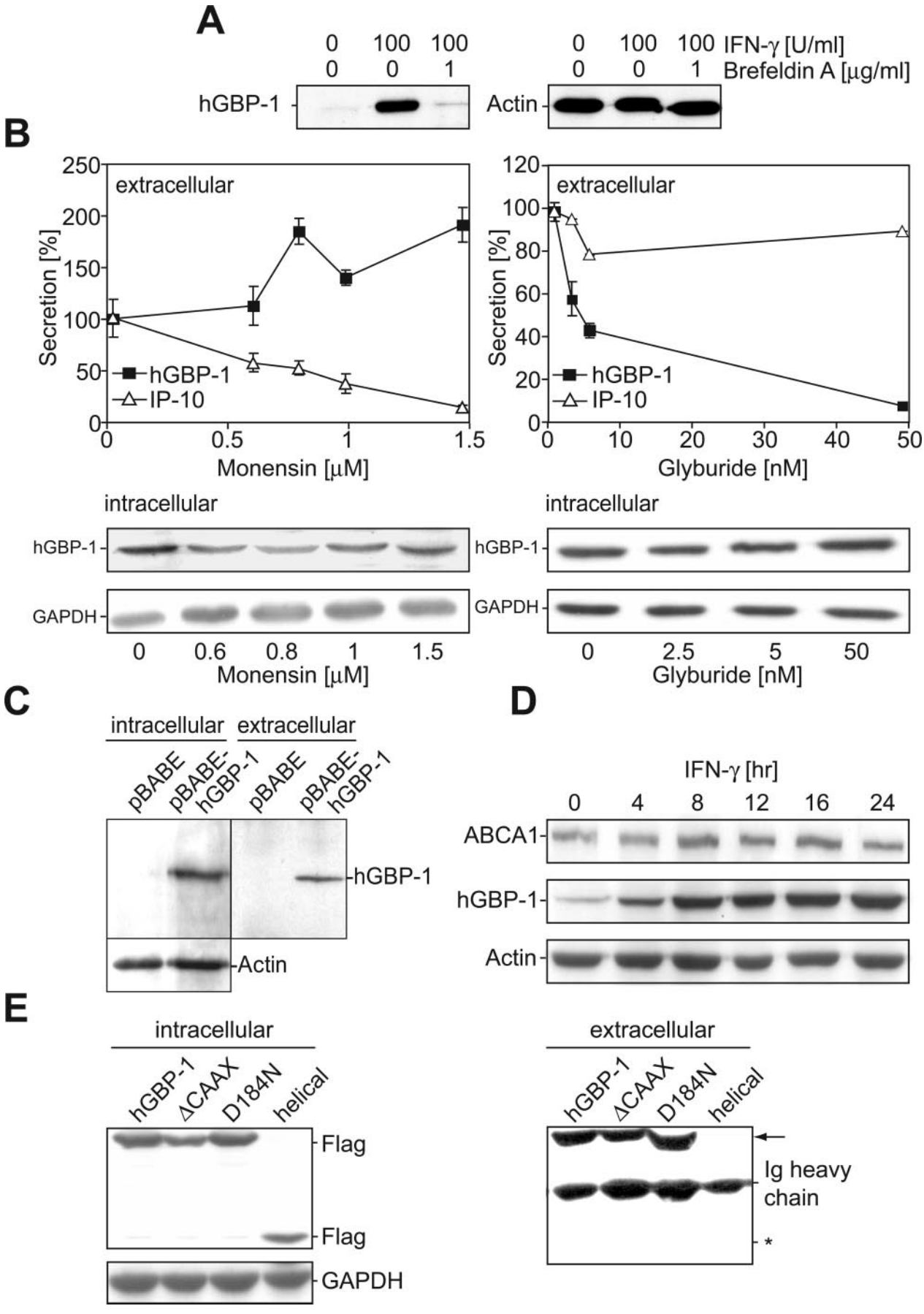
Results

hGBP-1 Is Secreted from Endothelial Cells

hGBP-1 expression was robustly induced by IFN-γ in HUVECs (Figure 1A, intracellular) as has been previously described.[8,10] Interestingly, hGBP-1 was also detected by immunoprecipitation in the CM of the same cells (Figure 1A, extracellular). This indicated that hGBP-1 may be released from these cells.

A specific sandwich ELISA was developed to quantitatively determine the amount of hGBP-1 released by HUVECs. A linearly increasing calibration curve was obtained with freshly prepared cell culture media supplemented with increasing concentrations (up to 100 ng/ml) of purified recombinant hGBP-1-His (C-terminal 6× His-tag; Figure 1B, left, black rhombus). In contrast, ELISA signals did not significantly increase in control experiments with increasing concentrations of other proteins such as hGBP-3-His, the closest homologue of hGBP-1[2] (Figure 1B, white circles), His-eGFP (Figure 1B, black squares) and BSA (Figure 1B, white triangles), or in experiments in which an isotypic monoclonal control antibody was used for coating (Figure 1B, white squares). The recombinant control proteins were of high purity and had equal concentrations as confirmed by Coomassie staining (Figure 1B, right).

ELISA showed that hGBP-1 concentrations in the CM of HUVECs increased in a concentration-dependent (Figure 1C, left) and time-dependent (Figure 1C, right) manner. In general, 10^6 HUVECs released 25 to 66 ng of hGBP-1 into the CM within 24 hours of stimulation with 100 U/ml IFN-γ. To exclude that hGBP-1 was present in the CM due to cell lysis, we determined in a first step the relative amount of hGBP-1 released in relation to total hGBP-1 expressed and to the number of cells with compromised permeability. To this goal the total CM was harvested from 8×10^5 IFN-γ-treated, and untreated HUVECs and cell lysates were prepared from the same cells, respectively. hGBP-1 was immunoprecipitated from the CM and from decreasing amounts of cell lysate (Figure 2A, left) under identical conditions (see Material and Methods). In parallel, increasing amounts of recombinant hGBP-1-His were subjected to the same procedure as a standard for quantification (Figure 2A, right). Comparison with the standard indicated that bands obtained with the CM of 8×10^5 cells and the cell lysates of 4×10^5 cells were still in the nonsaturated range. Therefore, the intensities of these bands were quantified (Figure 2A, band intensity). From the values obtained it could be calculated that the band intensity of total (intra- and extracellular) hGBP-1 synthesized by 8×10^5 cells was 53.1 ($14.26 + 19.42 \times 2$) arbitrary units. The band intensity of extracellular hGBP-1 was 14.26. This indicated that 26.9% of total hGBP-1 was released from IFN-γ-stimulated HUVECs.


A
0 100 100 IFN-γ [U/ml]
0 0 1 Brefeldin A [μg/ml]
hGBP-1
Actin
B
extracellular
Secretion [%]
hGBP-1
IP-10
Monensin [μM]
Glyburide [nM]
intracellular
GAPDH
0 0.6 0.8 1 1.5
0 2.5 5 50
C
intracellular
extracellular
pBABE
pBABE-hGBP-1
Actin
D
IFN-γ [hr]
0 4 8 12 16 24
ABCA1
E
hGBP-1
ΔCAAX
D184N
helical
Flag
GAPDH
Ig heavy chain
*

Cells with compromised permeability barrier were quantitatively determined with propidium iodide staining of both, the cells in the monolayer (Figure 2B) and of detached cells harvested from the cell culture supernatant (data not shown). Counting of propidium iodide-positive cells of three independent experiments showed that in total (monolayer and detached cells) 7.9 to 8.5% of untreated (Figure 2B, control) and 6.9 to 8.5% of IFN-γ-treated (Figure 2B, IFN-γ) HUVECs revealed permeability barrier defects. In addition, analysis of LDH activity in the CM confirmed that cell lysis was at the same low levels under the different conditions (Figure 2C). Lysis-associated permeability defects of maximally 8.5% (down to 6.9%) of the cells cannot account for the release of at least 25% of total hGBP-1, indicating that hGBP-1 may be released via specific secretion pathways.

hGBP-1 Is Secreted via a Nonclassical Secretion Pathway

Analysis of the hGBP-1 primary sequence using the protein sequence analysis software SignalP V3.0 (*http://www.cbs.dtu.dk/services/SignalP/*)[24] did not indicate the presence of a leader peptide sequence (Figure 3A, top; Table 1). In contrast, the N-terminal sequence of the classically secreted IFN-γ-inducible protein (IP)-10[25] revealed a high probability of a leader peptide cleavage site (Figure 3A, bottom; C-score) coinciding with a drop in hydrophobicity (Figure 3A, bottom; S-score) at the potential cleavage site. This is characteristically observed in proteins with leader peptide sequences. In addition, a leader peptide-associated D-score greater than 0.43 was obtained with several classically secreted test proteins (Table 1). In contrast, hGBP-1 had a D-score less than 0.43 similarly to other proteins that are secreted via alternative secretion pathways (Table 1).

Furthermore, hGBP-1 was not enriched in the Golgi of IFN-γ-treated HUVECs under physiological conditions, in the absence of AlF (Figure 3B, IFN-γ, arrows). Instead, hGBP-1 appeared with a cytoplasmic granular staining pattern (Figure 3B, IFN-γ, arrows). The same granular cytoplasmic distribution was observed with a constitutively expressed chimeric fusion protein of green fluorescence protein (GFP, N-terminal) and hGBP-1 (C-terminal), also in the absence of IFN-γ stimulation (Figure 3B, GFP-hGBP-1, arrows). Lack of a signal peptide and the absence of significant hGBP-1 concentrations in the Golgi indicated that nonclassical secretion pathways may trigger hGBP-1 release. To investigate this hypothesis in more detail, the potential secretion pathways were analyzed with specific inhibitory molecules. Brefeldin A and monensin inhibit the classical secretion pathway.[18,26–28] Brefeldin A is more commonly used but was found to inhibit IFN-γ-induced intracellular expression of hGBP-1 in HUVECs (Figure 4A). Therefore, monensin was applied, resulting in inhibited secretion of classically secreted IP-10 in IFN-γ-stimulated HUVECs (Figure 4B, top left, triangles). In contrast, in the same cells the secretion of hGBP-1 was not inhibited but was increased (Figure 4B, top left, squares). Expression levels of intracellular hGBP-1 were not significantly altered by monensin in the concentrations used (Figure 4B, bottom left).

Glyburide inhibits protein secretion via different transporter proteins, including the adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1).[29–33] Interestingly, hGBP-1 secretion was almost completely blocked by glyburide (Figure 4B, top right, squares), whereas the secretion of IP-10 was not significantly affected (Figure 4B, top right, triangles). Expression of intracellular hGBP-1 was not inhibited by glyburide (Figure 4B, bottom right). In addition, cell membrane permeability was not altered under these conditions as indicated by similar LDH activities in the CM of the different cell cultures (data not shown).

Subsequently, we analyzed whether an additional IFN-γ-induced factor may be required for hGBP-1 secretion. To this end we used HUVECs that constitutively expressed hGBP-1 after retroviral transduction (Figure 4C, intracellular, pBABE-hGBP-1). In addition, significant amounts of hGBP-1 were detected in the CM of these cells (Figure 4C, extracellular, pBABE-hGBP-1). hGBP-1 was not detected in the cell lysate (Figure 4C, intracellular, pBABE) and the CM (Figure 4C, extracellular, pBABE) of control cells, which were transduced with the plain vector. LDH activities were identical in the CM of hGBP-1- and control-transduced HUVECs (data not shown). These results showed that hGBP-1 does not increase cell permeability and no additional IFN-γ-induced factor is required for hGBP-1 secretion. The glyburide-sensitive transporter ABCA1, which was found to be constitutively expressed in HUVECs irrespective of IFN-γ stimulation (Figure 4D), may be a potential candidate to mediate secretion of hGBP-1. Successful IFN-γ stimulation of the cells was demonstrated by the time-dependent increase of hGBP-1 expression in the same cells (Figure 4D).

Figure 4. hGBP-1 is secreted by nonclassical pathways independent of additional IFN-γ-induced factors, its GTPase function, and isoprenylation. **A:** HUVECs were treated with brefeldin A using the indicated concentration 1 hour before treatment with IFN-γ (100 U/ml, 24 hours). hGBP-1 expression was detected by Western blot analysis with a monoclonal anti-hGBP-1 antibody. Actin was used as a loading control. **B:** HUVECs were treated with either monensin (**left**) or glyburide (**right**) using the indicated concentrations 1 hour before treatment with IFN-γ (100 U/ml, 24 hours). Concentrations of hGBP-1 (black squares) and IP-10 (white triangles) in the CM were analyzed by ELISA (**top**). Intracellular hGBP-1 was determined by Western blot analysis of cell lysates using GAPDH as a loading control (**bottom**). One representative experiment of three is shown. **C:** HUVECs were stably retrovirally transduced with either pBABE control vector (pBABE) or pBABE-hGBP-1 vector. Intracellular hGBP-1 expression was analyzed by Western blot analysis of cell lysates. Actin was used as a loading control. Extracellular hGBP-1 was assayed by immunoprecipitation of CM and subsequent Western blot analysis. **D:** HUVECs were stimulated with 100 U/ml IFN-γ for the indicated periods of time. Expression of ABCA1 and hGBP-1 was analyzed by Western blot analysis. Actin was used as a loading control. **E:** HUVECs were transiently transfected with plasmids expressing Flag-tagged hGBP-1, hGBP-1-ΔCAAX, hGBP-1-D184N, and the hGBP-1 helical domain. CM and cell lysates were harvested 30 hours after transfection. Intracellular expression was analyzed by Western blot analysis of cell lysates with anti-Flag antibodies (intracellular, **left**). GAPDH was used as a loading control. Secretion of the respective proteins was investigated by Flag-immunoprecipitation of the CM and subsequent anti-Flag Western blot analysis (extracellular, **right**). Secreted proteins are indicated by an **arrow**. The expected position of immunoprecipitated hGBP-1 helical domain is indicated by an **asterisk**. All experiments were performed in triplicate.

Finally, we investigated whether isoprenylation and/or GTPase activity may be required for hGBP-1 secretion. cDNA molecules of wild-type hGBP-1 and of previously characterized mutants of hGBP-1 [ΔCAAX-hGBP-1 (deleted isoprenylation signal), D184N-hGBP-1 (defective GTPase), isolated helical domain of hGBP-1[8,9]] were transiently expressed with a Flag-tag in HUVECs. Wild-type hGBP-1 and the different mutants were robustly expressed (Figure 4E, left, intracellular), at similar levels as observed in IFN-γ-stimulated cells (compare Figure 4E and Figures 4D and 5A). Immunoprecipitations from the CM of transfected cells with anti-Flag agarose beads and subsequent detection of precipitated proteins by an anti-Flag Western blot showed that ΔCAAX-hGBP-1, D184N-hGBP-1, and hGBP-1 were secreted with similar efficiency (Figure 4E, right, extracellular, arrow). Of note, the helical domain could not be detected in the CM (Figure 4E, extracellular, asterisk). The latter observation indicated that additional motifs from the globular domain may be required for the secretion and that unspecific protein release by cell lysis was very low in transfected cells.

hGBP-1 Is Selectively Secreted from Endothelial Cells

hGBP-1 may be secreted from different cell types. To investigate this, hGBP-1 expression was induced with IFN-γ in different human primary cell cultures (HUVECs, fibroblasts, smooth muscle cells) and cell lines (HeLa, HaCaT) (Figure 5A). Surprisingly, only in the CM of IFN-γ-stimulated HUVECs were high amounts of hGBP-1 detected (Figure 5B). In addition, human B, T, and monocytic cell lines and primary keratinocytes were analyzed and did not release hGBP-1 (data not shown). Analysis of LDH activity in the cell culture supernatants of the different cell cultures indicated that IFN-γ did not increase the permeability of the different cells (Figure 5C). Of note, LDH activity was 2.7-fold higher in the CM of HaCaT as compared to HUVECs (Figure 5C), whereas hGBP-1 concentrations were 14-fold higher in the HUVEC CM as compared to HaCaT (Figure 5B). Thus, hGBP-1 is selectively secreted from HUVECs.

hGBP-1 Is Present in Increased Concentrations in the CSF of Patients with Bacterial Meningitis

Finally, we investigated hGBP-1 concentrations in the CSF of patients with bacterial meningitis ($n = 32$) and in control patients ($n = 74$) in a blinded manner. Because of the lack of sufficient amounts of CSF, calibration curves were performed in human normal serum supplemented with increasing concentrations of recombinant hGBP-1-His (Figure 5D, left). A linear increase of signal intensities was observed up to 100 ng/ml (Figure 5D, black circles). Signals did not significantly increase with control proteins such as His-eGFP (Figure 5D, triangles), BSA (Figure 5D, white circles), hGBP-3-His (Figure 5D, squares), or bacterial lysates (data not shown).

Interestingly, hGBP-1 concentrations were significantly ($P < 0.001$, Mann-Whitney *U*-test) increased in the CSF of patients with bacterial meningitis ($n = 32$) as compared to the control patients ($n = 74$) (Figure 5D, right). hGBP-1 concentrations were clearly above background in the CSF of 71.9% of the patients with bacterial meningitis (mean, 516 ng/ml; median, 65 ng/ml) but only in 5.4% of the control patients (Figure 4D, right). These results indicated, that hGBP-1 may also be secreted *in vivo* and may be a useful surrogate marker for the diagnosis of bacterial meningitis.

Discussion

Here we showed for the first time, both *in vitro* and *in vivo*, that a large GTPase can be secreted. hGBP-1 was found to be released exclusively from endothelial cells via a nonclassical secretion pathway. Protein secretion via nonclassical pathways is generally less effective as compared to classical secretion. However, several evidences clearly proved that hGBP-1 is actively secreted and not passively released by cell lysis. 1) Cell permeability of IFN-γ-treated and retrovirally transduced hGBP-1-expressing cells was not increased as indicated by unaltered LDH activity in the CM in comparison to control cells. 2) Cell permeability was compromised in maximally 8.5% of the IFN-γ-treated HUVECs. In contrast, 26.9% of totally synthesized hGBP-1 was released. Apparently, hGBP-1 release is more than threefold higher, as would be expected by solely cell-lysis-mediated release. 3) hGBP-1 secretion was inhibited by glyburide. In contrast, hGBP-1 expression, cell lysis and release of classically secreted IP-10 were not affected in the same cells. 4) Only hGBP-1, but not its isolated helical domain, could be detected in the CM of HUVECs, which expressed these proteins at similar levels. The different recombinant proteins were detected via an identical immunological tag (Flag-tag), to exclude differential detection sensitivity. 5) IFN-γ treatment of HUVECs and HaCaT induced hGBP-1 expression in both cell types at similar levels. Of note, LDH activity in the CM indicated that cell lysis was higher (2.7-fold) in HaCaT, whereas significantly higher concentrations (14-fold) of hGBP-1 were detected in the CM of HUVECs. Altogether, we provided several different evidences suggesting that hGBP-1 is secreted from endothelial cells.

Golgi localization is a characteristic feature of classically secreted proteins. In this framework a recent report on Golgi localization of hGBP-1 in cells co-stimulated with IFN-γ and AlF suggested that hGBP-1 may be classically secreted.[13] However, under physiological conditions without AlF, no significant Golgi enrichment of hGBP-1 was detected. This does not exclude that Golgi translocation may be important for certain biological functions of hGBP-1, but clearly indicates that the amount of hGBP-1 in the Golgi may be too small to cause the release of significant amounts of hGBP-1 via the classical route. In addition, hGBP-1 does not have a signal peptide and its secretion was not inhibited, but rather increased by the classical pathway inhibitor monensin. Monensin blocks

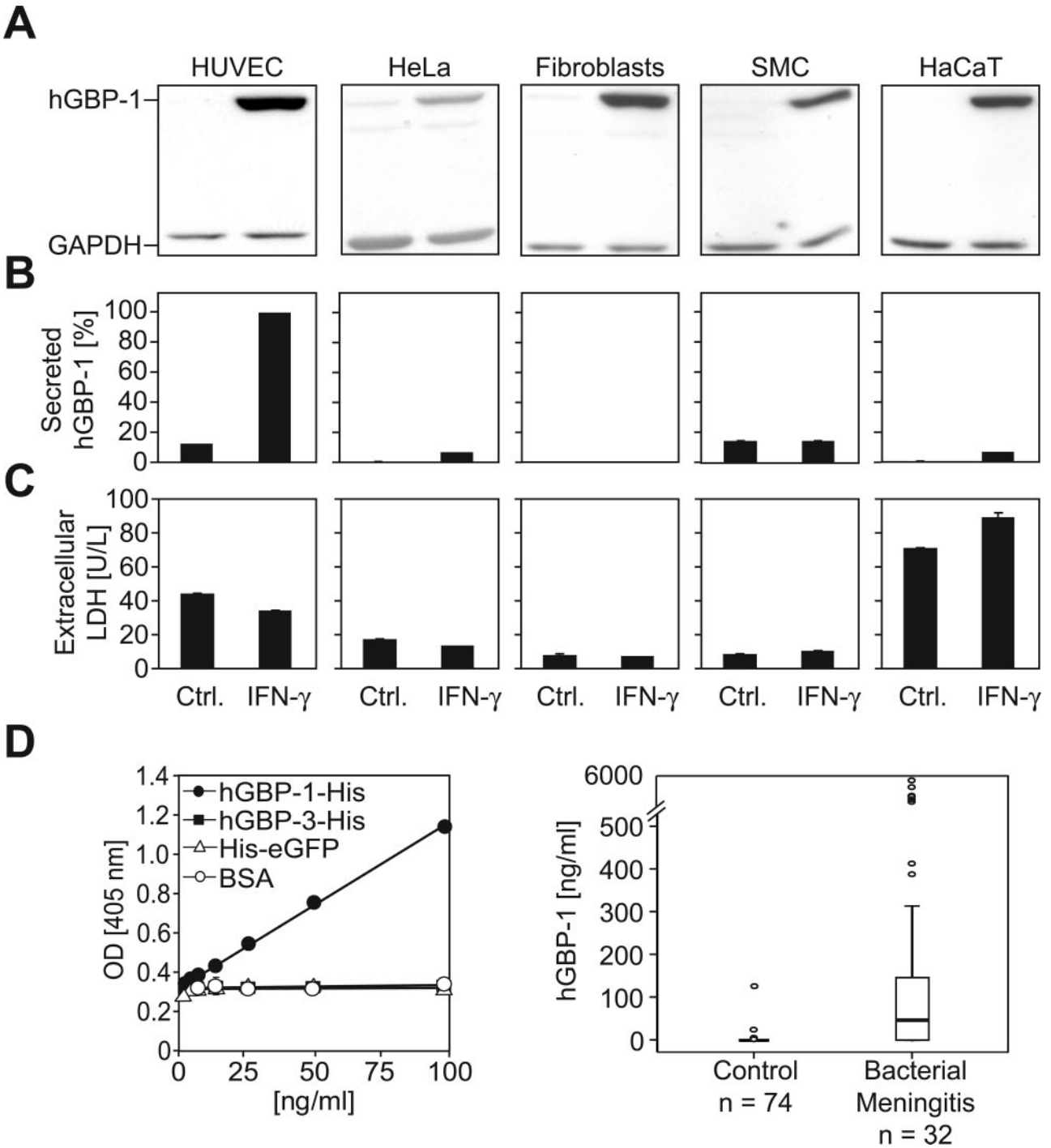


Figure 5. hGBP-1 is selectively secreted from endothelial cells and is present in increased concentrations in the CSF of patients with bacterial meningitis. Different primary cells [HUVECs, dermal fibroblasts, smooth muscle cells (SMCs)] and cell lines [HeLa, HaCaT (keratinocytes)] were either untreated (Ctrl.) or treated with IFN-γ (100 U/ml, 24 hours). **A:** Intracellular hGBP-1 was determined by Western blot of cell lysates. GAPDH was used as a loading control. hGBP-1 amounts (**B**) and LDH activities (**C**) were determined in the CM by hGBP-1-ELISA and the CytoTox nonradioactive cytotoxicity assay, respectively. In **B** the secreted hGBP-1 concentrations are given relative to the amount released by IFN-γ-treated HUVECs, which was set to 100%. Each ELISA value was determined in triplicate, means and SDs are shown. The latter were very low and therefore partly below graphical resolution. One representative experiment of three is shown. **D:** Human normal serum diluted in blocking buffer was supplemented with increasing concentrations of hGBP-1-His (black circles), hGBP-3-His (squares), His-eGFP (triangles), and BSA (white circles) and subjected to the hGBP-1-ELISA (**left**). hGBP-1 concentrations were determined by hGBP-1-ELISA in the CSF of patients suffering from bacterial meningitis ($n = 32$) and control patients ($n = 74$). A box plot is depicted for each group, the median is represented by the black horizontal bar (**right**). Circles indicate values that are more than 1.5 times beyond the distance between the upper and lower hinges of the box. The results of six patients with bacterial meningitis are not displayed within the linear scale. The specific hGBP-1 concentrations were as follows: 1.0, 1.2, 1.3, 2.1, 2.6, and 5.6 μg/ml.

the release of secretory vesicles from the Golgi apparatus,[34–36] and it is known that inhibitors of the ER/Golgi system can increase secretion of nonclassically secreted proteins.[27,37,38] Of note, isoprenylation, GTPase activity, and IFN-γ stimulation, which are required for Golgi translocation of hGBP-1,[13] were dispensable for its secretion.

Apparently, hGBP-1 secretion is independent of classical ER/Golgi pathways, which is a characteristic feature of nonclassically secreted proteins.[18,20] At present more than 20 proteins are known to be nonclassically secreted,[20] but no unifying transport concept has been detected. ABCA1 is the best characterized member of the different mediators of nonclassical secretion.[29,39] ABCA1 was found to be constitutively expressed in HUVECs, irrespective of IFN-γ stimulation, and the ABCA1 inhibitor glyburide blocked hGBP-1 secretion efficiently but did not affect the secretion of IP-10. Unfortunately, other ABCA1 inhibitors such as BSP and DIDS were not tolerated by primary endothelial cells (data not shown). Altogether, these results indicated that the ABC transporter pathway might be involved in the secretion of hGBP-1. However, the specific molecule(s) involved remain(s) to be identified. Preliminary evidence showed that centrifugation/filtration reduced hGBP-1 concentrations in the supernatants, whereas repeated freeze/thaw cycles increased the amounts that were detected by ELISA (data not shown). Both findings indicated that hGBP-1 may be associated with exosomes and/or microparticles in the supernatants.

Our search for potential pathological situations in which extracellular hGBP-1 may be involved was directed by the following considerations. Extracellular GTP has been implicated in neurite growth, and extracellular GTPases have been postulated as targets/regulators of extracellular GTP activity.[40,41] hGBP-1 expression and secretion is tightly associated with inflammatory activated endothelial cells.[2,8,22] hGBP-1 belongs to the GTPases of the innate immunity, which are regulating the defense against infectious agents.[1] Inflammatory activation of the nervous and vascular system in the course of infection is characteristically observed in meningitis. Meningitis is a severe disease, occurring by invasion of the subarachnoid space by infectious agents and associated with inflammation, break-down of the blood-brain barrier, and vasogenic edema.[42,43] In a blinded study with a newly established ELISA, significantly increased concentrations of hGBP-1 were detected in the CSF of 71.9% of patients with bacterial meningitis. In contrast, 94.6% of the CSF of control patients did not contain any detectable amounts of hGBP-1. These results demonstrated in a clinically relevant disease that hGBP-1 is also secreted under conditions of infection-associated inflammation *in vivo*.

Our work indicates for the first time that GTPases may have extracellular functions. The biological function of secreted hGBP-1 has to be determined in future studies. Apparently two different ways of extracellular hGBP-1 activities are conceivable: extracellular hGBP-1 may alter the extracellular GTP pool, which has been shown to be involved in cell growth and cell differentiation (indirect mechanism);[40,41] and extracellular hGBP-1 may activate cells or interfere with microorganisms by binding to specific receptors (direct mechanism). In addition to its extracellular functions, secreted hGBP-1 in the CSF may be a useful surrogate marker for bacterial meningitis.

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