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# Unique Features of Different Members of the Human Guanylate-Binding Protein Family

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## ABSTRACT

**Guanylate-binding proteins (GBPs) are the most abundant cellular proteins expressed in response to interferon-$\gamma$ (IFN-$\gamma$), with seven highly homologous members in humans, termed HuGBP-1 to HuGBP-7. To date, differential features that may indicate differential functions of these proteins have not been described. Here, we investigated the expression and subcellular localization of the different HuGBPs in endothelial cells (EC). IFN-$\gamma$, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), and interleukin-1$\beta$ (IL-1$\beta$) induced the expression of HuGBP-1, HuGBP-2, and HuGBP-3 at similar high levels. In contrast, expression of HuGBP-4 and HuGBP-5 was robustly induced only by IFN-$\gamma$ and not by TNF-$\alpha$ and IL-1$\beta$. Expression of HuGBP-6 and HuGBP-7 was not detected in EC under the various conditions examined. Investigating subcellular localization of the EC-expressed HuGBPs, HuGBP-1, HuGBP-3, and HuGBP-5 were exclusively detected in the cytoplasm, whereas HuGBP-2 and HuGBP-4 displayed a nucleocytoplasmic distribution. Treatment of the cells with IFN-$\gamma$ and aluminum fluoride caused rapid enrichment of HuGBP-1 and HuGBP-2 in the Golgi apparatus, as demonstrated by time-lapse microscopy and fluorescence analyses of GFP-tagged HuGBPs. HuGBP-3 and HuGBP-4 were never detected in the Golgi apparatus, whereas HuGBP-5 was constitutively enriched in this cytosolic compartment, irrespective of stimulation. These results assign a characteristic pattern of expression and subcellular localization to each of the HuGBPs, indicating for the first time that these proteins may have different cellular functions.**

## INTRODUCTION

GUANYLATE BINDING PROTEINS (GBPs) are among the most abundant cellular proteins expressed in interferon-$\gamma$ (IFN-$\gamma$)-treated cells.[1] Seven different GBPs (GBP-1 to GBP-**7***) with molecular weights of 67–73 kDa have been identified in human and six in mouse. Human GBPs (HuGBPs) exhibit a high degree of homology, which is highest between HuGBP-1 and HuGBP-3 (88% amino acid identity) and lowest between HuGBP-4 and HuGBP-5 (52% amino acid identity). GTPase activity and molecular weight classify the GBPs as a subfamily within the protein family of large GTPases. All of these proteins are characterized by their ability to oligomerize and display an oligomerization-dependent stimulation of GTP hydrolysis.[3]

HuGBP-1 is the best characterized GBP. HuGBP-1 expression is induced by type I and type II interferons (IFN), including IFN-$\gamma$, and also by interleukin-1$\beta$ (IL-1$\beta$), IL-1$\alpha$, and tumor necrosis factor-$\alpha$ (TNF-$\alpha$).[4–7] Each of these inflammatory cytokines can induce HuGBP-1 expression in many different cell types *in vitro*. *In vivo*, HuGBP-1 expression is almost exclusively associated with endothelial cells (ECs), suggesting

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*Identification of HuGBP-6 and HuGBP-7 occurred while this study was in revision.[2] Neither of the two HuGBPs was found to be expressed in HUVEC by RT-PCR, irrespective of stimulation (data not shown). However, expression of HuGBP-6 and HuGBP-7 was detected in lung and liver tissues, which served as a positive control (data not shown). Because HuGBP-6 and HuGBP-7 were not expressed in endothelial cells, we did not consider these proteins further in the present study. The following primer pairs were used for detection of HuGBP-6 and HuGBP-7: HuGBP-6 (forward) 5′-CCCAAATACGTA**ATGGAATCTGGACCCAA**-3′, (reverse) 5′-TTTACGCGTCGAC**TTAAAAGGGGAGCTTATGC**-3′; complementary sequence is in bold, restriction sites for cloning [SnaB1 (5′-TACGTA-3′) and Sal1 (5′-GTCGAC-3′)] are underlined; HuGBP-7: (forward) 5′-CCCAAATACGTA**ATGGCATCAGAGATCC**-3′, (reverse) 5′-TTTACGCGTCGAC**TCAGCTTATAATTTTCTTACCAG**-3′.

that this protein may exert a specific function in these cells.[4–6,8] In this framework, it has been shown that HuGBP-1 regulates the inhibition of EC proliferation and invasion in response to inflammatory cytokines.[6,9] In addition, it has been shown to exhibit antiviral activity against vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV).[10] Recently, we showed that HuGBP-1 is also secreted from ECs and is present in increased concentrations in the cerebrospinal fluid (CSF) of patients with bacterial meningitis.[11]

Few studies to date have investigated the subcellular localization of distinct GBPs. Three of the seven HuGBPs (HuGBP-1, HuGBP-2, and HuGBP-5) carry a CaaX-isoprenylation motif at their C-terminal end. Isoprenylation is involved in protein-protein interaction[12] and membrane association of proteins.[13] For example, isoprenylation of murine GBP-2 (MuGBP-2) causes the association of the protein with membranes surrounding cytoplasmic vesicles.[14] It was shown recently that HuGBP-1, which in general is homogeneously distributed throughout the cytoplasm in HeLa cells and human primary fibroblasts, becomes enriched in the Golgi apparatus in the presence of specific stimulatory substances (Golgi translocation process).[15] This translocation process required the presence of a functional isoprenylation signal, GTPase activity, stable induction of a protein structure resembling the transition state of the GTP-bound protein (achieved by addition of aluminum fluoride [AlF]), and IFN-$\gamma$ stimulation of the respective cells.[15]

At present, it is not known if HuGBP family members have redundant or different cell biologic functions. Recent studies described a similar expression pattern of MuGBP-1 through MuGBP-5 in different tissues *in vivo* and in response to stimulation with different cytokines *in vitro*.[16] In addition, some inbred mouse strains lack a functional MuGBP-1 but nevertheless appear healthy.[17,18] These results suggest that the expression of GBPs may be regulated coordinatively and that the different proteins may exert redundant activities. It has been concluded that this might hinder elucidation of the biologic functions of individual GBP family members.[16]

In the present study, we compared the expression and subcellular localization of HuGBP-1 through HuGBP-5, which are expressed in human cytokine-stimulated ECs. Surprisingly, we found that each of these HuGBPs has its own characteristic pattern of expression and localization. Identification of unique features of the different HuGBPs may provide new perspectives to elucidate cell biologic functions and phylogenetic relations of these proteins. This will improve our understanding of the complex response of eukaryotic cells to IFNs and inflammatory cytokines.

## MATERIALS AND METHODS

### *Cell culture*

Primary human umbilical vein endothelial cells of a single donor ($HUVEC_{single\ donor}$), a pool of donors ($HUVEC_{pool}$), and microvascular endothelial cells (MVEC) of a single donor were purchased from Cambrex (Verviers, Belgium). Cells were cultivated in EC basal medium (EGM-2MV) supplemented with 5% (v/v) fetal bovine serum (FBS) (Cambrex) at 37°C in a humidified atmosphere at 5% $CO_2$ and were routinely split at a ratio of 1:4 (one passage). Cell culture flasks (Nunc, Wiesbaden, Germany) were coated with 1.5% (w/v) bovine skin gelatin, type B (Sigma-Aldrich, Munich, Germany) diluted in phosphate-buffered saline (PBS) (Biochrom, Berlin, Germany). All cells used were mycoplasma free, as monitored by an ELISA detection kit (Roche, Mannheim, Germany). All experiments with HUVEC were carried out using two different cultures ($HUVEC_{pool}$ and $HUVEC_{single\ donor}$) and yielded identical results in all cases. Experiments were carried out between passages four and eight.

### *Stimulation of cells*

Recombinant human vascular endothelial growth factor ($VEGF_{121}$) was purchased from R&D Systems (Minneapolis, MN), human basic fibroblast growth factor (bFGF), IFN-$\gamma$, IL-1$\beta$, TNF-$\alpha$, and IFN-$\alpha$ were obtained from Roche. Prior to stimulation, cells were starved in EBM-2 basal medium supplemented with 0.5% FBS for 12 h. Subsequently, cytokines and growth factors (100 U/mL IFN-$\gamma$ or IFN-$\alpha$, 300 U/mL TNF-$\alpha$, 200 U/ml IL-1$\beta$, 10 ng/mL VEGF, and 10 ng/mL bFGF) were added in concentrations that were shown previously to induce maximal expression of HuGBP-1 (IFN-$\gamma$, IFN-$\alpha$, TNF-$\alpha$, IL-1$\beta$) or maximal EC proliferation (VEGF, bFGF).[6] All cytokines and growth factors were diluted in PBS containing 0.1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich). PBS/0.1% BSA was used as a control in all experiments.

### *Isolation of RNA*

Cells were harvested using trypsin/EDTA (0.05%/0.02%) in PBS (PAA-Laboratories, Pasching, Austria) 5 h after stimulation and washed twice in PBS. Total RNA was extracted using Trizol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. RNA concentration was determined photometrically (GeneQuant, Amersham Biosciences, Freiburg, Germany) at $\lambda = 260$ nm, and RNA integrity was controlled by nondenaturing agarose gel electrophoresis.

### *RT-PCR*

Reverse transcription was carried out in a total reaction volume of 20 $\mu$L with 1 $\mu$g total RNA, 200 U Superscript III reverse transcriptase (Invitrogen), and 0.1 $\mu$mol of an oligo-$dT_{(18)}$ primer (MWG, Ebersberg, Germany). PCR was carried out in a total reaction volume of 25 $\mu$L with 1 $\mu$L of either undiluted or diluted (1:10, 1:100, 1:1,000) cDNA, Platinum Taq DNA polymerase (0.625 U) (Invitrogen), 1.5 mM $MgCl_2$, 0.8 mM dNTPs, and 12.5 pM oligonucleotide primers for specific amplification of full-length HuGBP-1: (forward, 5′-CCCAAATACGTA**ATGGCATCAGAGATCCACAT**-3′, reverse, 5′-TTTACGCGTCGAC**TTAGCTTATGGTACATGCCTTT**-3′; complementary sequence in bold; restriction sites for SnaB1 (5′-TACGTA-3′) and Sal1 (5′-GTCGAC-3′) are underlined); HuGBP-2: (forward, 5′-CCCAAATACGTA**ATGGCTCCAGAGATCAACTT**-3′, reverse, 5′-TTTACGCGTCGAC**TTA-GAGTATGTTACATA-TTGGCTCC**-3′); HuGBP-3 (forward, 5′-CCCAAATACGTA**ATGGCTCCAGAGATCCACAT**-3′, reverse, 5′-TTTACGC-GTCGAC**TTAGATCTTTAGCTTATGCGACATATATC**-3′); HuGBP-4: (forward, 5′-CCCAAATACGTA**ATGGGTGAGA-**

**GAACTCTTCA**-3′, reverse, 5′-TTTACGCGTCGAC**TTAAATACGTGAGCCAAGATATTTT**-3′); HuGBP-5: (forward, 5′-CCCAAATACGTA-**ATGGCTTTAGAGATCCACAT**-3′, reverse,5′-TTTACGC-GTCGAC**TTAGAGTAAAACACATGGATCATC**-3′). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) encoding transcripts (forward, 5′-**AGCCACATCGCTCAGAACAC**-3′, reverse, 5′-**GAGGCATTGCTGATGATCTTG**-3′) were amplified as a control. Cycle parameters for all HuGBPs were as follows: initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 30 sec, 51°C for 30 sec, 72°C for 2 min, and a final extension for 7 min.

Amplification products were analyzed by agarose gel electrophoresis (1%) (Peqlab, Erlangen, Germany) and ethidium bromide (Roth, Karlsruhe, Germany) staining. Signal intensities of stained DNA fragments were quantified with the Gel-Doc system (Bio-Rad, Hercules, CA) and the AIDA software package (Raytest, Straubenhardt, Germany). Intergel variations were normalized according to the 2-kbp (92 ng) fragment of a molecular weight standard (Gene ruler, 1 kbp-DNA ladder) (Fermentas GmbH, Leon-Rot, Germany), and loading variations were adjusted by normalization to the GAPDH-specific amplification signal.

### *Expression of green fluorescent protein (GFP)-GBP and myc-GBP fusion proteins*

Transcripts encoding HuGBP-1, HuGBP-2, HuGBP-4, and HuGBP-5 (NCBI accession numbers gi: 60101452, gi: 48146240, gi: 15558942, and gi: 31377630, respectively) were amplified by RT-PCR from total RNA extracted from IFN-γ-stimulated HUVEC using the oligonucleotide primers described. HGBP-3 cDNA amplified from ECs contained an additional 96-bp in-frame insert compared with the hitherto described HuGBP-3 (gi: 24308156) sequence. This EC-associ-

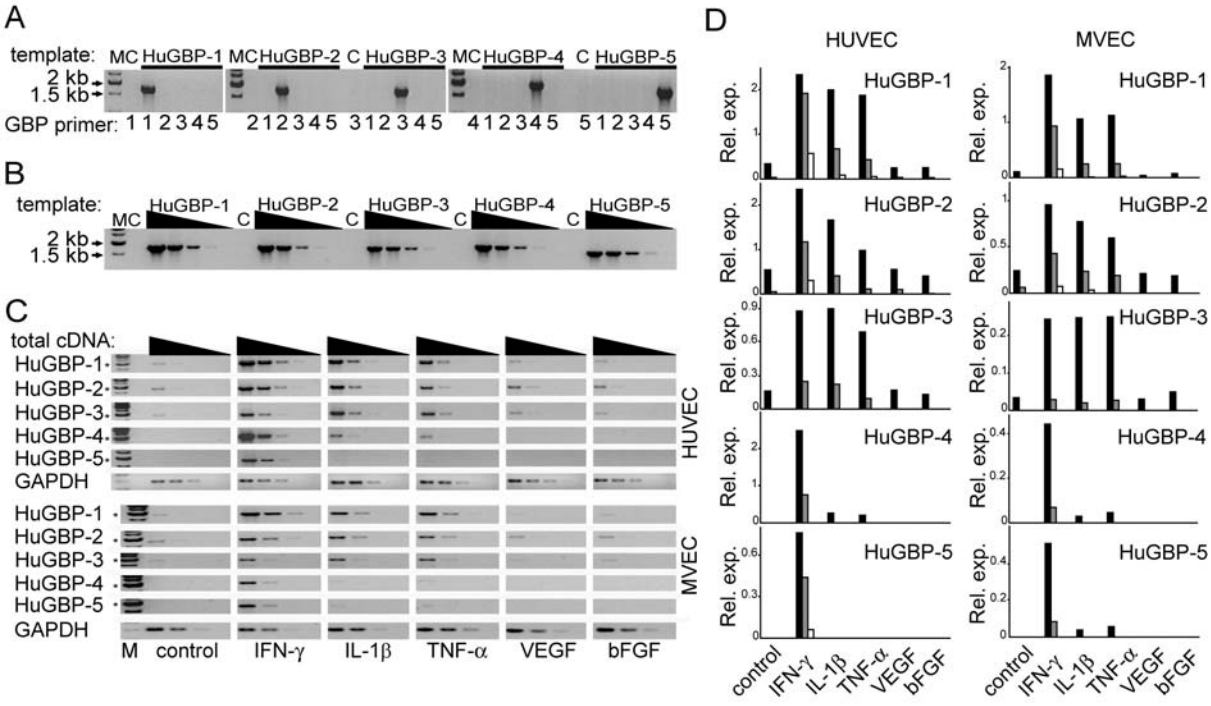


**FIG. 1.** Differential expression of HuGBP-1 through HuGBP-5 in ECs. (**A**) Primer specificity. Full-length cDNA encoding the different HuGBPs (0.1 ng, template) was added to herring sperm DNA (200 ng) and subjected to PCR using oligonucleotide primers specific for HuGBP-1, HuGBP-2, HuGBP-3, HuGBP-4 and HuGBP-5 (GBPx primer), respectively. Agarose gel electrophoresis (1%) followed by ethidium bromide staining showed a PCR product (HuGBP-1, 1779 bp; HuGBP-2, 1776 bp; HuGBP-3, 1788 bp; HuGBP-4, 1923 bp; HuGBP-5, 1761 bp) exclusively in those reactions where the respective primers matched the cDNA template. M, molecular weight standard; C, PCR reactions where template cDNA was omitted (control). (**B**) Sensitivity of HuGBP-specific PCR. Decreasing amounts of the different HuGBP cDNA templates (10 ng, 1 ng, 100 pg, 10 pg, and 1 pg) were amplified with the respective primers. In all cases, the detection limit after 30 cycles was 10 pg cDNA. (**C**) RT-PCR analysis of HuGBP expression in HUVEC (top) and MVEC (bottom). Cells were either untreated (control) or stimulated for 5 h with IFN-γ (100 U/mL), IL-1β (200 U/mL), TNF-α (300 U/mL), VEGF (10 ng/mL), or bFGF (10 ng/mL). After reverse transcription of isolated total mRNA, decreasing amounts of cDNA (undiluted, 1:10, 1:100, 1:1,000) were subjected to PCR for detection of transcripts encoding the different HuGBPs or GAPDH, respectively. (**D**) Densitometric evaluation of the amounts of intensities of RT-PCR fragments depicted in **C**. Relative expression levels were calculated by normalization of distinct signals to the one obtained from the 2-kbp (92 ng) fragment of the DNA molecular weight standard (**C,** asterisk) and the corresponding GAPDH signal. Corrected band intensities for the reactions with undiluted (black), 1:10 diluted (gray), and 1:100 diluted (white) cDNA templates are shown.

ated variant of HuGBP-3 has been deposited in the databases previously (gi: 60219669). In order to minimize mutations, Taq polymerase (Invitrogen) and Pfu polymerase (Stratagene, La Jolla, CA) were used in a mixture of 10:1, and cycle numbers were reduced to 25. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), digested with SnaB1 and Sal1 restriction endonucleases, inserted between SnaB1 and Sal1 sites within the multiple cloning site of the Moloney murine leukemia virus (MMLV)-derived retroviral vector pBabePuro.[19] Subsequently, a cDNA coding for the 11 amino acid myc-tag (MEQKLISEEDL) or a GFP-encoding cDNA (accession number, gi: 1543070) was inserted into the BamH1 and SnaB1 site or the SnaB1 site, respectively, in frame with the different HuGBP cDNAs. Each inserted sequence was verified by full-length DNA sequence analysis of both strands.

### *Transduction of HUVEC*

For generation of recombinant retroviral vector viruses encoding GFP-GBP fusion proteins, viral particles were generated in HEK 293T packaging cells as described,[19,20] with minor modifications. HEK 293T cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (PAA Laboratories). Plasmids encoding VSV glycoprotein (VSV-G),[21] murine leukemia virus (MLV) gag-pol genes,[22] and the pBabePuro-GFP-GBP constructs were cotransfected into the cells using calcium phosphate coprecipitation.[23] The culture medium was replaced 24 h after transfection, and virus-containing medium was collected after an additional 24 h. Cellular debris was removed by 0.45 $\mu$m filtration (Whatman, Brentford, U.K.). Viral particles were harvested by centrifugation (50,000*g* at 4°C for 2 h) and resuspended in 1:100 volume of EGM-2MV medium.

For infection, HUVECs were cultivated at 50% confluence in 6-well plates in 900 $\mu$L EGM-2MV/5% FBS. Subsequently, 100 $\mu$L of the virus-containing solution and polybrene (Sigma-Aldrich, final concentration 8 $\mu$g/mL) were added for 24 h. Forty-eight hours after infection, 0.3 $\mu$g/mL puromycin (Sigma-Aldrich) was added, and transduced cells were selected for 10 days, with medium removal every second day.

### *Western blot analysis*

For preparation of protein extracts, the cells were harvested by trypsinization, washed twice in PBS, and lysed in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, all obtained from Sigma-Aldrich, and complete protease inhibitor tablets from Roche). The protein concentration within cell extracts was determined using the DC assay (Bio-Rad). Total proteins (20 $\mu$g) were separated by electrophoresis in 10% SDS-PAGE and analyzed by Western blotting as described previously.[5] A monoclonal mouse anti-GFP antibody (Roche), diluted 1:1000 in 0.5% Western Blocking Solution (Roche) and 0.1% Tween 20 (Sigma-Aldrich), was used as a primary antibody. A sheep antimouse IgG antibody coupled to horseradish peroxidase (HRP) (1:5,000) (Amersham Biosciences) was used as the secondary antibody. Protein detection was performed using the enhanced chemiluminescence Western blotting detection system (ECL, Amersham) and Rx-films (Fuji, Tokyo, Japan).

### *Immunofluorescence analysis*

HUVECs were seeded in gelatin-coated Lab-Tek chamberslides (Nunc). Cells were left either untreated or stimulated for 24 h by the addition of IFN-$\gamma$, IFN-$\alpha$, IL-1$\beta$, or TNF-$\alpha$ in EBM-2/0.5% FBS at the respective concentrations without a preceding starvation period. AlF was freshly prepared by mixing 400 $\mu$L of 1 M sodium fluoride (NaF, Sigma-Aldrich) and 20 $\mu$L of 50 mM ($AlCl_3$, Sigma-Aldrich). After incubation for 5 min at ambient temperature, 42 $\mu$L AlF solution was added to 1 mL of cell culture medium. Forty minutes later, the cells were washed twice at 4°C with PBS and fixed in 3.7% buffered formalin (Sigma-Aldrich) or EtOH at 4°C, and GFP fluorescence was analyzed with a confocal laser scanning microscope (Leica, Bensheim, Germany/Zeiss, Oberkochen, Germany). Z-stacks were captured in steps of 0.4 $\mu$m.

For immunocytochemical detection of HuGBP-1, myc-HuGBPs, or the Golgi matrix protein GM130, cells were permeabilized after fixation in 0.1% saponin (Sigma-Aldrich), diluted in Tris-buffered saline (TBS) at ambient temperature for 30 min, incubated with a rat antihuman GBP-1 antibody (clone 1B1, diluted 1:100),[5] a mouse anti-myc-tag antibody (clone 9B11, diluted 1:1,500) (Cell Signaling, Danvers, MI), or a mouse anti-GM130 antibody (clone 35, diluted 1:1000) (BD Biosciences, San Jose, CA) overnight at 4°C. After washing, incubation continued with an AlexaFluor 488-conjugated goat anti-rat IgG, an AlexaFluor 488-conjugated goat antimouse IgG, or an AlexaFluor 546-conjugated goat antimouse IgG secondary antibody, respectively (diluted 1:500) (Invitrogen/Molecular Probes) for 1 h at ambient temperature.

### *Time-lapse analysis*

Stably transduced HUVECs expressing GFP-HuGBP-1 were seeded in gelatin-coated Lab-Tek chambered coverglasses. Cells were stimulated for 24 h with IFN-$\gamma$ (100 U/mL), and GFP-HuGBP was localized in the absence or presence of AlF for 40 min using a live cell observer microscope (Zeiss). Pictures were captured every 30 sec.

### *Phylogenetic tree analysis*

The phylogenetic tree was generated with the ClustalW[24] alignment of the European Bioinformatics Institute (*www.ebi.ac.uk/clustalw*) and TreeView, V. 1.6.6 (*taxonomy.zoology.gla.ac.uk/rod/rod.html*). The alignment was based on the full-length amino acid sequences of HuGBP-1 through HuGBP-5. The tree was generated without gap correction. Differences in protein length were scored as gap penalties.

## RESULTS

### *Differential expression of HuGBPs in ECs*

To investigate the expression of HuGBPs in ECs, distinct oligonucleotide primers were designed for the specific amplification of the mRNA encoding HuGBP-1 through HuGBP-5 by RT-PCR. With these primer pairs, the full-length cDNA of each of the five HuGBPs was amplified from IFN-$\gamma$-stimulated (100 U/mL for 5 h) HUVECs, cloned, and confirmed by se-

quence analysis (data not shown). Because of the high degree of sequence identity within the HuGBPs, each primer pair was tested for cross-reactivity (Fig. 1A). Toward this goal, the isolated cDNA templates encoding the different HuGBPs were added to the different PCR reactions. Each primer pair reacted specifically with its respective HuGBP-encoding template and did not cross-react with others (Fig. 1A). Subsequently, the respective primer pairs were tested for equal detection sensitivities. Decreasing amounts (10 ng, 1 ng, 100 pg, 10 pg, 1 pg) of the isolated HuGBP cDNA templates were added to different PCR reactions (Fig. 1B). With each primer pair, 10 pg of cDNA template represented the minimal detectable amount (Fig. 1B). This result demonstrated that the different primer pairs exhibited identical detection sensitivities.

After evaluation of specificity and sensitivity of the established RT-PCR approach, the expression of the different HuGBPs was investigated in primary human macrovascular (HUVEC) and microvascular (MVEC) ECs. Cells were either untreated (control) or treated with IFN-$\gamma$ (100 U/mL), IL-1$\beta$ (200 U/mL), TNF-$\alpha$ (300 U/mL), VEGF (10 ng/mL), or bFGF (10 ng/mL) for 5 h. As demonstrated in Figure 1C, in comparison to untreated cells, the expression of HuGBP-1 through HuGBP-5 was strongly induced by the addition of IFN-$\gamma$, both in HUVEC and MVEC (Fig. 1C, IFN-$\gamma$). IL-1$\beta$ and TNF-$\alpha$ induced the expression of HuGBP-1, HuGBP-2, and HuGBP-3 (Fig. 1C). In contrast, the expression levels of HuGBP-4 and HuGBP-5 in both EC types were not affected or only slightly induced by the addition of IL-1$\beta$ and TNF-$\alpha$ (Fig. 1C). The EC growth factors VEGF and bFGF did not significantly induce expression of any of the HuGBPs (Fig. 1C). GAPDH-encoding mRNA was amplified as a control, and the results obtained demonstrated that equal amounts of RNA were subjected to the different reactions (Fig. 1C).

A quantitative evaluation of the RT-PCR products by comparison of band intensities after normalization to GAPDH-specific signals confirmed that the expression of HuGBP-1, HuGBP-2, and HuGBP-3 in ECs was robustly induced by all cytokines used, whereas the expression of HuGBP-4 and HuGBP-5 was prominently induced only in the presence of IFN-$\gamma$ in both HUVEC and MVEC (Fig. 1D).

### *Differential localization of HuGBPs in ECs*

After analysis of expression patterns, the intracellular localization of the five HuGBPs was investigated by immunofluorescence studies and expression of GFP-HuGBP fusion proteins in ECs. Immunofluorescence analyses showed that in IFN-$\gamma$-treated ECs, endogenous HuGBP-1 showed a granular, cytoplasmic distribution (Fig. 2A, top row, IFN-$\gamma$). The protein translocated to the Golgi compartment when the cells were simultaneously treated with IFN-$\gamma$ and AlF (Fig. 2A, top row, arrow; see also Fig. 4). Both observations are in agreement with previous reports.[5,15] In addition, by means of retroviral transduction, a fusion protein of GFP (N-terminal) and HuGBP-1 (C-terminal) was constitutively expressed in HUVEC. In cells treated with IFN-$\gamma$ and AlF, this recombinant fluorescent fusion protein showed an identical translocation reaction to that

**FIG. 2.** AlF-induced Golgi translocation of HuGBP-1. (**A**) Immunofluorescence detection of endogenous HuGBP-1 (top) in HUVEC, using a monoclonal, HuGBP-1-specific antibody[5] compared with fluorescence analysis of a constitutively expressed GFP-HuGBP-1 fusion protein (middle) and GFP (bottom) in stably transduced HUVECs. Cells were either left untreated (control) or treated with AlF (40 min, 100 $\mu$M), IFN-$\gamma$ (24 h, 100 U/mL), or IFN-$\gamma$ (24 h, 100 U/mL) and AlF (40 min, 100 $\mu$M). Perinuclear, Golgi apparatus-associated localization of HuGBP-1 and GFP-HuGBP-1 is indicated by arrows. (**B**) Time course of AlF-induced HuGBP-1 translocation. GFP-HuGBP-1-expressing HUVECs were stimulated for 24 h with IFN-$\gamma$ (100 U/mL) alone (−AlF) or combined with AlF (40 min, 100 $\mu$M) (+AlF). After the addition of AlF, fluorescence microscopic pictures of the cells were captured every 30 sec for 40 min using a live cell observer (Zeiss). Pictures taken with a 5-min interval within the first 20 min are shown. Perinuclear localization of GFP-HuGBP-1 is indicated by arrows. (**C**) HUVEC transduced with a GFP-HuGBP-1-encoding retroviral vector were left either untreated (control) or stimulated for 24 h with IL-1$\beta$ (200 U/mL), TNF-$\alpha$ (300 U/mL), IFN-$\alpha$ (100 U/mL), or IFN-$\gamma$ (100 U/mL) and incubated for 40 min in the presence (+AlF) or absence (−AlF) of 100 $\mu$M AlF. GFP-HuGBP-1 was visualized by fluorescence microscopy. Perinuclear localization of GFP-HuGBP-1 is indicated by an arrow. Fluorescence observations were carried out at an excitation wavelength of 488 nm and an emission wavelength of 505–530 nm. Scale bars in **A, B,** and **C** represent 10 $\mu$m.

**FIG. 3.** Differential localization of HuGBPs in HUVEC. HUVEC, which expressed HuGBP-1 through HuGBP-5 (**A**) fused with GFP or (**B**) with a myc-tag were either left untreated (control) or stimulated for 24 h with IFN-$\gamma$ (100 U/mL) and subsequently were incubated for 40 min in the presence (IFN-$\gamma$ + AlF) or absence of 100 $\mu$M AlF (IFN-$\gamma$). GFP-HuGBP fusion proteins were visualized directly by fluorescence microscopy and myc-HuGBP-tagged proteins after immunofluorescence staining with a monoclonal anti-myc antibody, followed by detection using an AlexaFluor 488-conjugated goat anti-mouse IgG (excitation 488 nm, emission 505–530 nm). Nuclear localization of HuGBP-2 and HuGBP-4 fusion proteins is indicated by asterisks. AlF-induced Golgi-associated HuGBP-1, and HuGBP-2 fusion proteins are indicated by arrows. Constitutively Golgi-associated HuGBP-5 is indicated by arrowheads. Scale bar represents 10 $\mu$m.

**FIG. 4.** Golgi and nuclear localization of HuGBP-1 through HuGBP-5. (**A**) HUVECs expressing GFP-HuGBP-1 and HuGFP-2 fusion proteins were stimulated for 24 h with IFN-$\gamma$ (100 U/mL) and subsequently incubated for 40 min in the presence of 100 $\mu$M AlF. HUVECs expressing GFP-HuGBP-5 were left untreated. GFP-HuGBP fusion proteins were visualized directly by fluorescence microscopy (excitation 488 nm, emission 505–530 nm) and the Golgi matrix protein GM130 by immunofluorescence staining with a specific antibody, followed by detection with an AlexaFluor 546-conjugated goat antimouse IgG (excitation 543 nm, emission 560–615 nm). Colocalization signals in merged pictures are indicated by arrows. (**B**) HUVECs stably expressing GFP-HuGBP-1, HuGBP-2, and HuGBP-4 fusion proteins were stimulated with IFN-$\gamma$ and AlF as above or left untreated (Control). Z-stack images were captured every 0.4 $\mu$m at the indicated positions using confocal laser scanning microscopy. Scale bars in **A** and **B** represent 10 $\mu$m.

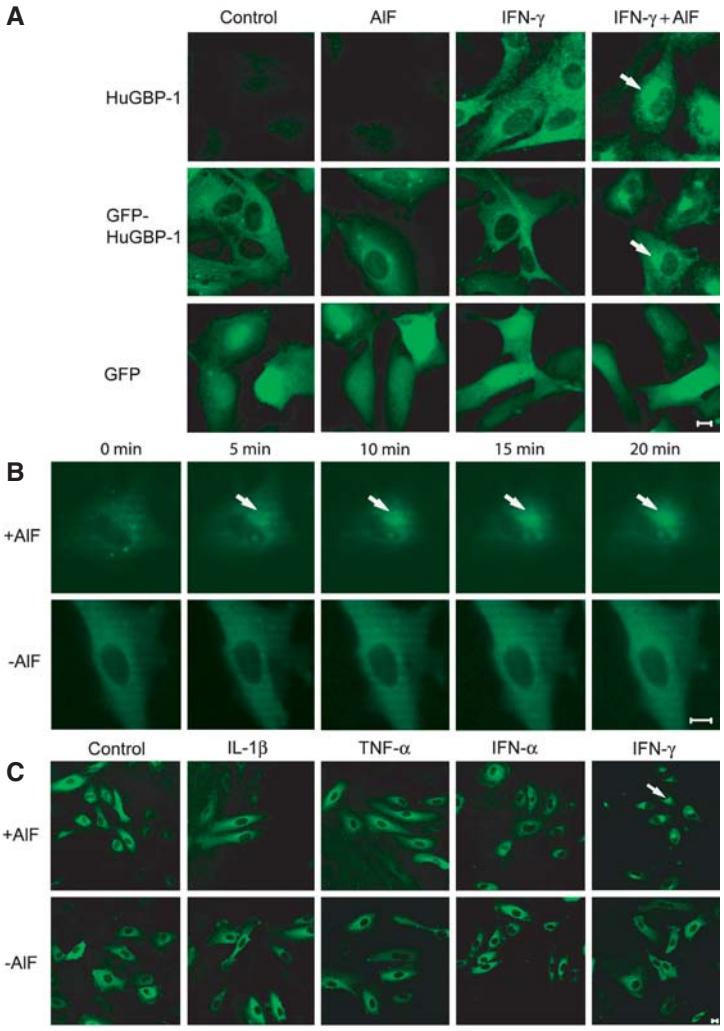


FIG. 2.

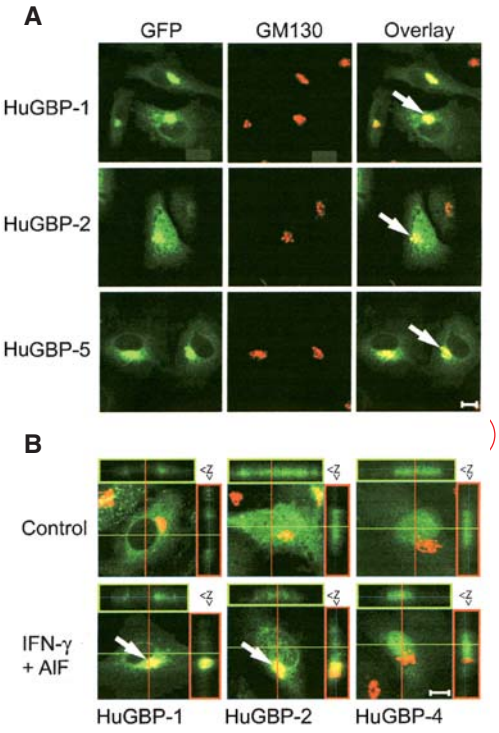


FIG. 4.

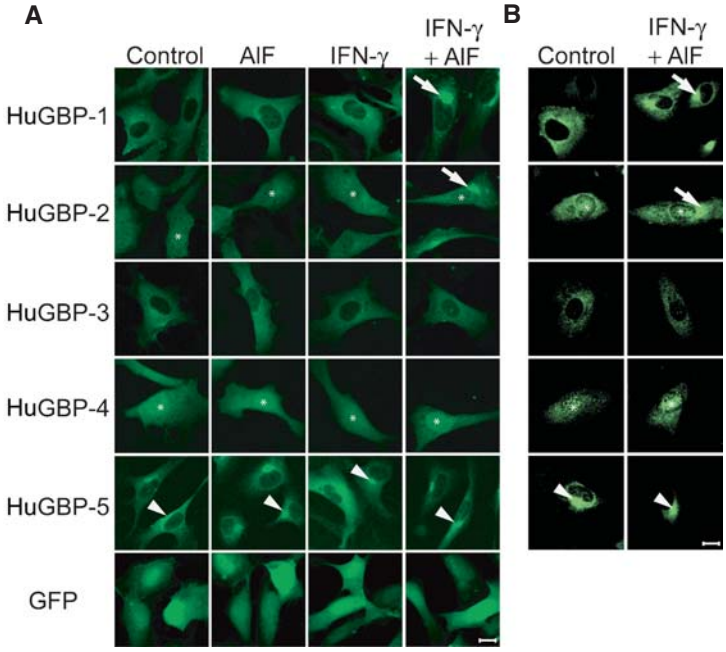


FIG. 3.

observed for endogenous HuGBP-1 (Fig. 2A, middle row, arrow). In contrast, retrovirally expressed GFP was uniformly distributed in the cytoplasm and the nucleus of the cells, irrespective of IFN-$\gamma$ and/or AlF treatment (Fig. 2A, bottom). Time-lapse studies of transduced HUVEC using a live cell imaging fluorescence microscope showed that GFP-HuGBP-1 was enriched in the Golgi apparatus of IFN-$\gamma$-treated cells at 5 min after addition of AlF (Fig. 2B, top row, arrows).

AlF-mediated translocation of GFP-HuGBP-1 into the Golgi apparatus was selectively supported by stimulation of ECs with IFN-$\gamma$ but not with other cytokines, such as IL-1$\beta$, TNF-$\alpha$, and IFN-$\alpha$ (Fig. 2C, top row). All cytokines were used in concentrations that have been shown to induce HuGBP-1 expression[6] (Fig. 1). The same results were obtained when 10-fold higher concentrations were used (data not shown).

In a next step, fusion proteins of GFP and HuGBP-1 through HuGBP-5 were expressed by means of retroviral transduction in HUVEC. Western blot analysis using an anti-GFP antibody showed a single band with the expected molecular weight of each fusion protein, respectively (GFP-HuGBP-1, 97 kDa; GFP-HuGBP-2, 97 kDa; GFP-HuGBP-3, 97 kDa, GFP-HuGBP-4, 102 kDa; GFP-HuGBP-5, 96 kDa) (data not shown). This confirmed that the expected fusion proteins were expressed exclusively and not truncated GFP-HuGBP fusion fragments, which could exhibit different subcellular localization patterns.

The transduced cells were subjected to cytokine treatment, and intracellular localization of GFP-HuGBP fusion proteins was investigated by fluorescence microscopy. Interestingly, distinct members of the HuGBP family showed a different subcellular localization in nonstimulated cells and reacted differentially in response to a combined IFN-$\gamma$ and AlF treatment (Fig. 3A). In nonstimulated cells GFP-HuGBP-1, GFP-HuGBP-3, and GFP-HuGBP-5 localized strictly in the cytoplasm, whereas GFP-HuGBP-2 and GFP-HuGBP-4 were present, in both the nucleus (Fig. 3A, asterisk) and the cytoplasm (Fig. 3A, control). Combined AlF and IFN-$\gamma$ treatment induced a Golgi translocation of GFP-HuGBP-1 and GFP-HuGBP-2 (Fig. 3A, arrows) but did not affect the subcellular localization of GFP-HuGBP-3, GFP-HuGBP-4, and GFP-HuGBP-5 (Fig. 3A). Of note, GFP-HuGBP-5 was constitutively associated with the Golgi apparatus, irrespective of endothelial cell treatment (Fig. 3A, arrowheads). Treatment with either AlF or IFN-$\gamma$ alone did not affect the localization of any of the constitutively expressed GFP-HuGBPs (Fig. 3A, AlF, IFN-$\gamma$). The respective myc-tagged HuGBPs were expressed as a control. Immunofluorescence analysis showed that each of these proteins had an identical intracellular localization as the respective GFP-tagged protein (Fig. 3B). This demonstrated that the GFP fusion did not interfere with cellular distribution and translocation of HuGBPs. Colocalization with the Golgi marker GM130[25] confirmed the Golgi localization of HuGBP-1, HuGBP-2, and HuGBP-5 (Fig. 4A, arrows). In addition, cytoplasmic localization of HuGBP-1 and nucleocytoplasmic localization of HuGBP-2 and HuGBP-4 were clearly confirmed by confocal sectioning of the cells in the Z-axis (Fig. 4B).

## DISCUSSION

In the present study we could show that HuGBP-1 through HuGBP-5 are characterized by a specific pattern of expression and subcellular localization in response to various cytokines (summarized in Fig. 5). ECs were employed in this study because HuGBP-1, the best characterized member of the HuGBP family, is closely associated with blood vessel ECs *in vivo*.[5] In addition, HuGBP-1 is biologically active in these cells, mediating the inhibition of proliferation and invasion in response to inflammatory cytokines.[6,9]

Expression of all five HuGBPs in ECs was robustly induced by treatment with IFN-$\gamma$. Moreover, the expression of HuGBP-1, HuGBP-2, and HuGBP-3, but not of HuGBP-4 and HuGBP-5, was significantly induced by IL-1$\beta$ and TNF-$\alpha$. Detection of mRNA may not necessarily reflect the presence of the respective proteins. However, transcriptional and translational HuGBP-1 levels were shown to be closely related in ECs.[6] High homology suggests that this may be also the case for the other HuGBPs. The expression patterns detected classify the EC-expressed HuGBPs into those that are strongly induced only by IFN-$\gamma$ and those that are induced to similar high levels also by the two other inflammatory cytokines, IL-1$\beta$ and TNF-$\alpha$ (IC-HuGBPs). Interestingly, IC-HuGBPs are more closely related to each other than to the other HuGBPs (Fig. 5) or MuGBPs, respectively[2,8] (data not shown). The expression of HuGBP-1 through HuGBP-5 in humans and mice is robustly induced by IFN-$\gamma$,[5,16] suggesting that this may be the ancestral response compared with the susceptibility to IL-1$\beta$ and TNF-$\alpha$ stimulation. The latter may be a more recently acquired capability of a common ancestor of the closely related IC-HuGBPs.

Before our study, intracellular localization of the GBPs was only investigated for MuGBP-1 and MuGBP-2,[14] and HuGBP-1.[5,15] MuGBP-1 was found to be homogeneously distributed in the cytoplasm, whereas MuGBP-2 shows a granular distribution and is localized in vesicles.[14] HuGBP-1 was found with a granular distribution in the cytoplasm[5] and translocated to the

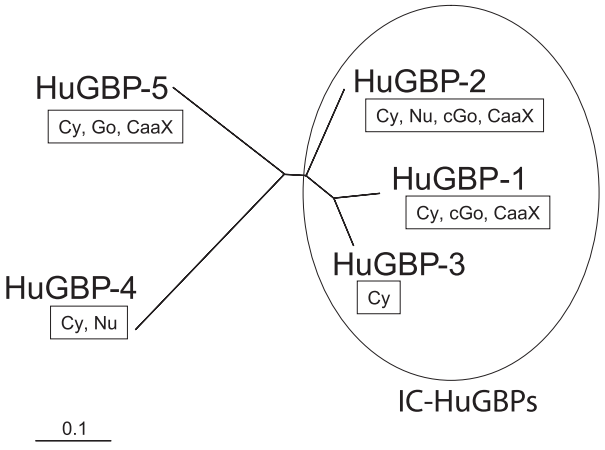


**FIG. 5.** Differential features and phylogenetic relations of HuGBPs in ECs. Each HuGBP is characterized by a specific signature with respect to expression in response to cytokines and subcellular localization (boxes; Cy, cytoplasmic; nu, nuclear, Go, present in the Golgi; cGO, conditionally present in the Golgi; CaaX, presence of an isoprenylation signal at the C-terminus). Those HuGBPs whose expression is induced by all inflammatory cytokines (IC), such as IFN-$\gamma$, TNF-$\alpha$, and IL-1$\beta$ are more closely related to each other (IC-HuGBPs) compared with HuGBP-4 and HuGBP-5. Phylogenetic distance is indicated by the bar and given in substitutions/site.

Golgi apparatus in the presence of AlF and IFN-$\gamma$.[15] Vesicle localization of MuGBP-2 and translocation of HuGBP-1 to the Golgi apparatus were both dependent on isoprenylation.[14,15] However, within the HuGBP family, only HuGBP-1, HuGBP-2, and HuGBP-5 contain a CaaX isoprenylation motif.[2] This indicated that different members of the HuGBP family may be localized in different cellular compartments and directed us to investigate the subcellular localization of HuGBP-1 through HuGBP-5 in more detail. In agreement with previously published results,[15] we found that IFN-$\gamma$ and AlF treatment induced translocation of HuGBP-1 into the Golgi apparatus also in ECs. A GFP tag fused to the N-terminal end of HuGBP-1 did not affect the translocation process and allowed the analysis of translocation kinetics in living cells. Time-lapse analysis showed that the translocation process is fast and is complete 5 min after the addition of AlF. In addition, we show here that Golgi translocation is strictly IFN-$\gamma$ dependent and does not occur after stimulation with IL-1$\beta$ and TNF-$\alpha$, which are also potent inducers of HuGBP-1 expression. Our previous work showed that two major functions of HuGBP-1 in ECs, namely, the inhibition of proliferation and matrix metalloproteinase-1 (MMP-1) expression, are independent of IFN-$\gamma$ stimulation.[6,9] This indicated that Golgi translocation is not required for these functions but may regulate additional functions of HuGBP-1, which have to be elucidated in future studies.

GFP-GBP fusion proteins were used to investigate the subcellular localization of the four other EC-expressed HuGBPs. The respective cDNA molecules were stably introduced into the cells by means of retroviral transduction in order to avoid EC activation commonly observed during transfection (unpublished observation). Analysis of HuGBP localization in nonstimulated cells and in cells treated with IFN-$\gamma$ or AlF or both showed that a specific localization pattern can be assigned to each of the HuGBPs (Fig. 5). HuGBP-1 is present in the cytoplasm and within this compartment can conditionally translocate into the Golgi apparatus. HuGBP-2 is present in the cytoplasm and the nucleus and in the presence of IFN-$\gamma$ and AlF can also translocate into the Golgi apparatus. HuGBP-3 and HuGBP-4 were never detected in the Golgi apparatus, irrespective of stimulation. However, HuGBP-3 was exclusively localized in the cytoplasm, and HuGBP-4 was also detected in the nucleus. HuGBP-5 was cytoplasmic, with constantly increased concentrations in the Golgi apparatus. In agreement with the starting hypothesis, only those HuGBPs that encoded a CaaX motif had the capability of Golgi localization. This clearly supported the observation of Modiano et al.,[15] who showed that CaaX is necessary for Golgi translocation of HuGBP-1.

In the present study, we detected, for the first time, differential features that are unique and specific for HuGBP-1 through HuGBP-5. This clearly suggests that the different HuGBPs may exert different and possibly cooperative biologic activities in eukaryotic cells. Our findings provide a valuable basis for further characterization of the biologic functions of HuGBPs.

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M.S. initiated the study, designed the experiments, and together with M.B. supervised the experiments. P.T. performed the experiments shown in Figs. 1, 2, 3, and 4. C.H., T.M., E.C., M.T., and E.N. provided reagents and technical help. M.S. and P.T. analyzed the results and prepared the manuscript.

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