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Guanylate binding protein-1 inhibits spreading and migration of endothelial cells through induction of integrin α_4 expression

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ABSTRACT **Human guanylate binding protein-1 (GBP-1) is a large GTPase that is induced by inflammatory cytokines and acts antiangiogenically through the inhibition of endothelial cell proliferation and migration. In this study, we detected that GBP-1-expressing cells show a significantly reduced spreading and migration on fibronectin matrices. Investigating possible mechanisms of these effects, we found that integrin α_4 (ITGA4) was consistently up-regulated at both the RNA and protein level in GBP-1-expressing cell cultures. Inhibition of cell spreading and migration by GBP-1 was dependent on the binding of ITGA4 to fibronectin. The inflammatory cytokines IL-1β and TNF-α induced ITGA4 expression in HUVECs and inhibited spreading and migration. Knockdown of GBP-1 by shRNA abrogated inflammatory cytokine induced ITGA4 expression and restored spreading and migration capabilities of the cells. These results show that inhibition of endothelial cell spreading and migration by inflammatory cytokines is mediated by GBP-1 through induction of ITGA4 expression. Endothelial cell migration is a key process during angiogenesis. Therefore, ITGA4 may be a novel molecular target to modulate angiogenesis in human disease.—Weinländer, K., Naschberger, E., Lehmann, M. H., Tripal, P., Paster, W., Stockinger, H., Hohenadl, C., Stürzl, M. Guanylate binding protein-1 inhibits spreading and migration of endothelial cells through induction of integrin α_4 expression. *FASEB J.* 22, 000–000 (2008)**

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THE FORMATION OF NEW BLOOD vessels is termed angiogenesis. It is associated with proliferation, migration, and invasion of endothelial cells (ECs). Angiogenesis is important in embryonal development, tissue repair, and pathological processes such as tumor growth and metastasis (1–3). Angiogenesis depends on molecular interactions between ECs and components of the surrounding extracellular matrix (ECM) and is tightly controlled by a balance between proangiogenic and antiangiogenic molecules. Interactions of ECs with ECM are regulated predominantly by integrins. Integrins comprise two noncovalently linked subunits termed α and β that form heterodimeric transmembrane proteins and bind to ECM proteins (4–7). The major proangiogenic molecules are vascular endothelial growth factor (VEGF) (8, 9) and basic fibroblast growth factor (bFGF) (10). Factors exerting antiangiogenic activities are thrombospondin (11), endostatin (12), angiostatin (13), and inflammatory cytokines (ICs), such as interferon (IFN) -γ, interleukin (IL) -1β, and tumor necrosis factor (TNF) -α. ICs inhibit EC proliferation and invasiveness (14–19). We have previously shown that the antiangiogenic effects of ICs are mediated by guanylate binding protein (GBP) -1 (17, 18, 20).

GBPs were originally identified as the most abundant proteins induced by IFN-γ (21). Seven different GBPs with a relative molecular mass of 65–71 kDa have been detected in humans, and 10 GBPs have been discovered in mice (22–24). The GBPs belong to a subfamily within the protein family of large GTPases that includes atlastins (25), dynamins (26), and Mx proteins (27). GBP-1 is the best characterized member of the human GBP family (28, 29). Expression of GBP-1 is induced by IFN-γ, IL-1β, and TNF-α in many different cell types *in vitro* (17, 20, 30, 31). *In vivo*, GBP-1 expression is highly associated with blood vessel ECs and rarely observed in other cells (20), indicating that GBP-1 may exert specific functions in ECs. Indeed, it has been shown that GBP-1 inhibits EC proliferation (17) and mediates the inhibition of EC invasiveness *via* down-regulation of matrix metalloproteinase (MMP) -1 expression (18).

Here, we show that GBP-1 also inhibits spreading and migration of ECs and that these effects are mediated *via* induction of ITGA4 expression by GBP-1. Our results further support the role of GBP-1 as an antiangiogenic regulatory molecule in inflammation.

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MATERIALS AND METHODS

Cell cultures

Primary human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell (designated as HUVEC1s) (Heidelberg, Germany) and Cambrex Bio Science (designated as HUVEC2s) (Verviers, Belgium) and were maintained in the corresponding endothelial cell basal medium [ECBM (PromoCell) or EBM (Cambrex)] supplemented with 2% fetal calf serum (FCS) at 37°C in a humidified atmosphere at 5% CO_2. HUVEC1s were routinely cultivated in uncoated culture flasks (Nunc, Wiesbaden, Germany), whereas HUVEC2s were cultured in flasks coated for at least 2 h with 1.5% bovine skin gelatin, type B (Sigma-Aldrich, Taufkirchen, Germany) in phosphate buffered saline (PBS) (Biochrom AG, Berlin, Germany). For routine cultivation, confluent cells were washed once with PBS, detached by using 1×0.5 g/L trypsin and 0.2 g/L ethylene-diamine-tetra-acetic acid in HBSS (trypsin/EDTA) (PAA, Colbe, Germany) for 2–3 min, and passaged in a 1:4 ratio (one passage). All experiments were carried out between passages 5 and 11.

For stimulation with recombinant proteins, cells were seeded on gelatin-coated flasks, incubated overnight in ECBM supplemented with 0.5% FCS (ECBM/0.5% FCS), and subsequently treated with inflammatory cytokines (200 U/ml IL-1β, 300 U/ml TNF-α, 100 U/ml IFN-γ) in the same medium for the indicated time spans. Recombinant human IL-1β, TNF-α, and IFN-γ were purchased from Roche (Mannheim, Germany). All cytokines were diluted in PBS containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich).

Retroviral transduction of HUVECs

Control (CR), GBP-1, and green fluorescent protein (GFP) -HUVECs were obtained by infection of HUVEC cultures with the retroviral vectors pBABEpuro, pBABEpuro-GBP-1, and pBABEpuro-GFP generated from PG13 packaging cells (32). For generation of HUVECs constitutively expressing shRNAs targeting GBP-1 or GFP, the respective shRNAs were cloned into the retroviral pBABEpuro vector (32). GBP-1-shRNA: 5′-GGTTGAGGATTCAGCTGACttcaagagaGTCAGCTGAATCCTCAACCTTTTTG-3′ (capital letters=sense and antisense strand, small letters=loop sequence), GFP-shRNA used as a control: 5′-GCAAGCTGACCCTGAAGTTCttcaagagaGAACTTCAGGGTCAGCTTGCTTTTTG-3′. Recombinant virus particles were generated in human embryonic kidney (HEK) 293T cells (24). Retroviral transduction of HUVECs was carried out as described previously (17).

Western blot analysis

Western blotting from cell lysates was performed as described previously (20, 33), with the exception that membrane blocking was carried out in PBS containing 5% skim milk overnight at 4°C with gentle shaking. The following primary antibodies diluted in PBS/2.5% skim milk were incubated for 1 h at room temperature (RT): monoclonal rat anti-human GBP-1 antibody (clone 1B1; 1:500; hybridoma supernatant), and monoclonal mouse anti-human GAPDH antibody (1:70,000) (Chemicon/Millipore, Schwalbach, Germany). Detection of the primary antibodies was performed using goat anti-rat and sheep anti-mouse immunoglobulin G (IgG) coupled to horseradish peroxidase (HRP) (1:5000) (GE Healthcare, Munich, Germany) for 45 min. HRP enzyme reaction was performed using enhanced chemiluminescence (ECL) reagents (GE Healthcare).

Cell spreading assay and quantification of cell surface area

Cell spreading assay with HUVECs was performed in 96-well plates (Nunc) that were coated either with 5 $\mu g/cm^2$ fibronectin (FN) (BD Biosciences, San Jose, CA, USA) or with a 120 kDa α-chymotryptic fragment of FN (FN-120) (Chemicon/Millipore, Temecula, CA, USA) for 1 h at 37°C. Nonspecific binding sites were blocked with PBS containing 1% BSA overnight at 4°C. Subsequently, the cells were washed once with PBS and detached by Accutase treatment (PAA). Cells (5×10^3) were seeded into the coated wells after resuspension in serum-free ECBM (SFM). Cells were allowed to adhere at 37°C and 5% CO_2 for 20 min. Unattached cells were removed by gentle washing with PBS containing 0.1% BSA. Attached cells were fixed with 3.7% neutral buffered formalin (Sigma-Aldrich) for 15 min, stained with 0.5% crystal violet in 2% ethanol for 10 min, and briefly washed with water. Images were acquired using an Olympus digital camera (Olympus, Munich, Germany) mounted on an Axiovert 25 microscope (Zeiss, Jena, Germany). The surface area of 100 cells per cell type was measured using the OPTIMAS 6.0 software package (Optimas Corporation, Washington, DC, USA), and the cells were grouped according to their size into 15 increments with increasing surface area (*e.g.*, increment 1: 0–400 μm^2; increment 2: 400–800 μm^2; . . . ; increment 15: 5600–6000 μm^2). Cells were classified as "spread" based on size cutoffs of either 1200 or 1600 μm^2. Mean values ± SD from at least 3 independent experiments were calculated and are depicted in the respective graphs.

Wound healing assay

CR-, GBP-1-, and shRNA-HUVECs were grown on FN- or FN-120-coated (5 $\mu g/cm^2$) 35-mm culture dishes (Nunc) until confluence and were starved in ECBM/0.5% FCS overnight. Confluent cell monolayers of CR- and GBP-1-HUVECs were scratched using a sterile 1-ml pipette tip. The monolayers were washed twice with SFM and cultured for 10 h in SFM. During this time span, the wounded area was repopulated with cells, resulting in reduced scratch width. shRNA-expressing HUVECs were stimulated with ICs for 48 h, as described above, before scratching. The cells were wounded and washed twice with ECBM/0.5% FCS after incubation with ICs. shRNA-expressing cells were cultivated for 10 h in ECBM/0.5% FCS because of an increased cell death when cultivated in SFM. Images of the same areas were taken immediately after scratching (time point 0) and every 2 h using an Olympus digital camera mounted on an Axiovert 25 microscope (Zeiss). The widths of the wounding scratches at different time points were measured and expressed as a percentage of the initial distance at time point 0, which was set to 100%. The results shown are depicted as means ± SD from at least 3 independent experiments.

RNA isolation and microarray analysis

Total RNA of CR- and GBP-1-HUVECs was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Residual traces of genomic DNA were removed with DNase I (Qiagen). RNA concentration and purity were determined photometrically (GeneQuant, Amersham Biosciences, Freiburg, Germany), and RNA integrity was controlled by nondenaturing agarose gel electrophoresis. Preparation of cRNA targets (5 to 10 μg total RNA), fragmentation, hybridization of HG-U133 plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA), washing, staining, and scanning were performed according to manufacturer's protocols (Affymetrix) with a commercial partner (Dr. Klein-Hitpass, Institute for Cell

Biology, University of Essen, Essen, Germany). Signal intensities and detection calls were determined using Affymetrix microarray suite, version 5.0. Comparison files were further filtered to detect differentially expressed genes. Filter criteria included a change of *I*, a signal log ratio of >1, a value of $P < 0.001$, and an overall signal intensity of >300; or a change of *D*, a signal log ratio of <−1, a value of $P > 0.999$, and an overall signal intensity of >300.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Reverse transcription of total RNA and amplification of GBP-1, ITGA4, and GAPDH cDNA were carried out as described previously (24). GBP-1 was amplified using the primers 5′-ATGGCATCAGAGATCCACAT-3′ (sense) and 5′-TTCGCTTATGGTACATGCCTTTC-3′ (antisense) and ITGA4 using the primers 5′-TTCGGAGCCAGCATACTACC-3′ (sense) and 5′-GCAGAATCAGACCGAAAAGC-3′ (antisense). For amplification of GAPDH, the primers 5′-AGCCACATCGCTCAGAACAC-3′ (sense) and 5′-GAGGCATTGCTGATGATCTTG-3′ (antisense) were used, as previously described (34). Gel pictures were acquired by the Gel-Doc system (Bio-Rad). Signal intensities of each PCR product were quantified with the AIDA software package (Raytest, Straubenhardt, Germany).

Ribonuclease protection assay (RPA)

In vitro transcription of the BD RiboQuant Multi probe human integrin template set (BD Biosciences) and RPA were carried out as described previously (35). Protected RNAs and the biotin-labeled multiple integrin probes were separated on a 6% tris-borate EDTA (TBE) urea gel (Anamed, Darmstadt, Germany) and blotted onto a positively charged nylon membrane (Bio-Rad, Hercules, CA, USA). Detection of RNA fragments was performed with the Chemiluminescent Nucleic Acid Detection Kit (Pierce, Rockford, IL, USA), and signal intensities were quantified with the AIDA software package.

FACS analysis

FACS analysis was performed as described previously (36). The following primary antibodies were incubated with the cells for 45 min on ice: monoclonal mouse anti-human ITGA4 (CD49d) antibody (clone HP2/1; 1:400) (Chemicon/Millipore) and monoclonal mouse anti-human ITGB1 (CD29) antibody (1:8000). Mouse IgG1 (R&D Systems, Minneapolis, MN, USA) was used as an isotype control in the corresponding concentrations. Primary antibodies were generally detected with an Alexa 488-conjugated goat anti-mouse secondary antibody (1:350) (Invitrogen). An R-phycoerythrin (PE)-conjugated goat anti-mouse secondary antibody (1:300) (Jackson ImmunoResearch, West Grove, PA, USA) was used for the detection of primary antibodies when GFP-expressing cells were stained. The use of a red fluorescing secondary antibody allowed differentiating ITGA4 and ITGB1 signals from GFP signals.

Statistical analysis

Student's *t* test was performed using the SPSS 14.0 for Microsoft Windows software (SPSS Inc., Chicago, IL, USA).

RESULTS

Ectopic expression of GBP-1 inhibits spreading and migration of ECs on FN matrices

FN is one of the most common proteins in the ECM and has been shown to be involved in the regulation of angiogenesis (37). In preliminary experiments, we realized that after seeding on FN matrices, spreading of GBP-1-expressing ECs was retarded as compared to GBP-1-nonexpressing cells (data not shown). To analyze the effect of GBP-1 on spreading in more detail, HUVECs were transduced with a retroviral vector encoding GBP-1 (GBP-1-HUVECs) or with the respective control vector (CR-HUVECs). GBP-1 expression was confirmed by Western blot analysis (**Fig. 1*A***). GBP-1- and CR-HUVECs were plated on FN matrices and were allowed to attach and to spread in SFM. Most of the CR-HUVECs were spread after 20 min on FN (Fig. 1*B*; CR), whereas the GBP-1-expressing cells still retained a small, round shape after this time (Fig. 1*B*; GBP-1). Quantification of the surface area of GBP-1- and CR-HUVECs was performed by grouping them into 15 increments according to their size (increment 1: 0–400 μm^2; increment 2: 400–800 μm^2; . . . ; increment 15: 5600–6000 μm^2). By this means, a clear difference in cell size was recognized between GBP-1- and CR-HUVECs (Fig. 1*C*). The percentage of GBP-1-expressing cells (27.0±5.6%) showing a surface area greater than 1200 μm^2 (increments 4 to 15) was decreased as compared to CR-HUVECs (87.8±3.6%) (Fig. 1*D*; ≥1200 μm^2). When using more stringent criteria of cell spreading (increments 5 to 15 or ≥1600 μm^2), the difference in numbers of spread CR-HUVECs (74.3±7.1%) compared to those of GBP-1-expressing cells (13.3±3.5%) was still highly significant ($P \leq 0.001$) (Fig. 1*D*; ≥1600 μm^2).

An important aspect of cell migration is membrane ruffling and protrusion of the leading edge (38, 39). Cell spreading mimics these events, which occur at the leading edge of a migrating cell. Migration of CR- and GBP-1-HUVECs was examined by performing *in vitro* wound healing assays. GBP-1-expressing cells migrated at a significantly slower rate into the wounded area in comparison to control cells (Fig. 1*E*). After 10 h, the scratch width of GBP-1-expressing cells was 81.2 ± 2.0% of the initial width, whereas CR-HUVECs reduced the scratch width to 45.6 ± 13.5% of the initial width (Fig. 1*F*). These results demonstrate that ectopically expressed GBP-1 inhibits spreading and migration of ECs on FN-coated surfaces.

GBP-1 up-regulates ITGA4 expression in ECs

To elucidate the molecular mechanisms of the inhibitory activities of GBP-1 on cell spreading and migration, we investigated GBP-1-regulated gene expression in ECs. Two different HUVEC cultures (HUVEC1 and HUVEC2) were subjected to the study, in order to exclude cell culture-specific variations of primary cells. In addition to the culture shown in Fig. 1, a second

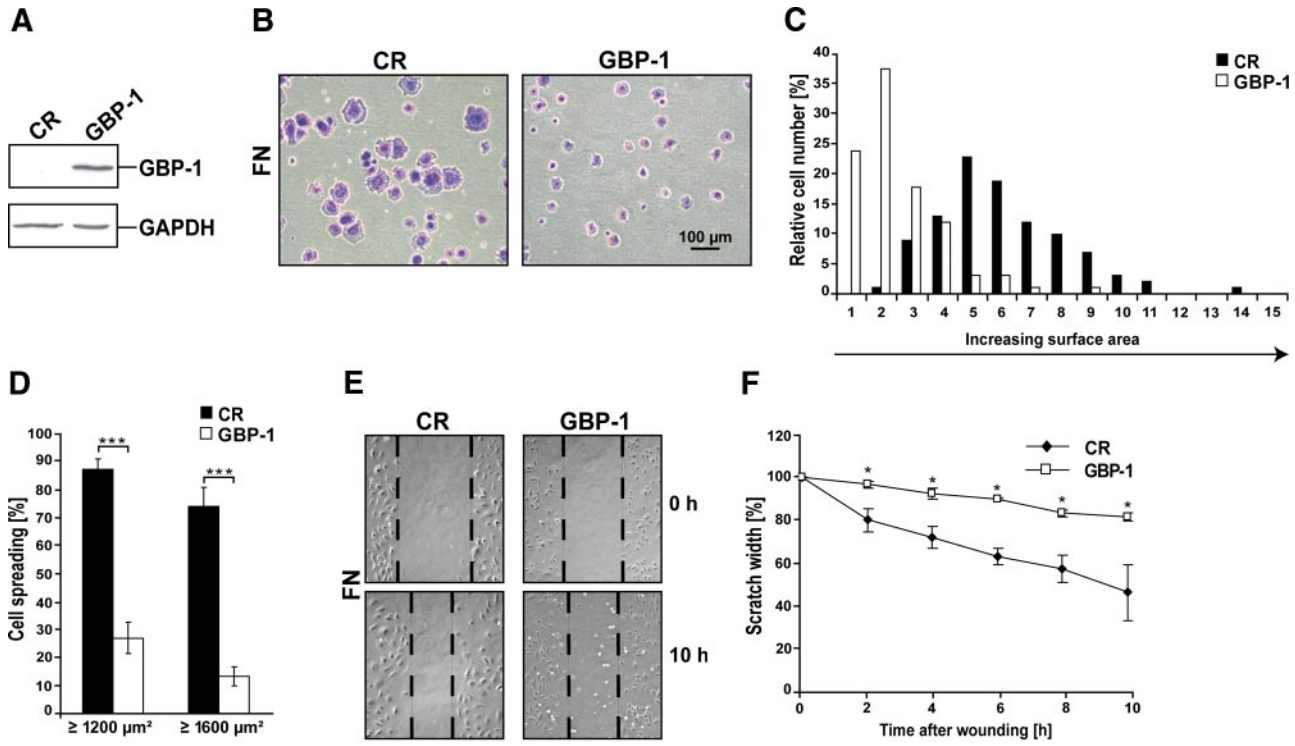

Figure 1. Expression of GBP-1 reduces spreading and migration of HUVECs on FN matrices. *A*) Western blot analysis of total cell lysates obtained from HUVEC1s transduced with the retroviral vector pBABEpuro (CR) or with pBABEpuro-GBP-1 (GBP-1). Immunochemical detection of GAPDH demonstrates that equal concentrations of proteins were blotted onto the membranes. *B*) CR- and GBP-1-HUVEC1s were plated on FN-coated dishes. Adherent cells were fixed with 3.7% neutral buffered formalin after 20 min and stained with 0.5% crystal violet. *C*) Surface area of 100 individual cells from each cell type, stained according to *B*, was determined as described in Materials and Methods. One increment corresponds to 400 μm^2. Representative size distribution of cell surface area as determined in one experiment is shown. *D*) Relative number of spread CR- and GBP-1-HUVECs is given as the percentage of cells with surface area $\geq$1200 μm^2 (left bars; increments 3–15) or $\geq$1600 μm^2 (right bars; increments 4–15) and is depicted in the diagram. Values are means $\pm$ SD from 4 independent experiments; $^{***}P \leq 0.001$. *E*) Migration activity of CR- and GBP-1-HUVEC1s on FN-coated surfaces was investigated using a wound healing assay. *In vitro* scratch wounds were generated by scraping confluent cell monolayers with a sterile pipette tip. Pictures from the same area acquired immediately after scratching (0 h) and after 10 h of culture (10 h) are shown. *F*) Width of the scratch wounds at different time points was determined relative to the initial width at time point 0 and is indicated as a percentage. Results are means $\pm$ SD from 3 independent experiments; $^{*}P \leq 0.05$.

culture was transduced with a retroviral vector encoding GBP-1 or with the respective control vector (CR). GBP-1 expression in this culture was also confirmed by Western blot analysis (**Fig. 2*A***). mRNA of both cultures was isolated, and the gene expression profiles of GBP-1-HUVECs were compared with CR-HUVECs by applying oligonucleotide-based microarray technology from Affymetrix (HG-U133 plus 2.0). Of the 38,500 genes analyzed, 6 were highly significantly ($P \leq 0.001$) regulated by GBP-1 in both HUVEC cultures tested (**Table 1**).

ITGA4 was the strongest up-regulated gene in both GBP-1-expressing cultures (Table 1). It has been reported that ITGA4 specifically dimerizes with the integrin β_1- and β_7-subunits. Of note, $\alpha_4\beta_1$ expression has been shown to reduce cell spreading (40–43) and to inhibit cell migration and invasion on FN (44–47). In contrast, other integrins, such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$, promoted those processes.

RT-PCR analysis (Fig. 2*B*) and RPA (Fig. 2*C*) confirmed the high induction of ITGA4 expression by GBP-1 in both HUVEC cultures. RPA additionally revealed that the expression of other integrin transcripts was either not consistently altered in both cultures (*itgav*), unchanged (*itga2*, *itga3*, *itga5*, *itga6*), or not detectable (*itga1*, *itga7*, *itga8,* and *itga9*) (Fig. 2*C*; left).

Subsequently, the presence of ITGA4 on the surface of CR- and GBP-1-HUVECs was analyzed by FACS. In agreement with the RNA expression analyses, the ITGA4 protein level was increased on the cell surface of GBP-1-expressing cells (2.8$\pm$0.7-fold HUVEC1; 2.2$\pm$0.2-fold HUVEC2), as compared to control cells (Fig. 2*D*; ITGA4; GBP-1). ITGB1 forms heterodimers with ITGA4 and belongs to the same integrin family (4, 5, 48). Therefore, ITGB1 was investigated as a control. ITGB1 was highly expressed in HUVECs, but its expression was not altered by ectopically expressed GBP-1 (Fig. 2*D*; ITGB1; GBP-1). Expression of GFP as a heterologous control protein, which was detectable in 98% of the cells (data not shown) affected neither ITGA4 nor ITGB1 expression (Fig. 2*D*; ITGA4 and ITGB1; GFP). Together, these data demonstrate that ectopically expressed GBP-1 is sufficient to induce ITGA4 expression in ECs at both the RNA and protein level.

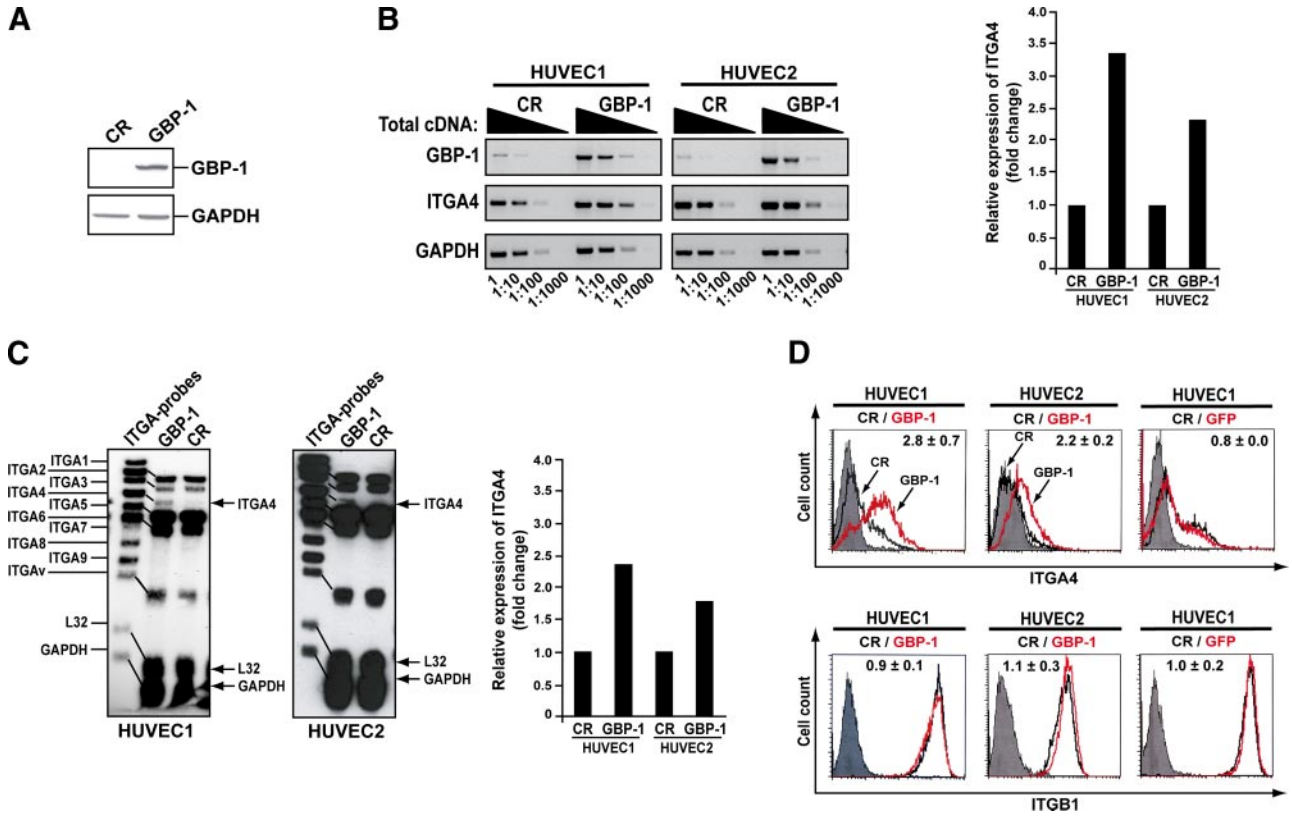

Figure 2. GBP-1 induces ITGA4 expression at RNA and protein level in HUVECs. *A*) Western blot analysis of total cell lysates obtained from HUVEC2s transduced with the retroviral vector pBABEpuro (CR) or with pBABEpuro-GBP-1 (GBP-1). Immunochemical detection of GAPDH demonstrates that equal concentrations of proteins were blotted onto the membranes. *B*) RT-PCR for analysis of GBP-1, ITGA4, and GAPDH expression in CR- and GBP-1-HUVECs (HUVEC1 and HUVEC2). Decreasing amounts of cDNA [undiluted (1), 1:10, 1:100, and 1:1000] were subjected to PCR after reverse transcription of isolated mRNA. Bands obtained with nonsaturated levels of ITGA4 (1:100) were densitometrically quantified and normalized to the corresponding GAPDH signal (1:100). Relative increase of ITGA4 expression in GBP-1-expressing cells as compared to CR-HUVECs is depicted in the diagram (right). *C*) Total mRNA of CR- and GBP-1-HUVECs was analyzed by Multiprobe RNase protection assay using a set of multiple biotinylated human integrin templates as probes. ITGA4 signal intensities were densitometrically quantified and normalized to the L32 signal. L32 is a housekeeping gene and encodes an essential protein for the 60S ribosomal subunit. Relative increase of ITGA4 expression in GBP-1-expressing cells as compared to CR-HUVECs is depicted in the diagram (right). *D*) FACS analysis of CR- (black), GBP-1- (red), and GFP-HUVECs (red) stained with a monoclonal antibody against ITGA4 (top panel) and ITGB1 (bottom panel), or an isotype-matched control antibody (gray). Fluorescence intensities from ITGA4- or ITGB1-stained cells were normalized to the isotype control. Normalized signals of ITGA4 and ITGB1 cell surface expression of GBP-1- or GFP-HUVECs relative to CR-HUVECs are given as means ± SD from at least 3 independent experiments.

The GBP-1-mediated inhibition of cell spreading and migration depends on the presence of the ITGA4 binding site in FN

ITGA4 expression correlated positively with the inhibition of cell spreading and migration on FN matrices. ITGA4 selectively binds to a distinct site near the C terminus of FN (type III repeat 14-IIICS) containing the CS-1 and CS-5 domains (**Fig. 3*A***; FN) (49, 50). All other integrins, such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$, bind to FN within the central integrin binding site (type III repeats 9–10) (Fig. 3*A*; FN) (51, 52). In case that ITGA4 causes the inhibition of cell spreading, both effects should be abrogated by plating the cells on a 120-kDa fragment of FN (FN-120). FN-120 lacks the C-terminal ITGA4 binding site but still harbors the central integrin recognition site (Fig. 3*A*; FN-120). Indeed, spreading of CR- and GBP-1-HUVECs on FN-120 was identical (Fig. 3*B, C*). Consistently, migration of GBP-1- and CR-HUVECs was also found to be similar on the FN-120 matrix (Fig. 3*D, E*). These results suggest that the GBP-1-mediated inhibition of cell spreading and migration depends on the binding of ITGA4 to its specific binding site in FN.

IL-1β and TNF-α induce ITGA4 surface expression and provoke an inhibition in cell spreading on FN matrices

Since IL-1β, TNF-α, and IFN-γ are known inducers of endogenous GBP-1 expression (17, 20, 31, 53), their effects on ITGA4 cell surface expression in ECs was investigated. To this aim, each of the ICs was applied in concentrations previously shown to induce GBP-1 expression (**Fig. 4*A***) and a clear biological response in HUVECs (20). ITGA4 cell surface expression was enhanced in IL-1β- (2.8±0.4-fold) and TNF-α-stimulated cells (2.0±0.4-fold) as compared to unstimulated cells

TABLE 1. *GBP-1-regulated genes in stably transduced HUVEC cultures*

Probe ID	Accession No.	Gene Title	Gene Symbol	Fold change HUVEC1	Fold change HUVEC2
Up-regulated					
213416_at	BG532690	Integrin, alpha 4	ITGA4	3.4	3.8
244741_s_at	BE855713	Hypothetical protein MGC9913	MGC9913	2.8	2.5
Down-regulated					
203868_at	NM_001078	Vascular cell adhesion molecule 1	VCAM1	–2.1	–2.1
206942_s_at	NM_002674	Promelanin-concentrating hormone	PMCH	–2.4	–2.4
219685_at	NM_021637	Transmembrane protein 35	TMEM35	–3.5	–2.1
205433_at	NM_000055	Butyrylcholin esterase	BCHE	–5.1	–2.0

$P \leq 0.001$ for GBP-1-regulated genes.

(Fig. 4*B*; ITGA4; IL-1β and TNF-α). Interestingly, ITGA4 surface expression was down-regulated in cells treated with IFN-γ (0.4±0.0-fold) in comparison to untreated cells (Fig. 4*B*; ITGA4; IFN-γ). ITGB1 surface expression was neither affected by IL-1β, TNF-α, nor by IFN-γ (Fig. 4*B*; ITGB1).

Subsequently, the effect of ICs on the spreading of HUVECs on FN and FN-120 was examined (Fig. 4*C*). Quantification of the cell surface area under the different conditions showed that the relative numbers of IL-1β- (26.0±1.7%), TNF-α- (21.7±2.5%), and IFN-γ-treated cells (20.3±0.6%) with surface area ≥1600 μm^2 were significantly decreased, as compared to untreated cells (82.3±3.8%) on FN matrices (Fig. 4*D*; FN; ≥1600 μm^2). On FN-120-coated surfaces, however, the spreading of IL-1β- and TNF-α-treated cells was not inhibited and was identical to that of unstimulated cells (Fig. 4*C*, *D*; FN-120; IL-1β and TNF-α). In contrast, spreading of

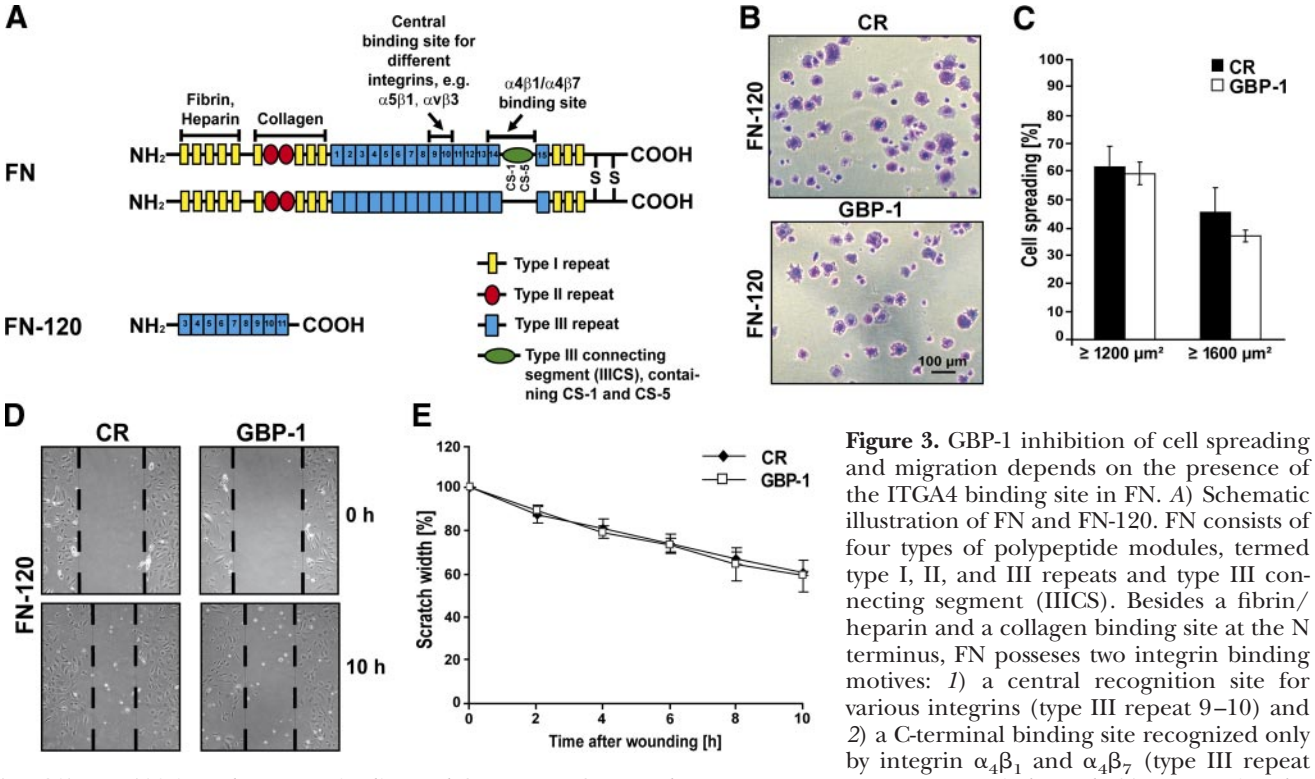

Figure 3. GBP-1 inhibition of cell spreading and migration depends on the presence of the ITGA4 binding site in FN. *A*) Schematic illustration of FN and FN-120. FN consists of four types of polypeptide modules, termed type I, II, and III repeats and type III connecting segment (IIICS). Besides a fibrin/heparin and a collagen binding site at the N terminus, FN posseses two integrin binding motives: *1*) a central recognition site for various integrins (type III repeat 9–10) and *2*) a C-terminal binding site recognized only by integrin $\alpha_4\beta_1$ and $\alpha_4\beta_7$ (type III repeat 14-IIICS). FN-120 is a chymotryptic digested fragment of FN and encompasses type III repeats 3 through 11. It contains the central integrin binding motif, but not the C-terminal binding region for integrin $\alpha_4\beta_1$ and $\alpha_4\beta_7$. *B*) CR- and GBP-1-HUVEC1s were plated on FN-120-coated surfaces. Adherent cells were fixed with 3.7% neutral buffered formalin after 20 min and stained with 0.5% crystal violet. *C*) Surface area of 100 individual cells from each cell type, stained according to *B*, was determined as described in Materials and Methods. Relative number of spread CR- and GBP-1-HUVECs is given as the percentage of cells with surface area ≥1200 μm^2 (left bars) or ≥1600 μm^2 (right bars) and is depicted in the diagram. Values are means ± SD from 3 independent experiments. *D*) Migration activity of CR- and GBP-1-HUVEC1s on FN-120-coated surfaces was investigated using a wound healing assay. *In vitro* scratch wounds were generated by scraping confluent cell monolayers with a sterile pipette tip. Pictures from the same area acquired immediately after scratching (0 h) and after 10 h of culture (10 h) are shown. *E*) Width of the scratch wounds at different time points was determined relative to the initial width at time point 0 and is indicated as a percentage. Results are means ± SD from 4 independent experiments.

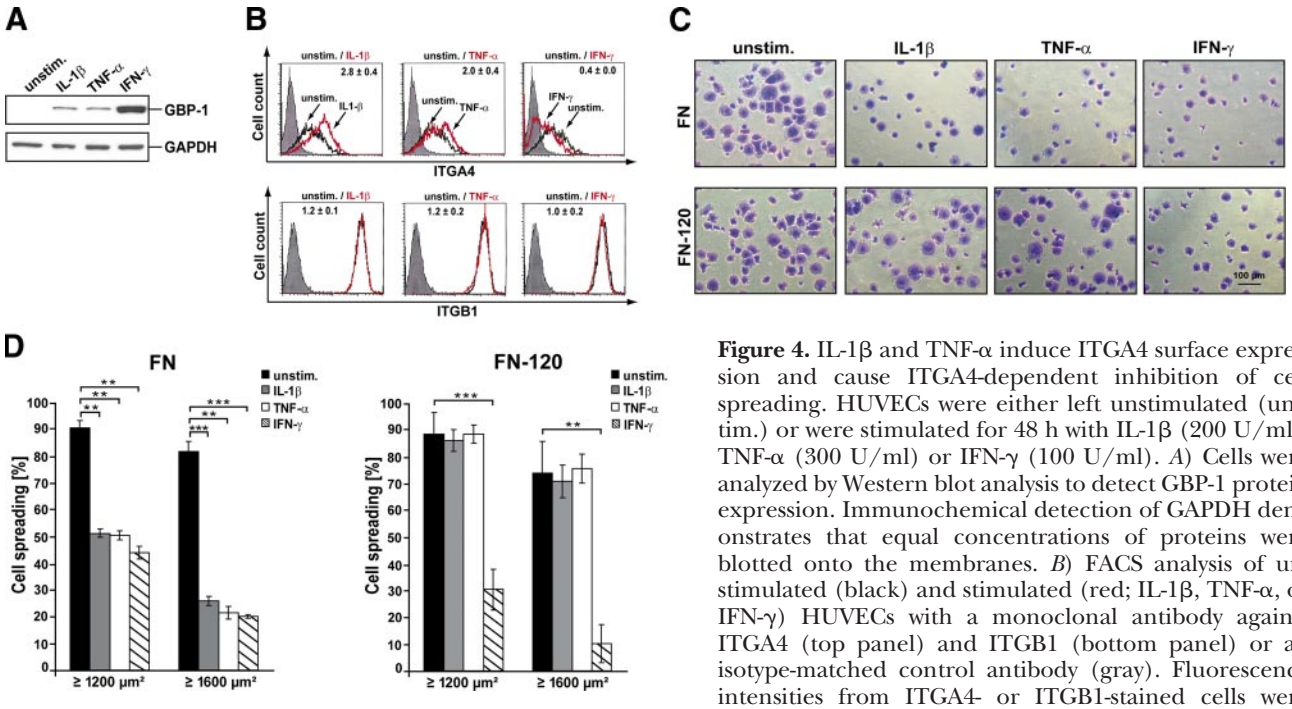

Figure 4. IL-1β and TNF-α induce ITGA4 surface expression and cause ITGA4-dependent inhibition of cell spreading. HUVECs were either left unstimulated (unstim.) or were stimulated for 48 h with IL-1β (200 U/ml), TNF-α (300 U/ml) or IFN-γ (100 U/ml). *A*) Cells were analyzed by Western blot analysis to detect GBP-1 protein expression. Immunochemical detection of GAPDH demonstrates that equal concentrations of proteins were blotted onto the membranes. *B*) FACS analysis of unstimulated (black) and stimulated (red; IL-1β, TNF-α, or IFN-γ) HUVECs with a monoclonal antibody against ITGA4 (top panel) and ITGB1 (bottom panel) or an isotype-matched control antibody (gray). Fluorescence intensities from ITGA4- or ITGB1-stained cells were normalized to the isotype control. Normalized signals for surface expression of ITGA4 and ITGB1 obtained from stimulated HUVECs relative to unstimulated cells were calculated; values are means ± SD from at least 3 independent experiments. *C*) Unstimulated and stimulated HUVECs (48 h) were plated on surfaces coated with FN (top panel) or FN-120 (bottom panel) and were allowed to spread for 20 min. Cells were fixed with 3.7% neutral buffered formalin and stained with 0.5% crystal violet. *D*) Surface area of 100 individual cells from each cell type, stained according to *C*, was determined as described in Materials and Methods. Relative number of spread unstimulated and stimulated HUVECs is given as percentage of cells with surface area ≥1200 μm^2 (left bars) or ≥1600 μm^2 (right bars) and is depicted in the diagram. Values are means ± SD from 3 independent experiments; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

IFN-γ-treated cells was still inhibited on FN-120 matrices (Fig. 4*C*, *D*; FN-120; IFN-γ).

These findings demonstrate that IL-1β and TNF-α induce ITGA4 surface expression and reduce HUVEC spreading dependent on ITGA4 binding to FN. IFN-γ also inhibited cell spreading, but in an ITGA4-independent manner. Therefore, in the following experiments, we focused on the effects of ITGA4 on spreading and migration of IL-1β- and TNF-α-treated cells.

GBP-1 mediates the IL-1β- and TNF-α-induced inhibition of cell spreading and migration *via* up-regulation of ITGA4 expression

GBP-1 knockdown experiments were carried out in order to demonstrate that GBP-1 is necessary to mediate the inhibitory effects of IL-1β and TNF-α on EC spreading. HUVECs were transduced with retroviral vectors encoding an shRNA targeting either GBP-1 (GBP-1-shRNA-HUVECs) or GFP as a control (ctrl-shRNA-HUVECs). GBP-1 expression was analyzed by Western blot analysis after treatment of transduced cells with IL-1β and TNF-α for 48 h (**Fig. 5*A***). Expression of GBP-1 in GBP-1-shRNA-expressing cells was decreased to 10% (IL-1β) and 7% (TNF-α) as compared to the expression level in ctrl-shRNA-HUVECs (Fig. 5*A*). In addition, ITGA4 mRNA levels (Supplemental Fig. 1), as well as cell surface expression of ITGA4 (Fig. 5*B*; ITGA4), were significantly lower in IL-1β- and TNF-α-treated cells that expressed GBP-1-shRNA as compared to the respective ctrl-shRNA-expressing cells. The expression of ITGB1 was not altered by the presence of either GBP-1-shRNA or ctrl-shRNA (Fig. 5*B*; ITGB1).

Subsequently, the spreading capability of these cells was assessed. Spreading of unstimulated ctrl-shRNA-HUVECs on FN within 20 min was greater as compared to ctrl-shRNA-HUVECs that had been stimulated for 48 h with IL-1β or TNF-α (Fig. 5*C*; ctrl-shRNA). Quantification of these effects indicated that the number of IL-1β- (27.3±5.6%) and TNF-α-treated (33.0±2.5%) ctrl-shRNA-HUVECs showing surface area ≥1600 μm^2 was highly significantly ($P \leq 0.001$) decreased in comparison to unstimulated cells (95.5±2.4%) (Fig. 5*D*; ctrl-shRNA; ≥1600 μm^2). These findings are in agreement with the data obtained for nontransduced HUVECs treated with IL-1β or TNF-α (compare Fig. 4*C, D*). This indicates that transduction of cells with retroviral vectors encoding a nonspecific ctrl-shRNA did not alter their spreading capability. In contrast, the specific knockdown of GBP-1 by introducing a GBP-1-specific shRNA abrogated the inhibitory effect of IL-1β and TNF-α on cell spreading (Fig. 5*C*, *D*; GBP-1-shRNA). IL-1β- and TNF-α-treated GBP-1-shRNA-HUVECs exhibited a similar spreading capability on FN-coated surfaces as unstimulated GBP-1-shRNA-HUVECs (Fig. 5*C*, *D*; GBP-1-shRNA).

In a final approach, we investigated the effect of GBP-1 knockdown on migration of IL-1β- and TNF-α-stimulated HUVECs on FN matrices. Stimulated

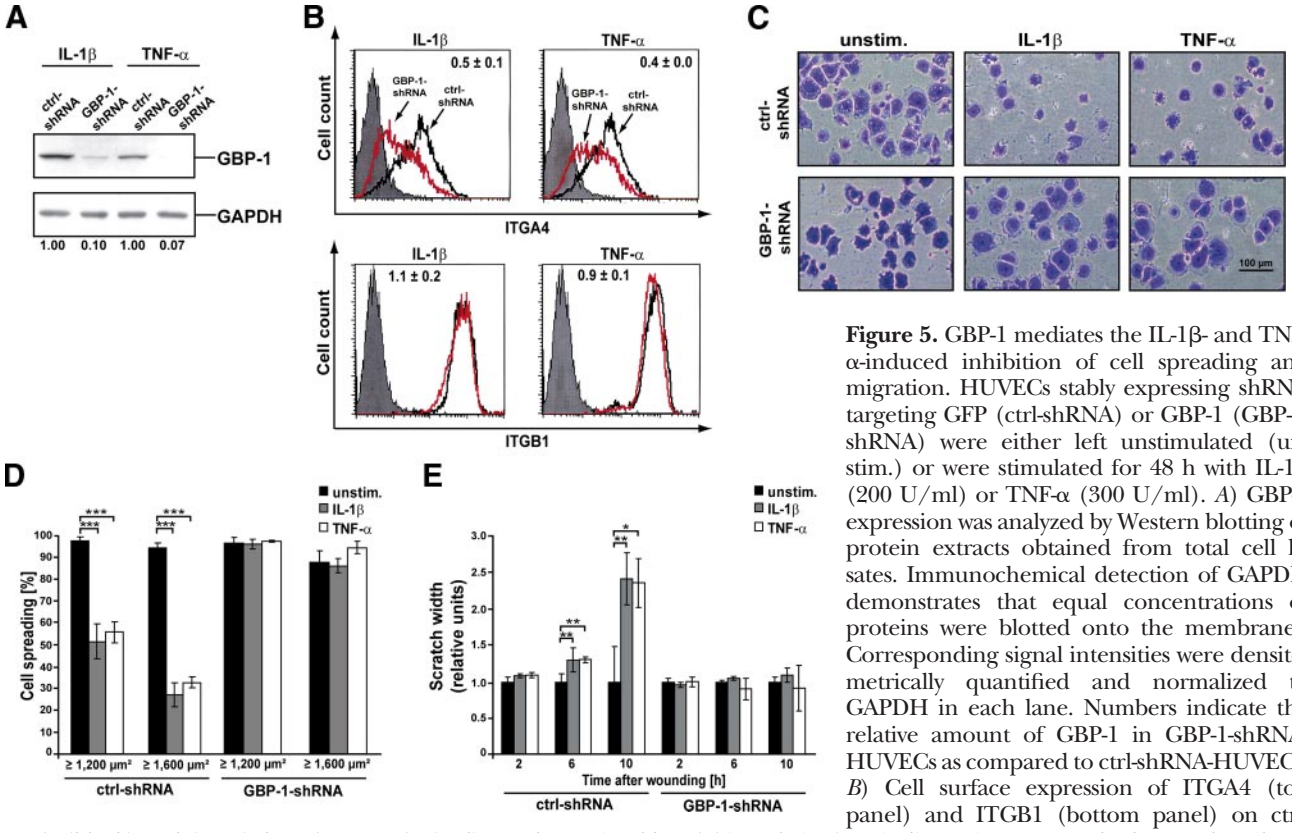

Figure 5. GBP-1 mediates the IL-1β- and TNF-α-induced inhibition of cell spreading and migration. HUVECs stably expressing shRNA targeting GFP (ctrl-shRNA) or GBP-1 (GBP-1-shRNA) were either left unstimulated (unstim.) or were stimulated for 48 h with IL-1β (200 U/ml) or TNF-α (300 U/ml). *A*) GBP-1 expression was analyzed by Western blotting of protein extracts obtained from total cell lysates. Immunochemical detection of GAPDH demonstrates that equal concentrations of proteins were blotted onto the membranes. Corresponding signal intensities were densitometrically quantified and normalized to GAPDH in each lane. Numbers indicate the relative amount of GBP-1 in GBP-1-shRNA-HUVECs as compared to ctrl-shRNA-HUVECs. *B*) Cell surface expression of ITGA4 (top panel) and ITGB1 (bottom panel) on ctrl-shRNA- (black) and GBP-1-shRNA-HUVECs (red) was determined by FACS analysis. Gray indicates isotype-matched control antibody staining. Fluorescence intensities from ITGA4- or ITGB1-stained cells were normalized to the isotype control. Normalized signals of GBP-1-shRNA-HUVECs relative to ctrl-shRNA-HUVECs are given as means ± SD from at least 3 independent experiments. *C*) Cells were fixed and stained with 0.5% crystal violet 20 min after adhesion to FN-coated surfaces. *D*) Surface area of 100 individual cells from each cell type, stained according to *C*, was determined as described in Materials and Methods. Relative number of spread unstimulated and stimulated ctrl-shRNA- or GBP-1-shRNA-HUVECs is given as percentage of cells with surface area ≥1200 μm^2 (left bars) or ≥1600 μm^2 (right bars) and is depicted in the diagram. Values are means ± SD from 4 independent experiments; ***$P \leq 0.001$. *E*) Migration activity of ctrl-shRNA- and GBP-1-shRNA-expressing HUVECs after stimulation with IL-1β or TNF-α on FN-coated surfaces was investigated using a wound healing assay. *In vitro* scratch wounds were generated by scraping confluent cell monolayers with a sterile pipette tip. Width of the scratch wounds at different time points was determined as a percentage of the initial width at time point 0. Diagram shows relative width of wounds of stimulated ctrl-shRNA- and GBP-1-shRNA-HUVECs *vs.* the respective unstimulated cells (set to 1) at 2, 6, and 10 h. Values are means ± SD from 4 independent experiments; *$P \leq 0.05$; **$P \leq 0.01$.

shRNA-expressing cells showed an increased cell death rate in SFM. Therefore, wounding assays with these cells were carried out in the presence of 0.5% FCS. Treatment of ctrl-shRNA-HUVECs with IL-1β and TNF-α significantly ($P \leq 0.05$) reduced the capability of these cells to migrate into the wounded area as compared to unstimulated cells (Fig. 5*E*; ctrl-shRNA). After 10 h the scratch widths in the IL-1β- and TNF-α-treated ctrl-shRNA-HUVEC cultures were larger (2.5±0.4- and 2.4±0.3-fold) as compared to that obtained in the unstimulated ctrl-shRNA-HUVEC culture (Fig. 5*E*; ctrl-shRNA; 10 h). Knockdown of GBP-1 restored migration capabilities of IL-1β- and TNF-α-treated cells (Fig. 5*E*; GBP-1-shRNA). Altogether, these results show that GBP-1 is both necessary and sufficient to mediate inhibition of spreading and migration of IL-1β- and TNF-α-treated cells.

DISCUSSION

GBP-1 is an antiangiogenic molecule. It is strongly expressed in inflammatory tissues, selectively in blood vessel ECs of tissue areas with high concentrations of ICs, and is absent in areas with high concentrations of angiogenic growth factors (17, 18, 20). ICs induce GBP-1 expression in ECs, and GBP-1 mediates the inhibition of cell proliferation and invasion, which are characteristic direct effects of ICs on ECs (17, 18). Here, we described a further antiangiogenic activity of GBP-1. We showed that GBP-1 inhibits spreading and migration of ECs on FN matrices. These inhibitory effects appear to be mediated by the up-regulation of ITGA4 expression in response to GBP-1.

The specific up-regulation of ITGA4 expression by GBP-1 was supported by several lines of experimental evidence. First, two different HUVEC cultures were used for comparative transcriptome analyses in order to compensate for the heterogeneity of EC primary cultures. Expression of the *itga4* gene was highly significantly increased by ectopically expressed GBP-1 in both HUVEC cultures. The expression of several other integrin α-subunits was either unchanged (*itga2, itga3, itga5, itga6*) or not consistently affected (*itgav*) by GBP-1. GFP, expressed as a heterologous control protein, did not induce ITGA4 expression. Second, ITGA4

up-regulation was confirmed by semiquantitative RT-PCR and RPA on the RNA level and by FACS analysis on the protein level. Interestingly, ITGA4 expression was not only up-regulated in response to ectopic expression of GBP-1, but also in response to treatment of the cells with IL-1β and TNF-α. Although each cytokine is known to exert different activities on ECs (54, 55), both induced expression of GBP-1 and ITGA4. In agreement with our findings, an increase of ITGA4 cell surface expression in ECs treated with TNF-α was reported previously (56). Finally, a specific knockdown of GBP-1 expression using shRNA abrogated the induction of ITGA4 mRNA transcription, as well as cell surface expression in the presence of IL-1β and TNF-α. These results congruently indicate that GBP-1 is necessary and sufficient to mediate the induction of ITGA4 expression in response to IL-1β and TNF-α.

In preliminary experiments with HUVECs expressing mutant GBP-1 proteins with a defective GTPase activity (57), we were able to show that ITGA4 induction was below 36% as compared to wild-type GBP-1-expressing cells (data not shown). This indicates that the GTPase activity is involved in the up-regulation of ITGA4 expression but may cooperate with additional structures/regions of GBP-1, which will need to be determined in future studies.

The capability of cytoplasmic GTPases to regulate gene expression has been generally accepted for the small GTPases Rac, Rho, and Ras (58–62). Expression of the *itga4* gene is regulated through several different transcription factors that can either stimulate [Ets family (63), Pax-6 (64), the Wilms tumor suppressor Wt1 (65), the glial cell missing factor GCMa (66), c-Myb (67)] or inhibit [ZEB (67)] *itga4* promoter activity. It will need to be determined in future studies whether GBP-1 regulates *itga4* expression *via* one of these factors or through post-transcriptional regulation of ITGA4 mRNA stability.

Interactions of ECs with components of the ECM, such as FN, are crucial for the regulation of angiogenesis. FN is present in provisional vascular matrices of angiogenic vessels, but not in the basal membrane of mature vessels, underscoring its important function in angiogenesis (68, 69). Numerous reports describe interactions of ECs with FN mainly mediated by the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$. These interactions promote EC proliferation, spreading, and motility during the growth of new blood vessels (70–73). ITGA4 is another FN binding integrin with distinct biochemical and biological functions from the others. ITGA4 binds to FN *via* a C-terminal binding site (type III repeat 14-IIICS) that is different from the central binding site used by $\alpha_5\beta_1$ and $\alpha_v\beta_3$. ITGA4 binding to full-length FN results in decreased cell spreading (40–43) and migration (44–47). Studies using integrin chimeras have demonstrated that these functional properties are conferred by the cytoplasmic domain of ITGA4 (40, 42), which directly and specifically binds to paxillin, a signaling adaptor protein, leading to reduced cell spreading (43). In agreement with these results, we demonstrated that ITGA4 up-regulation by ectopically expressed GBP-1 is sufficient to mediate the inhibition of EC spreading and migration on FN. These effects were abrogated on a 120-kDa fragment of FN (FN-120) lacking the C-terminal ITGA4 binding site. This demonstrates that ITGA4 binding to the type III repeat 14-IIICS motif is required for its inhibitory activity.

In addition, we were able to show that EC spreading and migration on FN matrices was significantly inhibited in response to IL-1β and TNF-α through GBP-1-mediated induction of ITGA4 expression. Cell spreading on FN matrices was also impaired when IFN-γ was applied, but in an ITGA4-independent manner. It is known that IL-1β, TNF-α, and IFN-γ exert differential activities on ECs. For example, IL-1β and TNF-α induce a prothrombotic and proinflammatory phenotype, whereas IFN-γ-induced activations are predominantly related to host defense against infections (54, 55). It is also clear that IL-1β and TNF-α in comparison to IFN-γ activate different signal transduction pathways. This is also evident in the regulation of GBP-1 expression. In the GBP-1 promoter, a promoter module of an IFN-α response element (ISRE) and a cRel motif have been described (31). In response to IL-1β and TNF-α, cooperative activation of both elements is observed and also required for the induction of GBP-1 expression. In contrast, in IFN-γ-treated cells exclusively the ISRE element is activated, and this is sufficient for the induction of GBP-1 expression. The different activities of IL-1β and TNF-α, as compared to IFN-γ on cellular activation and on the induction of GBP-1 expression may explain the different effects of GBP-1 on ITGA4 expression in IL-1β/TNF-α- *vs.* IFN-γ-stimulated ECs. It can be speculated that GBP-1 inhibits spreading and migration under inflammatory conditions, which are regulated by IL-1β and TNF-α, and may be engaged in different functions in infectious conditions regulated by IFN-γ. In this framework, it is interesting that GBPs have been shown to exert antiviral activity (74, 75) and have been found associated with antibacterial responses in mammalian cells (22).

Our work provides further insight into the function of GBP-1 in inflammation, showing that GBP-1 is necessary and sufficient to mediate the inhibitory effects of IL-1β and TNF-α on EC spreading and migration on FN matrices. Both of these important angiogenic processes can be inhibited by GBP-1 *via* induction of ITGA4 expression. These results indicate that ITGA4 may provide an antagonistic signal to ECM interactions of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ during inflammation. This may be a novel mechanism for termination of physiological and pathophysiological angiogenesis and thus may open new strategies for the modulation of angiogenesis in human diseases. FJ

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REFERENCES

1. Carmeliet, P., and Jain, R. K. (2000) Angiogenesis in cancer and other diseases. *Nature* **407,** 249–257
2. Folkman, J. (1971) Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* **285,** 1182–1186
3. Folkman, J. (2006) Angiogenesis. *Annu. Rev. Med.* **57,** 1–18
4. Hynes, R. O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69,** 11–25
5. Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* **110,** 673–687
6. Brakebusch, C., and Fassler, R. (2005) Beta 1 integrin function in vivo: adhesion, migration and more. *Cancer Metastasis Rev.* **24,** 403–411
7. Wiesner, S., Legate, K. R., and Fassler, R. (2005) Integrin-actin interactions. *Cell. Mol. Life. Sci.* **62,** 1081–1099
8. Carmeliet, P. (2005) VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69**(Suppl. 3), 4–10
9. Veikkola, T., Karkkainen, M., Claesson-Welsh, L., and Alitalo, K. (2000) Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res.* **60,** 203–212
10. Dow, J. K., and DeVere White, R. W. (2000) Fibroblast growth factor 2: its structure and property, paracrine function, tumor angiogenesis, and prostate-related mitogenic and oncogenic functions. *Urology* **55,** 800–806
11. Rodriguez-Manzaneque, J. C., Lane, T. F., Ortega, M. A., Hynes, R. O., Lawler, J., and Iruela-Arispe, M. L. (2001) Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 12485–12490
12. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* **88,** 277–285
13. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. (1994) Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79,** 315–328
14. Cozzolino, F., Torcia, M., Aldinucci, D., Ziche, M., Almerigogna, F., Bani, D., and Stern, D. M. (1990) Interleukin 1 is an autocrine regulator of human endothelial cell growth. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6487–6491
15. Frater-Schroder, M., Risau, W., Hallmann, R., Gautschi, P., and Bohlen, P. (1987) Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5277–5281
16. Friesel, R., Komoriya, A., and Maciag, T. (1987) Inhibition of endothelial cell proliferation by gamma-interferon. *J. Cell Biol.* **104,** 689–696
17. Guenzi, E., Töpolt, K., Cornali, E., Lubeseder-Martellato, C., Jörg, A., Matzen, K., Zietz, C., Kremmer, E., Nappi, F., Schwemmle, M., Hohenadl, C., Barillari, G., Tschachler, E., Monini, P., Ensoli, B., and Stürzl, M. (2001) The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. *EMBO J.* **20,** 5568–5577
18. Guenzi, E., Töpolt, K., Lubeseder-Martellato, C., Jörg, A., Naschberger, E., Benelli, R., Albini, A., and Stürzl, M. (2003) The guanylate binding protein-1 GTPase controls the invasive and angiogenic capability of endothelial cells through inhibition of MMP-1 expression. *EMBO J.* **22,** 3772–3782
19. Schweigerer, L., Malerstein, B., and Gospodarowicz, D. (1987) Tumor necrosis factor inhibits the proliferation of cultured capillary endothelial cells. *Biochem. Biophys. Res. Commun.* **143,** 997–1004
20. Lubeseder-Martellato, C., Guenzi, E., Jörg, A., Töpolt, K., Naschberger, E., Kremmer, E., Zietz, C., Tschachler, E., Hutzler, P., Schwemmle, M., Matzen, K., Grimm, T., Ensoli, B., and Stürzl, M. (2002) Guanylate-binding protein-1 expression is selectively induced by inflammatory cytokines and is an activation marker of endothelial cells during inflammatory diseases. *Am. J. Pathol.* **161,** 1749–1759
21. Cheng, Y. S., Colonno, R. J., and Yin, F. H. (1983) Interferon induction of fibroblast proteins with guanylate binding activity. *J. Biol. Chem.* **258,** 7746–7750
22. Degrandi, D., Konermann, C., Beuter-Gunia, C., Kresse, A., Wurthner, J., Kurig, S., Beer, S., and Pfeffer, K. (2007) Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense. *J. Immunol.* **179,** 7729–7740
23. Olszewski, M. A., Gray, J., and Vestal, D. J. (2006) In silico genomic analysis of the human and murine guanylate-binding protein (GBP) gene clusters. *J. Interferon Cytokine Res.* **26,** 328–352
24. Tripal, P., Bauer, M., Naschberger, E., Mörtinger, T., Hohenadl, C., Cornali, E., Thurau, M., and Stürzl, M. (2007) Unique features of different members of the human guanylate-binding protein family. *J. Interferon Cytokine Res.* **27,** 44–52
25. Zhu, P. P., Patterson, A., Lavoie, B., Stadler, J., Shoeb, M., Patel, R., and Blackstone, C. (2003) Cellular localization, oligomerization, and membrane association of the hereditary spastic paraplegia 3A (SPG3A) protein atlastin. *J. Biol. Chem.* **278,** 49063–49071
26. Diatloff-Zito, C., Gordon, A. J., Duchaud, E., and Merlin, G. (1995) Isolation of an ubiquitously expressed cDNA encoding human dynamin II, a member of the large GTP-binding protein family. *Gene* **163,** 301–306
27. Aebi, M., Fah, J., Hurt, N., Samuel, C. E., Thomis, D., Bazzigher, L., Pavlovic, J., Haller, O., and Staeheli, P. (1989) cDNA structures and regulation of two interferon-induced human Mx proteins. *Mol. Cell. Biol.* **9,** 5062–5072
28. Prakash, B., Praefcke, G. J., Renault, L., Wittinghofer, A., and Herrmann, C. (2000) Structure of human guanylate-binding protein 1 representing a unique class of GTP-binding proteins. *Nature* **403,** 567–571
29. Prakash, B., Renault, L., Praefcke, G. J., Herrmann, C., and Wittinghofer, A. (2000) Triphosphate structure of guanylate-binding protein 1 and implications for nucleotide binding and GTPase mechanism. *EMBO J.* **19,** 4555–4564
30. Decker, T., Lew, D. J., Mirkovitch, J., and Darnell, J. E., Jr. (1991) Cytoplasmic activation of GAF, an IFN-gamma-regulated DNA-binding factor. *EMBO J.* **10,** 927–932
31. Naschberger, E., Werner, T., Vicente, A. B., Guenzi, E., Töpolt, K., Leubert, R., Lubeseder-Martellato, C., Nelson, P. J., and Stürzl, M. (2004) Nuclear factor-κB motif and interferon-α-stimulated response element co-operate in the activation of guanylate-binding protein-1 expression by inflammatory cytokines in endothelial cells. *Biochem. J.* **379,** 409–420
32. Morgenstern, J. P., and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18,** 3587–3596
33. Naschberger, E., Lubeseder-Martellato, C., Meyer, N., Gessner, R., Kremmer, E., Gessner, A., and Stürzl, M. (2006) Human guanylate binding protein-1 is a secreted GTPase present in increased concentrations in the cerebrospinal fluid of patients with bacterial meningitis. *Am. J. Pathol.* **169,** 1088–1099
34. Lehmann, M. H., Weber, J., Gastmann, O., and Sigusch, H. H. (2002) Pseudogene-free amplification of human GAPDH cDNA. *BioTechniques* **33,** 766, 769–770
35. Lehmann, M. H., Schreiber, S., Vogelsang, H., and Sigusch, H. H. (2001) Constitutive expression of MCP-1 and RANTES in the human histiocytic lymphoma cell line U-937. *Immunol. Lett.* **76,** 111–113
36. Schellerer, V. S., Croner, R. S., Weinländer, K., Hohenberger, W., Stürzl, M., and Naschberger, E. (2007) Endothelial cells of human colorectal cancer and healthy colon reveal phenotypic differences in culture. *Lab. Invest.* **87,** 1159–1170
37. Nicosia, R. F., Bonanno, E., and Smith, M. (1993) Fibronectin promotes the elongation of microvessels during angiogenesis in vitro. *J. Cell. Physiol.* **154,** 654–661
38. Lauffenburger, D. A., and Horwitz, A. F. (1996) Cell migration: a physically integrated molecular process. *Cell* **84,** 359–369

39. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003) Cell migration: integrating signals from front to back. *Science* **302,** 1704–1709
40. Chan, B. M., Kassner, P. D., Schiro, J. A., Byers, H. R., Kupper, T. S., and Hemler, M. E. (1992) Distinct cellular functions mediated by different VLA integrin alpha subunit cytoplasmic domains. *Cell* **68,** 1051–1060
41. Goldfinger, L. E., Han, J., Kiosses, W. B., Howe, A. K., and Ginsberg, M. H. (2003) Spatial restriction of alpha4 integrin phosphorylation regulates lamellipodial stability and alpha4beta1-dependent cell migration. *J. Cell Biol.* **162,** 731–741
42. Kassner, P. D., Alon, R., Springer, T. A., and Hemler, M. E. (1995) Specialized functional properties of the integrin alpha 4 cytoplasmic domain. *Mol. Biol. Cell* **6,** 661–674
43. Liu, S., Thomas, S. M., Woodside, D. G., Rose, D. M., Kiosses, W. B., Pfaff, M., and Ginsberg, M. H. (1999) Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. *Nature* **402,** 676–681
44. Ignatoski, K. M., Maehama, T., Markwart, S. M., Dixon, J. E., Livant, D. L., and Ethier, S. P. (2000) ERBB-2 overexpression confers PI 3′ kinase-dependent invasion capacity on human mammary epithelial cells. *Br. J. Cancer* **82,** 666–674
45. Jia, Y., Zeng, Z. Z., Markwart, S. M., Rockwood, K. F., Ignatoski, K. M., Ethier, S. P., and Livant, D. L. (2004) Integrin fibronectin receptors in matrix metalloproteinase-1-dependent invasion by breast cancer and mammary epithelial cells. *Cancer Res.* **64,** 8674–8681
46. White, E. S., Thannickal, V. J., Carskadon, S. L., Dickie, E. G., Livant, D. L., Markwart, S., Toews, G. B., and Arenberg, D. A. (2003) Integrin alpha4beta1 regulates migration across basement membranes by lung fibroblasts: a role for phosphatase and tensin homologue deleted on chromosome 10. *Am. J. Respir. Crit. Care Med.* **168,** 436–442
47. Zhang, Y., Lu, H., Dazin, P., and Kapila, Y. (2004) Functional differences between integrin alpha4 and integrins alpha5/alphav in modulating the motility of human oral squamous carcinoma cells in response to the V region and heparin-binding domain of fibronectin. *Exp. Cell. Res.* **295,** 48–58
48. Ruegg, C., Dormond, O., and Mariotti, A. (2004) Endothelial cell integrins and COX-2: mediators and therapeutic targets of tumor angiogenesis. *Biochim. Biophys. Acta* **1654,** 51–67
49. Mould, A. P., Komoriya, A., Yamada, K. M., and Humphries, M. J. (1991) The CS5 peptide is a second site in the IIICS region of fibronectin recognized by the integrin alpha 4 beta 1. Inhibition of alpha 4 beta 1 function by RGD peptide homologues. *J. Biol. Chem.* **266,** 3579–3585
50. Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., and Carter, W. G. (1989) Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* **109,** 1321–1330
51. Pankov, R., and Yamada, K. M. (2002) Fibronectin at a glance. *J. Cell Sci.* **115,** 3861–3863
52. Plow, E. F., Haas, T. A., Zhang, L., Loftus, J., and Smith, J. W. (2000) Ligand binding to integrins. *J. Biol. Chem.* **275,** 21785–21788
53. Decker, T., Lew, D. J., and Darnell, J. E., Jr. (1991) Two distinct alpha-interferon-dependent signal transduction pathways may contribute to activation of transcription of the guanylate-binding protein gene. *Mol. Cell. Biol.* **11,** 5147–5153
54. Mantovani, A., Bussolino, F., and Dejana, E. (1992) Cytokine regulation of endothelial cell function. *FASEB J.* **6,** 2591–2599
55. Mantovani, A., Garlanda, C., Introna, M., and Vecchi, A. (1998) Regulation of endothelial cell function by pro- and anti-inflammatory cytokines. *Transplant. Proc.* **30,** 4239–4243
56. Brezinschek, R. I., Brezinschek, H. P., Lazarovits, A. I., Lipsky, P. E., and Oppenheimer-Marks, N. (1996) Expression of the beta 7 integrin by human endothelial cells. *Am. J. Pathol.* **149,** 1651–1660
57. Praefcke, G. J., Kloep, S., Benscheid, U., Lilie, H., Prakash, B., and Herrmann, C. (2004) Identification of residues in the human guanylate-binding protein 1 critical for nucleotide binding and cooperative GTP hydrolysis. *J. Mol. Biol.* **344,** 257–269
58. Boyer, L., Travaglione, S., Falzano, L., Gauthier, N. C., Popoff, M. R., Lemichez, E., Fiorentini, C., and Fabbri, A. (2004) Rac GTPase instructs nuclear factor-κB activation by conveying the SCF complex and IkBα to the ruffling membranes. *Mol. Biol. Cell* **15,** 1124–1133
59. Hernandez-Perera, O., Perez-Sala, D., Soria, E., and Lamas, S. (2000) Involvement of Rho GTPases in the transcriptional inhibition of preproendothelin-1 gene expression by simvastatin in vascular endothelial cells. *Circ. Res.* **87,** 616–622
60. Hill, C. S., Wynne, J., and Treisman, R. (1995) The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* **81,** 1159–1170
61. Kraynack, N. C., Corey, D. A., Elmer, H. L., and Kelley, T. J. (2002) Mechanisms of NOS2 regulation by Rho GTPase signaling in airway epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **283,** L604–L611
62. Yang, B. S., Hauser, C. A., Henkel, G., Colman, M. S., Van Beveren, C., Stacey, K. J., Hume, D. A., Maki, R. A., and Ostrowski, M. C. (1996) Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. *Mol. Cell. Biol.* **16,** 538–547
63. Rosen, G. D., Barks, J. L., Iademarco, M. F., Fisher, R. J., and Dean, D. C. (1994) An intricate arrangement of binding sites for the Ets family of transcription factors regulates activity of the alpha 4 integrin gene promoter. *J. Biol. Chem.* **269,** 15652–15660
64. Zaniolo, K., Leclerc, S., Cvekl, A., Vallieres, L., Bazin, R., Larouche, K., and Guerin, S. L. (2004) Expression of the alpha4 integrin subunit gene promoter is modulated by the transcription factor Pax-6 in corneal epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **45,** 1692–1704
65. Kirschner, K. M., Wagner, N., Wagner, K. D., Wellmann, S., and Scholz, H. (2006) The Wilms tumor suppressor Wt1 promotes cell adhesion through transcriptional activation of the α4integrin gene. *J. Biol. Chem.* **281,** 31930–31939
66. Schubert, S. W., Lamoureux, N., Kilian, K., Klein-Hitpass, L., and Hashemolhosseini, S. (2008) Identification of integrin-α4, Rb1, and syncytin a as murine placental target genes of the transcription factor GCMa/Gcm1. *J. Biol. Chem.* **283,** 5460–5465
67. Postigo, A. A., Sheppard, A. M., Mucenski, M. L., and Dean, D. C. (1997) c-Myb and Ets proteins synergize to overcome transcriptional repression by ZEB. *EMBO J.* **16,** 3924–3934
68. Jin, H., and Varner, J. (2004) Integrins: roles in cancer development and as treatment targets. *Br. J. Cancer* **90,** 561–565
69. Ruegg, C., and Mariotti, A. (2003) Vascular integrins: pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis. *Cell. Mol. Life Sci.* **60,** 1135–1157
70. Kim, S., Bell, K., Mousa, S. A., and Varner, J. A. (2000) Regulation of angiogenesis in vivo by ligation of integrin α5β1 with the central cell-binding domain of fibronectin. *Am. J. Pathol.* **156,** 1345–1362
71. Kim, S., Harris, M., and Varner, J. A. (2000) Regulation of integrin α vβ 3-mediated endothelial cell migration and angiogenesis by integrin α5β1 and protein kinase A. *J. Biol. Chem.* **275,** 33920–33928
72. Livant, D. L., Brabec, R. K., Kurachi, K., Allen, D. L., Wu, Y., Haaseth, R., Andrews, P., Ethier, S. P., and Markwart, S. (2000) The PHSRN sequence induces extracellular matrix invasion and accelerates wound healing in obese diabetic mice. *J. Clin. Invest.* **105,** 1537–1545
73. White, E. S., Livant, D. L., Markwart, S., and Arenberg, D. A. (2001) Monocyte-fibronectin interactions, via α(5)β(1) integrin, induce expression of CXC chemokine-dependent angiogenic activity. *J. Immunol.* **167,** 5362–5366
74. Anderson, S. L., Carton, J. M., Lou, J., Xing, L., and Rubin, B. Y. (1999) Interferon-induced guanylate binding protein-1 (GBP-1) mediates an antiviral effect against vesicular stomatitis virus and encephalomyocarditis virus. *Virology* **256,** 8–14
75. Carter, C. C., Gorbacheva, V. Y., and Vestal, D. J. (2005) Inhibition of VSV and EMCV replication by the interferon-induced GTPase, mGBP-2: differential requirement for wild-type GTP binding domain. *Arch. Virol.* **150,** 1213–1220

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