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Novel Role of Ras-GTPase Activating Protein SH3 Domain-Binding Protein G3BP in Adhesion and Migration of 32D Myeloid Progenitor Cells

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Abstract: Rho GTPases are involved in homing and mobilization of hematopoietic stem and progenitor cells due to their impact on cytoskeleton remodeling. We have previously shown that inhibition of Rho, Rac and Cdc42 clearly impairs adhesion of normal and leukemic hematopoietic progenitor cells (HPC) to fibronectin and migration in a three-dimensional stromal cell model. Here, we identified the Ras GTPase-Activating Protein SH3 Domain-Binding Protein (G3BP) as a target gene of Rho GTPases and analysed its role in regulating HPC motility. Overexpression of G3BP significantly enhanced adhesion of murine 32D HPC to fibronectin and human umbilical vein endothelial cells, increased the proportion of adherent cells in a flow chamber assay and promoted cell migration in a transwell assay and a three-dimensional stromal cell model suggesting a strong impact on the cytoskeleton. Immunofluorescent staining of G3BP-overexpressing fibroblasts revealed a Rho-like phenotype characterized by formation of actin stress fibers in contrast to the Rac-like phenotype of control fibroblasts. This is the first report implicating a role for G3BP in Rho GTPase-mediated signalling towards adhesion and migration of HPC. Our results may be of clinical importance, since G3BP was found overexpressed in human cancers.

Keywords: G3BP, Rho GTPases, 32D progenitor cells, adhesion, migration, homing, toxin B, lethal toxin.

INTRODUCTION

Proteins of the Ras superfamily such as Ras, Rac, Rho, and Cdc42 have a determinant role in cell growth, differentiation, and malignant transformation and control cell adhesion and motility by remodelling of the cortical actin cytoskeleton [1]. GTPases are molecular switches that cycle between an active GTP-bound and an inactive GDP-bound state. Activation is mediated by guanine nucleotide exchange factors (GEFs) that favor GTP binding while deactivation is regulated by GTPase-activating proteins (GAPs) that accelerate hydrolysis of GTP to GDP *via* a catalytic domain [2]. A major down-regulator of Ras, RasGAP, contains additional structural motifs including an SH3 domain, that is involved in cytoskeleton reorganization, cell adhesion and induction of gene expression in a Ras-dependent manner [3, 4]. The Ras-GTPase activating protein SH3 domain binding protein (G3BP) has been identified as one of only four proteins known to interact with RasGAP *via* its SH3 domain [5]. G3BP is composed of 466 amino acids and has a predicted molecular mass of 52 kDa. Three isoforms of the gene G3BP-1 and two alternatively spliced isoforms of mouse and human G3BP-2 (G3BP-2α and G3BP-2β) have been described. While the isoforms G3BP-2α and -2β have been mapped to 4q12-4q24, G3BP1 is located on 5q14.2-5q33.3 [6]. G3BP is overexpressed in several human cancer cell lines and various cancer tissues and stimulates S-phase entry in cultured cells [7]. In addition, G3BP expression has been positively correlated with the presence of lymph node metastasis and has been shown to promote invasion of cancer cells [8].

The bone marrow (BM) microenvironment constitutes a homing compartment for transplanted hematopoietic stem and progenitor cells. Homing is defined as the specific migration of circulating hematopoietic stem and progenitor cells through the vasculature to the BM, which is a prerequisite for the engraftment in specialized BM niches that support and regulate maintenance, proliferation and differentiation of hematopoietic stem and progenitor cells [9, 10].

Previously, we investigated the contribution of different Rho GTPase members to migration of human $CD34^+$ stem and progenitor cells: in a three-dimensional BM stromal cell environment, migration was significantly inhibited by cell permeable, Rho GTPase-blocking bacterial toxins: Toxin B derived from *C. difficile* inactivates Rho, Rac and Cdc42 [11], whereas lethal toxin of *C. sordellii* predominantly inactivates Rac and also to some degree Cdc42, but not Rho [12]. Here, we identify G3BP as one of several genes downregulated by toxin B and lethal toxin and characterize its impact on adhesion and migration of HPC.

MATERIALS AND METHODOLOGY

Cell Culture and Chemicals

RAT-1 cells were maintained in DMEM medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf se-

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rum (FCS; Hyclone, Greiner, Frickenhausen, Germany) with 1% glutamine (200 mM) and 1% penicillin/streptomycin (Invitrogen). The murine myeloid progenitor cell line 32D (No. ACC411, DSMZ, Braunschweig, Germany) was cultured in RPMI 1640 (Gibco-Life Technologies, Paisley, UK) supplemented with 10% FCS and 10 µg/ml murine interleukin-3 (IL-3; Peprotech, London, UK). The human $CD34^+$ cell line TF-1 (No. CRL-2003, American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 with 10% FCS in the presence of human IL-3 (R&D Systems, Wiesbaden, Germany) and granulocyte macrophage-colony stimulating factor (GM-CSF; 10 ng/ml each; Essex Pharma, Munich, Germany). TF-1 cells were incubated with 100 ng/ml toxin B or lethal toxin in IMDM/10% FCS for 6 hours at 37°C (toxins were kindly provided by H. Barth and K. Aktories, Institute of Pharmacology and Toxicology, University of Freiburg, Germany). The murine BM stroma cell line M2-10B4 (kindly provided by D. Hogge, Terry Fox Laboratory, Vancouver, Canada), modified to produce human IL-3 and GM-CSF, was maintained in RPMI 1640 with 10% FCS, 0.06 mg/ml hygromycin B (Calbiochem, Bad Soden, Germany), 0.4 mg/ml geneticin, and 8 mM HEPES (both from Gibco-Life Technologies). Human umbilical vein endothelial cells (HUVEC) were maintained in medium 199 (M199) with Earle's salts (Sigma Chemical, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, Steinheim, Germany), 1% antibiotic-antimycotic solution (Sigma-Aldrich), 2 mM L-glutamine, 120 µg/ml heparin (Sigma-Aldrich), and 100 µg/ml endothelial cell growth supplement (Collaborative Biomedical, Bedford, MA).

Microarray Analysis

Hybridization of total RNA to the HuGeneFL oligonucleotide microarray (Affymetrix Inc., Santa Clara, CA) was performed as described previously [13]. Briefly, at least 8 µg of total RNA were reverse transcribed by Superscript II reverse transcriptase (Invitrogene, Grand Island, NY) using T7-$(dT)_{24}$ primer containing a T7 RNA polymerase promoter site. After synthesis of the second cDNA strand, this product was used in an *in vitro* transcription reaction to generate biotinylated complementary cRNA. Fifteen micrograms of fragmented cRNA was hybridized to a HuGeneFL microarray for 16 hours at 45° C with constant rotation at 60 rpm according to the Affymetrix protocol. The fluorescence intensity was scanned by the Affymetrix GeneChip Scanner and normalized by global scaling to the average fluorescence intensity for the entire microarray. Data analysis was performed with the GeneSpring software version 4.2 (Silicon Genetics, San Carlos, CA) and Microarray Analysis Suites 4.01 (MAS 4.01, Affymetrix, Inc.). Selection of differentially expressed genes required at least a 2-fold change in normalized expression values.

Cloning of Full-Length G3BP

A 1412 bp cDNA encoding full-length G3BP of human origin was obtained by PCR cloning using the One-step RT-PCR Kit (Invitrogen) and specific oligonucleotides G3BP_fwd (5'-AGCAATGGTGATGGAGAAGCC-3') and G3BP_rev (5'- CATGAAGATTACTGCCGAGGA-3'). The PCR product was cloned into the pCR2.1 vector with the TA-cloning kit (Invitrogen) according to the manufacturer's instructions. An expression vector for G3BP was produced using the Gateway recombination technology (Invitrogen). Briefly, the cDNA encoding full-length G3BP was first subcloned into pENTR1A using an *Eco*RI restriction site and then transferred into the GFP expressing destination vector pinco [14] by LR recombination following the manufacturer's instructions (Invitrogen). The resulting vectors are referred to as control (pinco empty vector) and pinco_G3BP (full length G3BP in pinco vector).

Retroviral Infection

Ecotropic Phoenix packaging cells were transiently transfected with the indicated retroviral vectors as described before [15]. For the infection target cells were plated onto retronectin-coated (Takara-Shuzo, Shiga, Japan) non–tissue culture treated 24-well plates and exposed to the retroviral supernatant for 3 hours at 37°C in the presence of 4 µg/mL polybrene (Sigma-Aldrich). Cells were centrifuged at 2,200 rpm for 45 minutes. Infection was repeated four times and infection efficiency had to be at least 70% for each sample as assessed by detection of green fluorescent protein–positive cells by FACS.

Small Hairpin RNA

The small hairpin RNA (shRNA) sequences encoding inverted repeats of 21 nucleotides (nts) separated by a 10-nt spacer were designed using publicly available software tools (www.ambion.com/techlib/misc/silencer_siRNA_template.html). Three shRNAs against different regions of the G3BP sequence were tested. The inverted repeats corresponded to bp 173-194 (shRNA1), bp 450-471 (shRNA2) and bp 679-700 (shRNA3) of the murine G3BP cDNA and had at least 3 nt differences from any other murine gene. A shRNA derived from prokaryotic lacZ gene served as unspecific control. The oligos containing *Hpa*I and *Bbs*I restriction sites and hairpin DNA were annealed and ligated into the *Bbs*I-*Hpa*I-digested expression vector vPGKpuroU6FH [15]. The construct was controlled by sequencing and designated as si1-3. Retroviral infection of 32D cells was carried out as described above. Efficiency of the shRNA was confirmed by Western blotting.

Western Blotting

Western blotting was done according to widely used protocols with the following antibodies: anti-G3BP (BD Transduction Laboratories, San Diego, USA) and anti-α-tubulin (Dianova, Hamburg, Germany). All antibodies were diluted in 5% low fat dry milk. Blocking was performed in 5% low fat dry milk, washing was carried out in TBS containing 0.1% Tween20 (TBS-T). Densitometry was performed using Quantity One Software from BioRad (Munich, Germany).

Cell Adhesion Assay

Fibronectin (FN, 5 µg/cm^2; BD Biosciences, Heidelberg, Germany) diluted in PBS was adsorbed to wells of 6-well plates overnight at 4°C. Non-specific bindings were blocked with PBS containing 2 % BSA for 1 hour at 37°C. RAT-1 or 32D cells were washed once in PBS and plated at 1 x 10^6 cells in 3 ml DMEM with 10% FCS. Cells were allowed to adhere to the coated plates for 3 hour at 37°C in a humidified atmosphere of 5 % CO_2. After incubation, non adherent cells were washed away with PBS by gentle agitation and adherent cells were counted.

Flow Chamber Assay

HUVEC of the second or third passage were grown to subconfluency on glass slides and activated by TNF-α (100 U/ml)

for 7 hours prior to the assay. The glass slides were assembled as the lower wall of the flow chamber (Circular Parallel Plate Flow Chamber kit; GlycoTech, Gaithersburg, MD, USA) and the flow chamber was mounted on the stage of an inverted phase-contrast microscope (Carl Zeiss, Jena, Germany). 32D cells (10^6 cells/ml; mock-transfected or pinco_G3BP) were perfused through the chamber at the desired flow rate generated with an automated syringe pump (B. Braun Medical, Emmensbruegge, Switzerland). Initial application of the cells was performed with a shear stress of 0.1 dyn/cm^2. After 10 min, shear stress was increased to 2 dyn/cm^2 for 20 min. Subsequent to each perfusion period, adherent cells were documented by photographs of three independent fields using a gauged grid and counted [16].

Transwell Assay

Transwell units (8 μM pore size; BD Falcon**,** Heidelberg, Germany) were coated with FN (5 μg/cm^2) diluted in PBS over night at 4°C. RAT-1 cells or 32D cells were washed once in PBS and seeded in the upper chamber at 1 x 10^4 cells in the adequate medium. Cells were allowed to spontaneously migrate through the pores of the membrane for 3 hours at 37°C. After incubation, filters were washed with PBS by gentle agitation and migrated cells attached to the lower surface of the membrane were fixed with 3.7% formaldehyde and stained with coomassie staining solution (2% Coomassie Brilliant Blue; 45% methanol; 10% acetic acid). Stained cells were documented by photographs of three independent fields and counted.

Spheroid Assay

M2-10B4 spheroids were grown in 1% agarose-coated 96-well plates as previously described [17]. Briefly, 2.5 x 10^4 cells were inoculated per well in 200 μl Iscove's modified Dulbecco's medium (IMDM; Biochrom, Berlin, Germany) supplemented with 10% FCS. After 4 days, 1 x 10^4 32D cells were added for cocultivation. Spheroid co-cultures were harvested 24 hours later, washed with PBS, and dissociated with a 0.25% trypsin and 0.1% EDTA solution (1:3 in PBS; PAN Biotech, Aidenbach, Germany). Cell suspensions were filtered and incubated with FITC-conjugated anti-human CD45 Ab (or anti-human IgG1Ab as control) to determine the percentage of hematopoietic cells in the spheroids. Analysis was performed on FACScan (BD Bioscience) using CellQuest and PC-Lysis software.

Immunofluorescent Staining of Actin Cytoskeleton

2 x 10^4 RAT-1 cells were transduced with bicistronic retroviral vectors expressing the enhanced green fluorescent protein (GFP) and full-length G3BP (pinco_G3BP) or GFP alone (pinco_control) and were seeded on fibronectin-coated (50 μg/ml) coverslips and allowed to adhere for 3 hours at 37°C. Non adherent cells were removed by gentle washing with PBS and adherent cells were fixed with 3.7% formaldehyde/PBS for 15 min at room temperature. After washing with PBS cells were permeabilized for 10 min with PBS containing 0.5% Triton-X. After blocking with AbDil solution (0.1% Triton X in PBS, 2% BSA, 0.1% sodiumacide) actin cytoskeleton was stained using phalloidin-TRITC (0.5μg/ml in AbDil; Sigma-Aldrich).

Statistical Analysis

Data were compared by a two-tailed Student *t* test; *p* values < 0.05 were considered to be significant.

RESULTS

G3BP is a Target Gene of Rho GTPases

The cell permeable toxins from *Clostridium spec.* lethal toxin and toxin B have previously been shown to significantly impair migration, homing and engraftment of hematopoietic stem cells [17, 18]. To further elucidate the molecular mechanisms of this inhibition, we treated human CD34$^+$ TF-1 cells with lethal toxin or toxin B and analyzed changes in gene expression using high-density microarrays. Within a list of 48 and 116 genes showing differential expression after treatment with toxin B or lethal toxin, respectively, we identified several genes regulated by both toxins implicated in cellular motility, cell-cell contact or GTPase signalling which are listed in Table **1**. We focussed on RasGAP SH3 domain binding protein (G3BP) since the encoding gene is located on the long arm of chromosome 5, a chromosomal region frequently deleted in myelodysplastic syndromes and acute myeloid leukemia. The observed 2.5-fold downregulation of G3BP mRNA in toxin-treated TF-1 cell compared to control cells was validated on the protein level by Western blot analysis using a monoclonal anti-G3BP antibody Fig. (**1**).

Table 1. Genes Regulated by Both Toxin B and Lethal Toxin (Identified by Gene Expression Profiling of Toxin Treated TF-1 Cells)

Gene	Genbank	Toxin B Fold Change	Lethal Toxin Fold Change	Description
G3BP	U32519	2.5 (-)	2.7 (-)	Ras-GTPase-activating protein SH3-domain-binding protein
DCN	M14219	2.1 (-)	2.3 (-)	Decorin
CAPZA2	U03851	2.3 (-)	3.1 (-)	Capping protein (actin filament) muscle Z-line, alpha 2
P311	U30521	1.6 (-)	2.3 (-)	P311 protein
CD164	D14043	1.2 (-)	2.0 (-)	CD164 antigen, sialomucin
IQGAP1	L33075	1.1 (-)	3.1 (-)	IQ motif containing GTPase activating protein 1
PLEC1	Z54367	3.6 (+)	2.7 (+)	Plectin 1, intermediate filament binding protein, 500kD

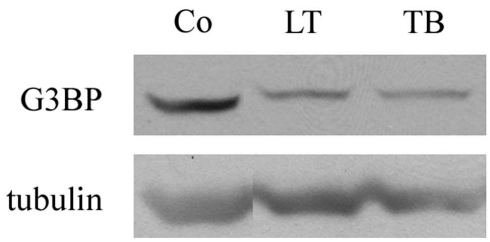

Fig. (1). Inhibition of small GTPases with *Clostridium* toxin B and lethal toxin inhibits expression of G3BP. TF-1 cells were treated for 6 hours with lethal toxin (LT) or toxin B (TB) to inhibit small GTPases of the Rho family. Western blot analysis of total cell lysates from treated cells or untreated controls (Co) was performed and anti-G3BP antibody (1:500) was used to determine G3BP expression level. Inhibition of GTPases by *Clostridium spec.* toxins markedly downregulated expression of G3BP protein. Tubulin was used as a loading control.

G3BP Enhances Adhesion of 32D Progenitor Cells Under Shear Stress

Having identfied G3BP as a target gene involved in Rho GTPase signalling in human hematopoietic progenitor cells (HPC) we evaluated its impact for the homing of HPC to the bone marrow. The BM endothelium is the first anchoring site for homing cells, supporting rolling and firm adhesion of circulating leukocytes by constitutive expression of adhesion molecules and stimulating cytokines [9]. We thus studied the interaction of murine 32D progenitor cells with HUVEC, a well-established vascular endothelial cell system [19]. 32D cells were infected with bicistronic retroviral vectors expressing the enhanced green fluorescent protein (GFP) and full-length G3BP (pinco_G3BP) or GFP alone (pinco_control) with an efficiency of > 90% as determined by FACS Fig. (**2A**) and perfused over a HUVEC monolayer in a flow chamber applying low and elevated shear stress. Cells adhering to the endothelial cell monolayer were documented and counted as described after 10 min at 0.1 dyn/cm^2 and after another 20 min at 2 dyn/cm^2. G3BP increased the number of rolling HPC by 23 ± 16 % (n = 4; $p< 0.05;$ 0.1 dyn/cm^2) and firmly adhering cells by 28 ± 15 % (n = 4; $p< 0.05;$ 2 dyn/cm^2) compared to mock-transfected controls Fig. (**2B**).

TNF-activated HUVEC support rolling and firm attachment of HPC under physiological shear-flow by displaying high levels of E-selectin, ICAM-1, and VCAM-1 [20]. As VCAM-1 is a vascular ligand for the integrin very late antigen (VLA)-4, we studied the effect of G3BP overexpression on HPC adhesion on fibronectin, one of the major extracellular matrix (ECM) component containing binding sites for VLA-4 (CD49d/CD29) and VLA-5 (CD49e/CD29). G3BP signifi-

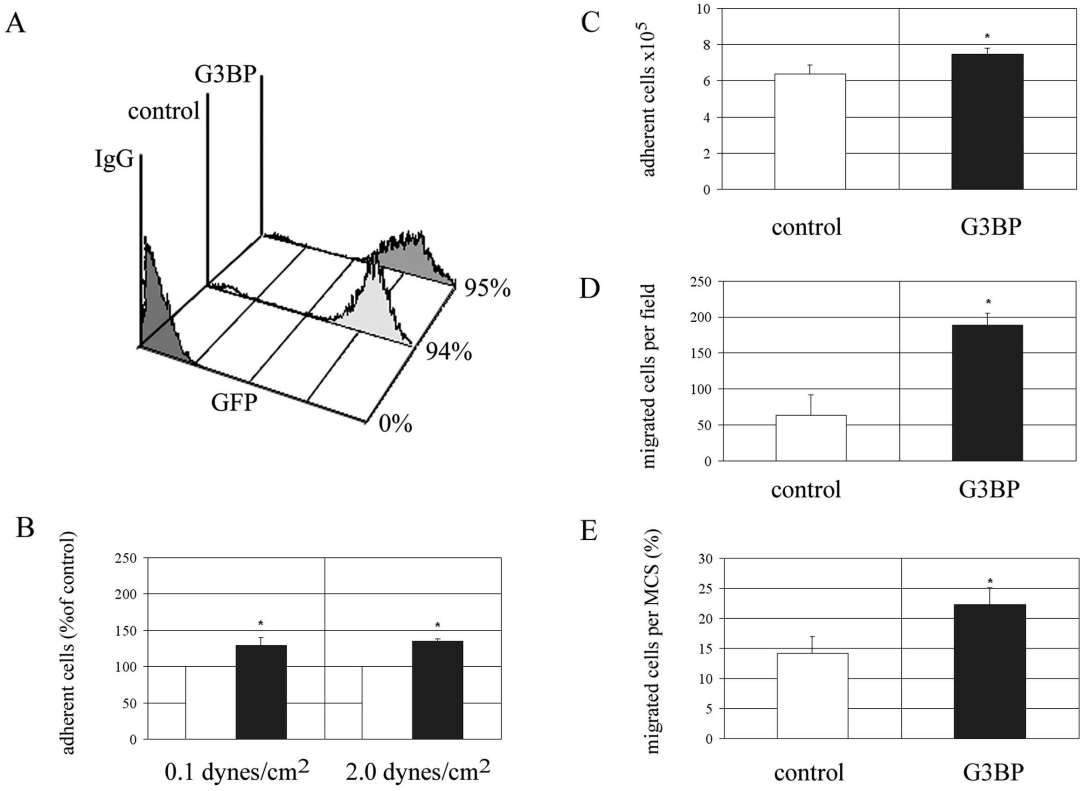

Fig. (2). Impact of G3BP on integrin-mediated adhesion and migration of HPC. Infection efficiency confirmed by FACS measurement of GFP-positive cells was > 90% in mock- and G3BP-transduced cells (**A**). Adhesion of 32D to a cellular endothelial surface (HUVEC monolayer) under low and elevated shear stress was tested in a flow chamber assay. G3BP significantly enhanced the number of firmly arrested cells compared to controls by 23 ± 16 % at 0.1 dyn/cm^2 and 28 ± 15 % at 2.0 dyn/cm^2 (n = 4) (**B**). G3BP was able to induce adhesion of 32D cells to fibronectin compared to controls (7.5 x 10^5 ± 0.5 x 10^5 vs. 6.4 x 10^5 ± 0.2 x 10^5 adherent cells, n = 4) (**C**). G3BP increased the number of migrated 32D cells from 63 ± 29 to 189 ± 16 cells/ field (n = 4) (**D**). Migration of 32D cells into multicellular spheroids (MCS) was also increased by G3BP. The number of cells incorporated into the spheroids increased significantly from 14.1 ± 2.8% to 22.3 ± 2.8% (n = 4) (**E**). **P* < 0.05.

cantly enhanced adhesion of 32D cells to fibronectin compared to the corresponding controls Fig. (**2C**); 7.5 x 10^5 ± 0.5 x 10^5 vs. 6.4 x 10^5 ± 0.2 x 10^5 adherent cells, n = 4; $p< 0.05$. Enhanced interaction of HPC with ECM may improve transendothelial migration and trafficking within the BM microenvironment [9]. G3BP significantly induced spontaneous migration of 32D cells through the pores of a fibronectin-coated transwell filter resulting in 189 ± 16 vs. 63 ± 29 migrated cells/field Fig. (**2D**), n = 4; $p< 0.05$. Our data provide the first evidence that G3BP contributes to the regulation of adhesion and migration of HPC.

G3BP Stimulates Migration of 32D Progenitor Cells into Stromal Cell Spheroids

Multicellular spheroids (MCS) serve as a model for engraftment in the stem cell microenvironment. We have previously shown that migration of human $CD34^+$ stem and progenitor cells in a three-dimensional BM stromal cell environment is significantly inhibited by cell permeable Rho GTPase-blocking bacterial toxins [17] Since G3BP proved to be a target gene downregulated upon toxin treatment we aimed to confirm the impact of G3BP on motility of murine 32D cells by comparing G3BP- to mock-infected controls. Migration was quantified by counting the number of cells incorporated in the spheroids. G3BP enhanced the number of incorporated HPC significantly from 14.1 ± 2.8% to 22.3 ± 2.8% (n = 4; $p< 0.05$; Fig. (**2E**). These results support the data obtained in the transwell assay and assures a role of G3BP in the regulation of migration of HPC.

The impact of G3BP on adhesion and migration in 32D cells shown above was confirmed by shRNA experiments. All three shRNA vectors (sh1-3) applied were able to suppress protein expression in 32D cells as shown by western blotting Fig. (**3A**). Sh2 resulted in a 75% reduction of G3BP protein compared to the control vector and was chosen for further experiments Fig. (**3B**). Suppression of G3BP by sh2 lead to a significantly decreased adhesion compared to controls (4.1 ± 0.3 x10^5 vs. 7.9 ± 0.5 x 10^5 adherent cells; $p< 0.05$; Fig. (**3C**). As expected, shRNA accordingly inhibited migration in the transwell assay in a significant way 230 ± 70 vs. 127 ± 16 migrated cells/field; $p< 0.05$; Fig. (**3D**). These data confirm that G3BP plays an essential role in adhesion and migration of HPC.

G3BP Induces Cytoskeletal Rearrangements in RAT-1 Cells

The RasGAP SH3 domain is involved in cytoskeletal reorganization and cell adhesion [3, 4]. We thus aimed to study the effect of G3BP on cytoskeletal rearrangements by comparing the morphology of RAT-1 cells infected with pinco_G3BP to mock-infected control cells Fig. (**4A**). Control cells spread on the fibronectin coated surface and displayed formation of lamellipodia at the edges Fig. (**4B**, upper panel). Actin fibers are visible but not organized in stress fibers. In contrast, overexpression of G3BP led to a dramatic induction of stress fibers Fig. (**4B**, lower panel) and increased the number of RAT-1 fibroblasts adhering to a fibronectin-coated surface from 5.2 x 10^5 ± 0.1 x 10^5 to 7.5 x 10^5 ± 0.2 x 10^5 when counted 3 hours after plating Fig. (**4C**, n = 3, $p< 0.05$). Our findings are consis-

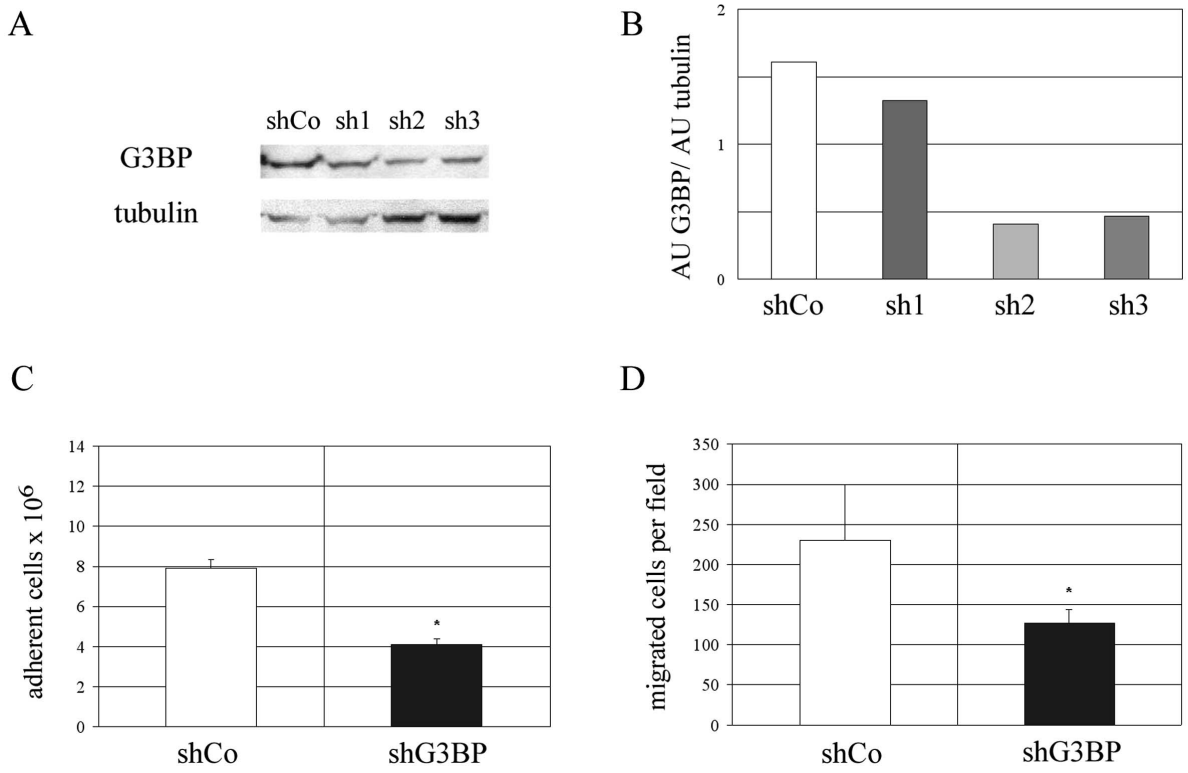

Fig. (3). RNAi of G3BP suppresses adhesion and migration in 32D cells. Three shRNA vectors (si1-3) applied were able to suppress G3BP expression in 32D cells as shown by Western blotting (**A**). The most effective one (si2) was chosen for further experiments verified by densitometry of the Western Blot shown above (**B**). The suppression of G3BP by introduction of si2 lead to a significantly reduced adhesion compared to controls (4.1 ± 0.3 x10^5 vs. 7.9 ± 0.5 x 10^5 adherent cells; n = 3) (**C**). As expected, RNAi significantly inhibited migration in the transwell assay (230 ± 70 vs. 127 ± 16 migrated cells/field; n = 3) (**D**). *$P < 0.05$.

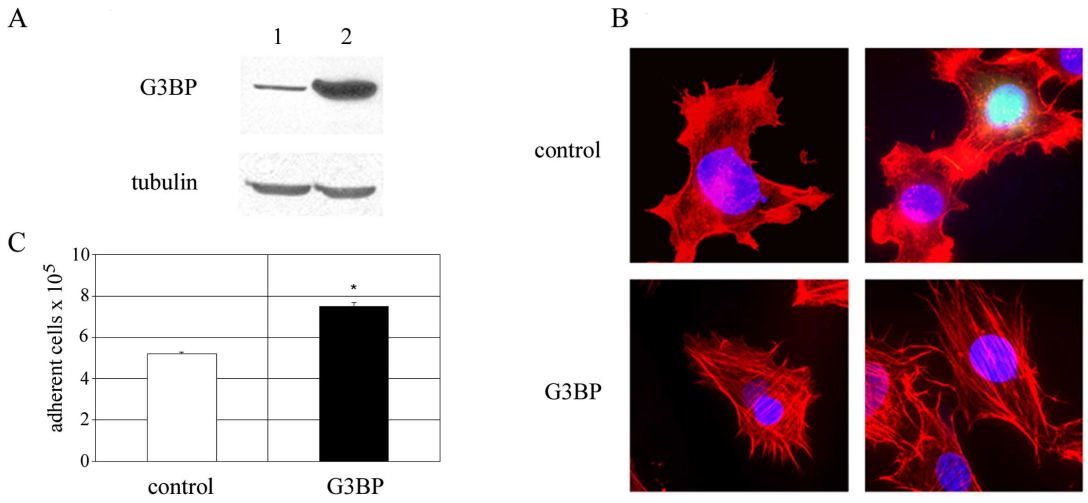

Fig. (4). Retroviral overexpression of G3BP in RAT-1 fibroblasts. Full length G3BP was cloned in the retroviral expression vector pinco_GFP. The viral construct successfully infected RAT-1 fibroblasts. Overexpression of G3BP was confirmed by western blotting (**A**). G3BP induces formation of actin stress fibers (lower panel) while mock-transfected controls (upper panel) display more lamellipodia-like protrusions (**B**). Adhesion of G3BP overexpressing cells (■) was compared to mock-infected controls (□) as described. After 3 hours, a significantly higher number of adherent G3BP-overexpressing cells was counted compared to controls (**C**; $7.5 \times 10^5 \pm 0.2 \times 10^5$ vs. $5.2 \times 10^5 \pm 0.1 \times 10^5$, n = 3). **P* < 0.05.

tent with the previously described Rho-like phenotype in resting fibroblasts upon overexpression of RasGAP [3].

DISCUSSION

Suppression of migration, short-term homing, hematopoietic cell regeneration and cell cycling by toxin B and lethal toxin has been reported [17,18], demonstrating impairment of stem cell functions by both toxins, similar to that published for selective inhibition of Rac and Cdc42. Here, we provide evidence that the Ras GTPase activating protein SH3 domain-binding protein (G3BP) is significantly downregulated by toxin B and lethal toxin. Adhesion and migration of HSC are considered to be important components of efficient HSC homing, a prerequisite of therapeutic stem cell transplantation. In order to home to the BM, HPC have to roll along and firmly adhere to the blood vessel, cross the endothelium barrier and finally traffic within the BM microenvironment. [9] We utilized a variety of appropriate *in vitro* adhesion and migration assays to study the impact of G3BP on each sequential homing step [17, 20, 21]. Overexpression of G3BP in the murine HPC line 32D enhanced rolling and adhesion to endothelial cells under physiological shear flow, improved transmigration through a fibronectin-coated permeable membrane and enhanced migration into three-dimensional spheroids composed of BM stromal cells. We further demonstrate that knock-down of endogenous G3BP by shRNA caused impaired adhesion and migration of murine 32D HPC. Thus, our results indicate that at least a part of the toxin-induced effects on HPC are attributable to G3BP. Similar effects have been observed by inhibition of Rac or RhoA in primary HPC [22]. Furthermore, deletion of both Rac1 and Rac2 causes massive defects in stem cell proliferation and survival *in vitro* and Rac1-deficient stem cells have reduced homing efficiency to the BM and fail to engraft [23,24].

Integrin-dependent activation of Rho GTPases has been extensively studied in fibroblasts plated on fibronectin. Engagement of integrins with ECM leads to activation of Cdc42 and subsequently Rac. Together, these GTPases mediate cell spreading and membrane ruffling, while Rho is activated independently to induce stress fibers [25,26]. In this setting, overexpression of G3BP in RAT-1 fibroblasts produced a Rho-like phenotype characterized enhanced formation of stress fibers and lead to extension of thin processes instead of lamellipodia consistent with inhibition of Rac. This is in agreement with data demonstrating that RasGAP per se is able to trigger stress fiber formation by stimulating Rho activity whereas blocking of the SH3 domain of RasGAP specifically abrogates Rho-dependent cytoskeletal reorganization [3]. Additional results obtained with the *Drosophila* homologue of G3BP suggest a role in both Ras and Rho signalling by serving as a link between these two GTPases [27].

CONCLUSION

In conclusion, we identify G3BP as one of several genes significantly downregulated by toxin B and lethal toxin and for the first time define its major contribution to adhesion and motility of 32 D HPC and cytoskeletal alterations in fibroblasts.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS' CONTRIBUTIONS

KS carried out the molecular biology studies and the immunfluorescent staining, participated in the functional assays and helped to draft the manuscript. FA MKampfmann participated in the molecular biology studies and the functional assays. BR carried out the functional assays. MKomor and

WKH performed the microarray analysis. MR participated in the molecular biology studies and the immunfluorescent staining and helped to draft the manuscript. RH participated in the functional assays and helped to draft the manuscript. GB designed and coordinated the experiments and drafted the manuscript.

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