The N-terminal domain of G3BP enhances cell motility and invasion by post-transcriptional regulation of BART

**Running title:** G3BP accelerates cell motility and invasion

**Key words:** stress granule, post-transcriptional regulation, RNA-binding protein

**Authors/Affiliations:**

Keisuke Taniuchi\(^1, 2\), Isao Nishimori\(^2\), Michael A. Hollingsworth\(^1\)

\(^1\)Eppley Institute for Research in Cancer and Allied Diseases, and Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198-6805, USA

\(^2\)Department of Gastroenterology and Hepatology, Kochi University Medical School, Nankoku, Kochi 783-8505, Japan
G3BP accelerates cell motility and invasion

Corresponding author:

Keisuke Taniuchi M.D., Ph.D.
Department of Gastroenterology and Hepatology
Kochi University Medical School
Nankoku, Kochi 783-8505, Japan
Phone: +81.88.880.2338
Fax: +81.88.880.2338
Email: jm-ktaniuchi@kochi-u.ac.jp
Abstract

The regulation of mRNA stability plays an important role in the control of gene expression during cell motility and invasion. We previously reported that GTPase activating protein (SH3 domain) binding protein (G3BP), a marker of cytoplasmic stress granules that are formed in stressed cells and regulate mRNA stability, binds and degrades the mRNA of Binder of Arl Two (BART) that inhibits retroperitoneal invasion and hepatic metastasis of pancreatic cancer cells. Here, we report that overexpression of the amino (N)-terminal region of G3BP, including the binding region for BART mRNA, dominant-negatively inhibits formation of the complex between endogenous G3BP and BART-mRNA, and increases the expression of BART. This, in turn, inhibits the invasiveness of pancreatic cancer cells. On the other hand, the carboxy (C)-terminal region of G3BP is associated with phosphorylation of eIF2α that initiates stress granule assembly, but does not modulate the post-transcriptional regulation of BART mRNA. N-terminal G3BP also plays a role in regulating secreted matrix metalloproteinases, transcription factors, and a variety of genes involved in cell adhesion and motility. These results suggest that N-terminal G3BP contributes to post-transcriptional regulation of cell motility and invasive capacity of pancreatic cancer.
Introduction

G3BP was originally identified based on its ability to bind the Src homology 3 (SH3) domain of Ras GTPase-activating protein p120 (RasGAP), which functions as a physiological negative regulator of Ras signaling in exponentially growing cells (1, 2, 3). The phosphorylation of G3BP and its association with RasGAP are affected by extracellular stimuli (3); however, functional consequences of the binding of G3BP to RasGAP have not been fully elucidated. The N-terminus of G3BP is a nuclear transport factor-2 (NTF-2)-like domain homologous to NTF-2, and is followed by acidic and proline-rich regions, an RNA-binding domain with an RNA-recognition motif (RRM), and multiple arginine-glycine rich (RGG) motifs (1). The NTF-2-like domain of G3BP influences the cellular localization of proteins and its oligomerization with itself or with other partners (4). Indeed, in vitro binding assays suggest that the NTF-2-like domain of G3BP is responsible for RasGAP binding (5). Assembly of stress granules (SGs) can be dominantly induced by G3BP overexpression (4). G3BP binds to Caprin-1 through the NTF-2-like domain and this complex is localized in cytoplasmic granules that contain a major part of the cytoplasmic RNA (6). This complex induces the formation of cytoplasmic SGs that form in response to a variety of cellular stresses (6). SGs contain mRNAs, certain translation initiator factors, and RNA-binding proteins that can be directed to translation initiation or to decay pathways (4). G3BP is likely to regulate the transport and translation of
G3BP accelerates cell motility and invasion

mRNAs of proteins involved in cellular proliferation and migration in multiple cell types (6). This suggests that the NTF-2-like domain of G3BP influences gene expression by affecting the steady-state levels of mRNA following post-transcriptional repression or induction through binding to partner proteins and SG formation.

Another interesting feature of G3BP is that the RRM domain mediates the binding of G3BP to specific RNA sequences so that G3BP can exert its function as a dinucleotide-specific single-strand-specific endoribonuclease (7). G3BP can bind to the 3’ untranslated region (3’UTR) of human c-Myc mRNA through the RRM domain in a phosphorylation-dependent manner to increase its degradation in vitro (3). G3BP is the only known endoribonuclease that requires site-specific phosphorylation for its catalytic activity (3). A small number of general pathways appear to be responsible for degrading most mRNAs; therefore, regulatory mechanisms are thought to target the initial events that direct the mRNA into one of these pathways, to either maintain it in a translationally inactive state, or permit its translation (8). The major decay initiating events are deadenylation, endonucleolytic cleavage and decapping (9). c-Myc transcript levels, which are considered to be regulated by G3BP, are subject to post-transcriptional regulation by a sequential pathway involving deadenylation followed by degradation of the mRNA body (10). In contrast, G3BP can stabilize tau mRNA, a microtubule-associated protein highly regulated during neuronal cell
G3BP accelerates cell motility and invasion

differentiation (11). In addition to endoribonuclease activity, G3BP functions to stabilize mRNA. These studies that examine the intersection of G3BP with RNA metabolism have proposed that the RRM domain of G3BP plays an important role in regulating mRNA translation or decay, or both.

We previously reported that BART inhibits retroperitoneal invasion and liver metastasis of pancreatic ductal adenocarcinoma (PDAC) cells in an orthotopic xenograft model (12). Our findings provide an explanation for the oncogenic function of G3BP to induce tumor invasion and metastasis by mediating BART mRNA decay. Here, we provide evidence that the N-terminal G3BP (G3BP1-180) functions in the post-transcriptional regulation of BART mRNA for the promotion of cell motility and invasion in PDAC. The C-terminal G3BP (G3BP241-466) functions in SG assembly, but the expression of BART and c-Myc mRNA is not regulated. Moreover, G3BP1-180 plays a critical role in establishing a pattern of gene expression that favors PDAC cell motility and invasive behavior.
G3BP accelerates cell motility and invasion

Materials and Methods

Identification of the BART-binding region in G3BP

We produced six deletion constructs of G3BP (G3BP1-420, G3BP1-330, G3BP1-240, G3BP1-180, G3BP1-135 and G3BP1-80 of accession number NM_005745) by cloning the pcDNA3.1(+)/myc-HisA vector (Invitrogen, Carlsbad, CA) into appropriate sites. COS7 cells were transiently transfected with each plasmid using FuGENE6 (Roche, Penzberg, Germany). After 48 h of transfection, cell extracts were immunoprecipitated with agarose-conjugated anti-myc antibody. Each myc-tagged immunoprecipitate was incubated with biotinylated BART 3'UTR (748-1703 of accession number NM_012106) or GAPDH RNA immobilized on streptavidin magnetic beads for 2 h at 4 °C. Bound proteins in the pull-down material were washed using strong permanent magnets (MPC-S; Dynal, Oslo, Norway) and analyzed by western blotting using anti-myc antibody.

Real-time quantitative RT-PCR

Total RNA was extracted using the RNeasy kit with DNase I treatment, and reverse transcribed as described above. The resultant cDNA was amplified by real-time PCR using Assays-on-Demand primers and TaqMan probes for BART (Hs00183708_m1), c-Myc (Hs00153408_m1) and GAPDH (Hs99999905_m1) (Applied
G3BP accelerates cell motility and invasion

Biosystems, Foster City, CA). Each sample was run in triplicate for both BART and GAPDH in a 20 µL reaction using TaqMan Universal PCR Master Mix according to the manufacturer’s instructions (Applied Biosystems). The gene-specific RT-PCR products were measured continuously using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Transcript levels of BART and c-myc were normalized to GAPDH in each sample using the standard curve method as described by the manufacturer.

Matrigel invasion assay

The two-chamber invasion assay (24-well plates, 8 µm pore size, membrane coated with a layer of Matrigel extracellular matrix proteins; Becton Dickinson, San Jose, CA) was used to assess cell invasion. 5.0×10^4 cells were seeded in serum-free medium into the upper chamber and allowed to invade toward 5% FCS as a chemoattractant in the lower chamber. After 20 h of incubation, the number of invading cells at the bottom of the membrane was estimated by counting three independent visual fields in a microscope.
Results

Overexpression of G3BP in PDAC

G3BP expression in cell lysates from PDAC cell lines and from a line of immortalized normal pancreatic epithelial cells (HPNE) (13) was determined by Western blot analysis (Fig. 1A). All PDAC cell lines showed high levels of G3BP expression compared to HPNE cells. G3BP expression in PDAC and in normal tissue sections was also investigated using an anti-G3BP monoclonal antibody (Fig. 1B-E). In concordance with the results of the Western blot, immunohistochemical analysis revealed strong signals of G3BP in the cytoplasm of cancer cells, but its expression was hardly detectable in normal pancreatic tissues. Among the five PDACs examined by immunohistochemistry, all specimens showed strong signals of G3BP. These observations suggest that G3BP is overexpressed in PDAC compared to normal pancreas.

The acidic domain of G3BP is essential for binding to BART mRNA

G3BP binds to the 3’UTR of BART (748-1703 of accession number NM_012106) and degrades BART mRNA (12). To define the region of G3BP that is required for its binding to BART-3’UTR, the binding ability of six myc-tagged fragments of G3BP (Fig. 2A; 1-420, 1-330, 1-240, 1-180, 1-135, and 1-80) to BART-3’UTR were investigated. Each fragment was transiently expressed in COS7 cells and high levels of
expression were confirmed by Western blotting (Fig. 2B). Myc-tagged proteins in total cell lysates were 
immunoprecipitated with anti-myc antibody and incubated with in vitro-transcribed biotinylated BART- 
3'UTR or control GAPDH RNA immobilized on streptavidin magnetic beads. The immunoprecipitated 
complexes were then pulled down with magnets. G3BP\textsubscript{1-420}, G3BP\textsubscript{1-330}, G3BP\textsubscript{1-240} and G3BP\textsubscript{1-180} were able 
to interact with BART-3’UTR, but G3BP\textsubscript{1-135} and G3BP\textsubscript{1-80} were unable to interact (Fig. 2C). GAPDH 
mRNA failed to pull down the G3BP fragments (data not shown). These experiments suggest that the region 
spanning amino acid residues 136-180, located in the acidic domain, is sufficient for binding BART-3’UTR.

Inhibition of G3BP endoribonuclease activity for BART-3’UTR transcript by synthesized polypeptides 
of G3BP\textsubscript{136-180}

To investigate the functional significance of the G3BP\textsubscript{136-180} fragment that includes the region that binds to 
the BART-3’UTR transcript, we used synthetic 45 amino-acid polypeptides (G3BP-peptide\textsubscript{36-180}). BART- 
3’UTR was incubated with recombinant G3BP with or without the G3BP-peptide\textsubscript{136-180}, followed by pull- 
down of the BART transcript. When the G3BP-peptide\textsubscript{136-180} was added it was able to inhibit formation of 
the complex between recombinant G3BP and BART-3’UTR (Fig. 2D), thus indicating that this synthesized 
peptide functions in a dominant-negative manner. To determine dominant-negative effects of the G3BP- 
peptide\textsubscript{36-180} on the RNase activity of G3BP, we performed an in vitro mRNA decay assay (Fig. 2E). In vitro-
transcribed BART-3’UTR was efficiently cleaved after 30 min of incubation with recombinant G3BP in the absence of G3BP-peptide_{136-180}, but mRNAs for GAPDH were not cleaved. G3BP-peptide_{136-180} treatment strongly decreased cleavage of the BART-3’UTR by G3BP, but had no effect on control GAPDH. These results suggest that the interaction of G3BP and BART-3’UTR is mediated through the region of G3BP_{136-180}, and that G3BP_{136-180} is necessary for specific G3BP RNase activity on BART mRNA.

**Exogenous overexpression of N-terminal G3BP dominant-negatively increases BART expression in PDAC cells**

To examine the global function of G3BP_{136-180} on post-transcriptional regulation, we established PANC-1 derivative clones from a PDAC cell line. These clones constitutively expressed either exogenous N-terminal G3BP (G3BP-N, 1 to 180) with the NTF-2-like domain and G3BP_{136-180}, or the C-terminal G3BP (G3BP-C, 241 to 466), including the RNA-binding domains (Fig. 3A). Western blot analysis shows the expression levels of the exogenous G3BP fragments, compared to mock-control clones (Fig. 3B).

BART and c-Myc mRNAs are specifically associated with G3BP in PDAC cells (12). This was confirmed by immunoprecipitation (IP) experiments in PANC-1 cells using anti-G3BP or control mouse IgG monoclonal antibodies (Fig. 3C). The immunoprecipitated transcripts were then converted to cDNA by
reverse transcription, and reverse transcription-PCR (RT-PCR) was performed. We found that antibodies to G3BP specifically immunoprecipitated both BART and c-Myc mRNAs. Nontarget GAPDH mRNA was also amplified, albeit inefficiently, and to the same extent in both IP groups. These findings reveal the presence of low levels of contaminating, nonspecific mRNA in all IP samples, yet verifies that equal amounts of input material was used, and demonstrates that the G3BP-IP materials were enriched compared to the control IgG-IPs. Following this, quantitative RT-PCR analysis was performed to identify the levels of BART mRNA in the G3BP-deletion mutant clones and to thereby determine the functions of N-terminal G3BP on the post-transcriptional regulation of BART. We found that BART mRNA was upregulated in N-terminal G3BP-PANC-1 clones, but not in mock or C-terminal-clones by real-time quantitative RT-PCR (Fig. 3D). The same result was observed by Western blotting (Fig. 3E). Interestingly, G3BP-N cells also increased c-Myc mRNA as compared to mock-control clones; however, G3BP-C failed to alter c-Myc mRNA levels (Fig. 3F). These results indicate that G3BP-N functions in a dominant-negative manner by stabilizing BART mRNA and that G3BP-N also affects the mRNA levels of c-Myc that binds to the C-terminal RNA-binding domain of G3BP.

Exogenously overexpressed N- and C-terminal G3BP colocalize with cytoplasmic RNA in PDAC cells

To demonstrate the mechanism of overexpressed G3BP-N in regulating the target transcripts via inhibition of endogenous G3BP activity, we investigated the subcellular colocalization of myc-tagged N- and C-
terminal G3BP proteins, and total cellular RNA in G3BP-N and G3BP-C PANC-1 cells. Ethidium bromide staining of total cellular RNA demonstrated that the bulk of cytoplasmic RNA colocalized with the transfected myc-tagged N- and C-terminal G3BP (Fig. 4A). In addition, RNase treatment eliminated all ethidium bromide staining in the cytoplasm (Supplementary Fig. 1). These results suggest that not only G3BP-C including the RRM and RGG domains, but also G3BP-N binds to RNA. Importantly, G3BP-N was diffusely distributed in the cytoplasm, whereas the C-terminal form was recruited to the cytoplasmic RNP granules (Fig. 4A). In line with these results, overexpressed N-terminal G3BP\textsubscript{1-141} is diffusely distributed in the cytoplasm of HeLa cells (6), and C-terminal G3BP\textsubscript{334-466} containing the RNA-binding domains is recruited to SGs in COS cells (4). G3BP is a marker for SGs, which form in stressed cells (14), and when overexpressed, G3BP induces SG formation (4). The C-terminal RNA-binding domains become concentrated in SGs under oxidative stress with sodium arsenite (SA), and their ability to bind RNA is reportedly sufficient to concentrate them in SGs (4). To verify that the C-terminal form is present in SGs, cells were immunostained under conditions of oxidative stress caused by treatment with SA (Fig. 4B). In G3BP-C cells, the C-terminal form colocalized in SGs with the endogenous SG-specific protein TIA-1, with or without SA treatment (white arrows and arrowheads in Fig. 4B). This colocalization was confirmed in the confocal Z stack (Fig. 4C). Thus, RNP granules induced by the overexpression of G3BP-C might consist of SGs. TIA-1 was localized at SA-induced cytoplasmic granules in G3BP-N cells (yellow arrows in Fig. 4B).
G3BP accelerates cell motility and invasion

However, SA did not induce colocalization of transfected G3BP-N with endogenous TIA-1 at SGs in G3BP-N cells (Fig. 4B). These results suggest that exogenously overexpressed G3BP-N is not associated with SG formation and does not inhibit SA-induced stress granule formation.

Exogenous overexpression of C-terminal G3BP induces eIF2α phosphorylation

SG assembly is usually initiated by the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), a translational initiation factor that exists as a component of the eIF2-GTP-tRNAMet ternary complex (15).

To further analyze if overexpressed G3BP-N and G3BP-C function in SG formation, we investigated the phosphorylation of eIF2α in stable transfectants by immunoblotting (Fig. 5A). Overexpression of N-terminal G3BP did not induce phosphorylation of eIF2α; however, C-terminal G3BP did induce eIF2α phosphorylation. Subsequently, we decided to test the capacity of endogenous G3BP to bind to exogenously overexpressed N- or C-terminal G3BP. When SG formation is initiated, endogenous G3BP forms multimers (16). Extracts prepared from mock-control, G3BP-N and G3BP-C PANC-1 cells were subjected to immunoprecipitation with anti-myc or anti-G3BP antibodies (Fig. 5B). Endogenous G3BP was efficiently co-immunoprecipitated with the myc-tagged G3BP-C-terminus, but not with the myc-tagged G3BP-N-terminus. Combined with the result of Fig. 4B, these data suggest that overexpressed N-terminal G3BP did not induce self-association of G3BP and subsequent SG assembly; however, it did increase BART mRNA in...
G3BP accelerates cell motility and invasion

a dominant-negative manner (Fig. 3D, E). As reported previously (4), the G3BP-C terminus plays a role in inducing SG formation; however, it is not associated with the post-transcriptional regulation of BART or c-Myc mRNAs (Fig. 3D-F).

Exogenous overexpression of N-terminal G3BP inhibits cell motility and invasion in PDAC

To investigate the functional significance of N- and C-terminal G3BP for cell motility and invasion in PDAC, we employed in vitro tumor culture assays. G3BP-C PANC-1 cells appeared more spindle-shaped and showed a mesenchymal morphology as compared to mock-control and G3BP-N cells, both of which showed epithelial cell morphology (Fig. 6A). We next measured directed motility into an artificial wound using a confluent monolayer culture of G3BP-N or G3BP-C PANC-1 clones, and compared them to mock-control cells (Fig. 6B, C). G3BP-N cells showed inhibition of motility activity into the wound area as compared to mock and C-terminal PANC-1 cells. In spite of the spindle-shaped morphological change of the G3BP-C cells, no other significant changes were seen. To verify these results, a motility assay on uncoated membranes was performed to quantify motile cells. Fig. 6D showed that G3BP-N PANC-1 cells were significantly less motile than the mock-control or G3BP-C cells. In addition, in two-chamber invasion assays, G3BP-N clones were significantly less invasive than the mock-control or G3BP-C cells (Fig. 6E). To rule out bias due to differences in proliferation, we performed MTT assays and the results showed that N- and C-
terminal G3BP clones did not affect cell proliferation (Supplementary Fig. 2). These results suggest that the
dominant-negative expression of myc-tagged N-terminal G3BP decreases cellular motility and invasiveness
of PDAC cells. Thus, in contrast to the G3BP-C terminus, the G3BP N-terminus appears to accelerate
cellular motility and invasiveness.

**Transcriptional effects of N-terminal G3BP in PDAC**

To determine the transcriptional program and pathways regulated by the expression of exogenous G3BP-N,
which might underlie the differences in cell motility and invasion, we performed cDNA microarray gene
expression profiling experiments with three G3BP-N PANC-1 clones and three mock control PANC-1
clones (Table 1). Raw data from the microarray experiments were uploaded onto the Gene Expression
defined as genes exhibiting a 2-fold increase in the average ratio in signal intensity. Five genes among the
fourteen upregulated genes were related to cell adhesion, motility and invasion. Many of these genes have
been previously described as modulators of cell invasion in various tumor cell models. The group of
downregulated genes consists mainly of genes associated with cell motility and invasion. We confirmed our
microarray data of the vast majority of genes tested at the RNA level by RT-PCR analysis (Supplementary
Fig. 3). Our microarray results suggest that N-terminal G3BP predominantly contributes to the regulation of
G3BP accelerates cell motility and invasion

genes involved in cell motility, invasion and adhesion in PDAC.
G3BP accelerates cell motility and invasion

Discussion

We previously reported that G3BP plays a role in inducing invasion and metastasis of PDAC cells by BART mRNA decay (12). BART was originally identified as a binding partner of ARL2 (17), a small G-protein implicated in the regulation of microtubule dynamics or folding (18). To investigate the mechanisms by which G3BP modulates cell motility and invasion in PDAC cells, we overexpressed N-terminal G3BP1-180 and C-terminal G3BP241-466. We found that overexpressed G3BP-N, which contains the NTF-2-like domain and G3BP136-180, dominantly negatively increases BART expression and thereby inhibits cell motility and invasion of PDAC cells. Thus, it is likely that the N-terminal domain of endogenous G3BP enhances the invasive ability of PDAC cells by post-transcriptionally regulating the level of specific transcripts. The association of a particular mRNA with G3BP may be highly regulated and might depend on interaction with other proteins (3, 6, 19). The interaction of G3BP with cyclin-dependent kinase 7 (CDK7) mRNA is dependent on the interaction of RasGAP with Filamin C (FLN-C) (19). When RNAi interrupts the interaction between RasGAP and FLN-C, CDK7 mRNA is released from G3BP (19). We also reported that intracellular CD24 binds to G3BP and inhibits G3BP RNase activity in PDAC cells (12). Thus, binding proteins of G3BP may form a link between G3BP and the mRNAs, and influence cellular mRNA stabilization and protein synthesis. Additionally, the NTF-2-like domain plays an important role in recruiting
G3BP accelerates cell motility and invasion

G3BP to the vicinity of local mRNA concentrations, and in facilitating its interaction with selected mRNA species. Our results suggest that a fragment of the NTF-2-like domain as well as a minimum binding region of the acidic domain of G3BP could contribute to the recruitment of G3BP to BART mRNA for subsequent BART mRNA degradation.

In mammalian cells, phosphorylation of eIF2α can trigger the assembly of SGs and the self-aggregation of G3BP (15). In the present study, exogenously overexpressed C-terminal G3BP was able to upregulate the phosphorylation of eIF2α and induced SG assembly. Despite the colocalization of overexpressed G3BP-C fragments with RNA observed by immunostaining of G3BP-C cells (Fig. 4A), no significant changes in the mRNA expression of BART or c-Myc were detected (Fig. 3D-F). SGs may be sites of mRNA sorting whereby the structure and composition of individual mRNPs might determine whether mRNAs are repacked into translationally competent mRNPs or degraded (14). G3BP has been shown to bind c-Myc (3) and tau mRNA (11), inducing either degradation through endoribonuclease activity or stabilization, respectively. Thus, the C-terminal G3BP may not always function in mRNA degradation in SGs but it may instead be sometimes associated with mRNA stabilization.

SG assembly can be dominantly induced by full-length G3BP overexpression (4). Moreover, G3BP binds to
Caprin-1 through the NTF-2-like domain and this complex induces the formation of cytoplasmic SGs (6). In addition, G3BP1-141 containing the NTF-2-like domain is recruited to SGs in COS cells (4). In the present study, G3BP1-180 containing the NTF-2-like domain and the BART mRNA-binding region did not induce G3BP self-association in PDAC cells. Since G3BP self-association is required for SG formation, it is likely that the N-terminal G3BP1-180 does not play a role in SG formation in PDAC cells. Nevertheless, G3BP1-180 is essential for degrading specific mRNAs, because exogenously overexpressed G3BP1-180 dominantly negatively increased the expression of mRNAs for BART and c-Myc. We conclude that G3BP1-180 is involved in mRNA degradation in PDAC cells although the exact mechanism by which it modulates mRNA degradation remains to be clarified. In this respect, further studies are needed to demonstrate the mechanism by which G3BP binds and recruits BART mRNA to SGs.

To identify the factors and pathways regulated by N-terminal G3BP and to obtain leads into the mechanisms behind the inhibition of cell invasion by dominant-negatively overexpressed N-terminal G3BP, we performed global expression analyses (Table 1). Fourteen genes were upregulated and six genes were downregulated after N-terminal G3BP was overexpressed in PANC-1 cells. Strikingly, five of the upregulated transcripts coded for proteins involved in cellular adhesion and/or invasion. Of these, CLU, a secreted glycoprotein with stress-induced expression in various diseased and aged tissues, decreases cell...
G3BP accelerates cell motility and invasion

proliferation and metastatic behavior of prostate cancer cells (20). AHR participates in signaling pathways regulating cell-cell and cell-substratum adhesion (21). HPX and hemopexin domains of human proteins fulfill functions in the activation of MMPs, the inhibition of MMPs, dimerization, binding of substrates or ligands, cleavage of substrates, and endocytosis (22). NSEP1, a member of the Y-box binding factor family of evolutionarily conserved DNA/RNA binding factors, decreases collagen type I mRNA and protein levels (23). PAI-1, a member of the serine protease inhibitors (serpins) family that blocks the activity of urokinase-type plasminogen activator (uPA), is downregulated in PDAC (24) and decreases tumor invasiveness and metastatic potential in cell lines derived from colon cancer, fibrosarcoma (25), and murine melanoma (26).

Concerning the downregulated genes, four of the identified transcripts code for proteins involved in cellular adhesion and/or invasion. These include MFGE8 identified as a marker of breast cancer (27) with angiogenesis-promoting activity (28) and anticoagulant activity (29). HSPG2 is a major component of blood vessel basement membranes and positively regulates angiogenesis (30). Interestingly, APOE induces expression of HSPG2 (31). LGALS1 increases motility of glioma cells and the reorganization of the actin cytoskeleton associated with increased expression of RhoA, a protein that modulates actin polymerization and depolymerization (32). Accordingly, the G3BP N-terminal region could play a role in regulating secreted MMPs, transcription factors, and a variety of genes involved in cell adhesion and motility, which in turn would enhance cell motility and invasion capacity of PDAC cells. Future studies are essential to determine
G3BP accelerates cell motility and invasion

the endoribonuclease activity of G3BP towards specific mRNAs identified in this cDNA microarray analysis.

Furthermore, the invasive activity of these genes in PDAC cells should be determined by the use of RNAi knockdown techniques.

The data presented here demonstrates that G3BP1-180 containing the novel RNA-binding residue G3BP136-180 contributes to post-transcriptional regulation of specific mRNAs for the promotion of cell motility and invasion in PDAC. Moreover, the N-terminus of G3BP plays a critical role in establishing a pattern of gene expression that favors PDAC cell motility and invasive behavior. Thus, G3BP1-180, especially G3BP136-180, could represent a novel target for blocking tumor invasion and metastasis in PDAC.
Acknowledgements

We thank Michel Ouellette for providing the HPNE cells and David Kelly for cDNA microarray analysis.

We also thank Thomas Caffrey and Tomoko Minakuchi for their technical help. The NIH funded this work with a grant to M.H. (R01CA057362).
G3BP accelerates cell motility and invasion

References


22. Piccard H, Van den Steen PE, Opdenakker G. Hemopexin domains as multifunctional liganding modules


27. Larocca D, Peterson JA, Urrea R, Kuniyoshi J, Bistrain AM, Ceriani RL. A Mr 46,000 human milk fat protein...
G3BP accelerates cell motility and invasion


32. Camby I, Belot N, Lefranc F, Sadeghi N, de Launoit Y, Kaltner H, et al. Galectin-1 modulates human glioblastoma cell migration into the brain through modifications to the actin cytoskeleton and levels of
G3BP accelerates cell motility and invasion

G3BP accelerates cell motility and invasion

Figure legends

Figure 1. Overexpression of G3BP in PDAC.

A, G3BP expression in PDAC cell lines in comparison with a normal pancreatic epithelial cell line (HPNE), examined by Western blot analysis (left panels). PDAC cells (1, PANC-1; 2, BxPC3; 3, S2-013; 4, SUIT-2; 5, COLO357; 6, HPAF; 7, MIA-Paca2 and 8, Capan2) and HPNE cells (N) were used. β-actin was used as a quantitative control. Scanning and densitometric analyses were performed in a representative experiment (right panel).

B-E, Immunohistochemistry was performed on clinical samples of PDAC using anti-G3BP antibody. PDAC specimens (B-D) and one normal pancreatic tissue specimen (E) are shown.

Figure 2. Identification of the BART mRNA-binding region in G3BP.

A, Schematic representation of six COOH-terminal myc-tagged G3BP deletion mutants.

B, Western blotting with anti-myc antibody on COS7 whole cell lysates transfected with myc-tagged G3BP deletion mutants.

C, Identification of the region in G3BP that binds to BART mRNA. The biotinylated BART-3’UTR transcript was incubated with each immunoprecipitated myc-tagged G3BP deletion protein from COS7 cells.
Association of the biotinylated BART-3’UTR with myc-tagged G3BP was assessed by pull-down of the RNA using streptavidin-conjugated beads, followed by western blotting with anti-myc antibody.

**D.** Reduction of the complex formed between recombinant G3BP protein and biotinylated BART-3’UTR transcript by treatment with G3BP<sub>136-180</sub> peptides.

**E.** BART 3’UTR or control GAPDH RNA was incubated with recombinant G3BP with or without pretreatment with G3BP<sub>136-180</sub> peptides. The RNA was electrophoresed and visualized by SYBR Gold staining. Closed arrowhead, BART-3’UTR; arrow, control GAPDH.

**Figure 3.** Stable overexpression of N- or C-terminal G3BP in PANC-1 cells.

**A.** Schematic representation of the G3BP-N-terminal deletion mutant containing the NTF-2-like and acidic domains, and the C-terminal deletion mutant containing the RRM and RGG motifs.

**B.** Exogenously overexpressed G3BP fragments were detected in two sets of transfected clones (G3BP-N and G3BP-C) by Western blot analysis with anti-myc antibody. Mock-transfected PANC-1 cells (Mock) served as controls.

**C.** The association of endogenous G3BP with endogenous BART and c-Myc mRNAs in PANC-1 cells was tested by IP. The transcripts in the IP materials were detected by RT-PCR. Mouse IgG monoclonal antibody was used as a control.
G3BP accelerates cell motility and invasion

**D**, Quantitative RT-PCR analysis of BART mRNA in stable control, G3BP-N, and G3BP-C cells of PANC-1. Quantification of mRNA levels was done in triplicates and repeated three times. *Columns*, mean; *bars*, SD. *p* value compared with mock-control and G3BP-C cells.

**E**, Western blot with anti-BART antibody showing two representative clones of each of G3BP-N and G3BP-C cells of PANC-1.

**F**, Quantitative RT-PCR analysis of c-Myc mRNA in stable control, G3BP-N, and G3BP-C cells of PANC-1. Quantification of mRNA levels was done in triplicates and repeated three times. *Columns*, mean; *bars*, SD. *p* value compared with mock-control and G3BP-C cells.

**Figure 4.** Overexpressed C-terminal G3BP is localized in cytoplasmic SGs in PDAC cells.

**A**, Immunocytochemical staining of exogenous myc-tagged G3BP fragments and total cellular RNA in PANC-1 transfectants, as determined using anti-myc antibody (green) and ethidium bromide (red), respectively and DAPI staining (blue, nucleus). Bars: 10 µm.

**B**, Stable G3BP-N and G3BP-C cells of PANC-1 were exposed to 500 µM arsenite (SA) for 30 min. Immunocytochemical staining, as determined with anti-myc antibody (green), anti-TIA-1 antibody (violet) and DAPI staining (blue). White Arrows and arrowheads indicate myc-tagged G3BP-C colocalizing with
G3BP accelerates cell motility and invasion

endogenous TIA-1 in SGs after treatment with or without SA, respectively. Yellow arrows indicate SGs in which TIA-1 is localized. Bars: 10 µm.

C, Confocal Z stack with white boxes (a and b in Fig. 4B) depicting the position where SGs associate with myc-tagged G3BP-C and endogenous TIA-1, as determined with anti-myc antibody (green), anti-TIA-1 antibody (violet) and DAPI staining (blue). Arrows and arrowheads indicate myc-tagged G3BP-C colocalizing with endogenous TIA-1 in SGs after treatment with or without SA, respectively.

Figure 5. Overexpressed C-terminal G3BP induces phosphorylation of eIF2α and self-aggregation of endogenous G3BP in PDAC cells.

A, Western blot analysis of endogenous phosphorylated eIF2α in G3BP-deletion mutants. Mock-control cells were exposed to 500 µM arsenite for 30 min under oxidative stress and used as a positive control.

B, Whole-cell extracts prepared from the mock-control and G3BP-deletion mutants were immunoprecipitated with anti-myc antibody or anti-G3BP antibody. Mouse IgG monoclonal antibody was used as a control. Immunoprecipitates were analyzed by Western blotting with anti-myc and anti-G3BP antibodies. Arrows, myc-tagged G3BP; asterisk, co-immunoprecipitated endogenous G3BP with myc-tagged C-terminal G3BP.
**G3BP accelerates cell motility and invasion**

**Figure 6.** Overexpression of N-terminal G3BP dominant-negatively suppresses cell motility and invasion in PDAC cells.

- **A,** Morphology of PANC-1 control, G3BP-N, and G3BP-C cells as analyzed by phase-contrast microscopy.

- **B,** Wound healing assay with PANC-1 control, G3BP-N, and G3BP-C cells. The dashed lines indicate the border of the scratch (time 0) made by a plastic pipette tip.

- **C,** The number of cells that migrated into the initially cell-free scratch was counted. Cells in four defined areas per group per experiment were quantified. Data are representative of three independent experiments. *Columns,* mean; *bars,* SD. *p* < 0.005, **p** < 0.004 compared with mock-control and G3BP-C cells, respectively.

- **D,** Transwell motility assay in control, G3BP-N, and G3BP-C cells. Migrated cells in four fields per group were counted. Data are representative of three independent experiments. *Columns,* mean; *bars,* SD. *p* < 0.005, **p** < 0.002 compared with mock-control cells.

- **E,** Quantification of the two-chamber invasion assay with PANC-1 control, G3BP-N, and G3BP-C cells. Invaded cells in four fields per group were counted. Data are representative of three independent experiments. *Columns,* mean; *bars,* SD. *p* < 0.002 compared with mock-control cells.
Figure 1

A

IB: G3BP
IB: β-actin

Relative signal (normalized to β-actin)

B

C

D

E

on June 19, 2017. © 2011 American Association for Cancer Research. mcr.aacrjournals.org Downloaded from mcr.aacrjournals.org on June 19, 2017. © 2011 American Association for Cancer Research.
Figure 2

A

<table>
<thead>
<tr>
<th>1</th>
<th>NTF2</th>
<th>ACIDIC</th>
<th>RRM</th>
<th>RGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>140</td>
<td>240</td>
<td>341</td>
<td>411</td>
</tr>
<tr>
<td>428</td>
<td>450</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

G3BP1-420
G3BP1-330
G3BP1-240
G3BP1-180
G3BP1-135
G3BP1-80

136-180

B

IB: Myc

C

IB: Myc

D

Pulldown: BART-3'UTR

<table>
<thead>
<tr>
<th>Recombinant G3BP:</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3BP-peptide 136-180:</td>
<td>-</td>
<td>5 μM</td>
<td>10 μM</td>
</tr>
</tbody>
</table>

IB: G3BP

Input

E

<table>
<thead>
<tr>
<th>BART-3'UTR</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

G3BP-peptide 136-180 (10 μM):

kb

- 1.5
- 1.0
- 0.5
- 0.3
- 0.1

Downloaded from mcr.aacrjournals.org on June 19, 2017. © 2011 American Association for Cancer Research.
Figure 3

A

G3BP-N-terminus (1-180)

G3BP-C-terminus (241-466)

B

IB: Myc

IB: β-actin

C

IP: Control G3BP

BART

c-Myc

GAPDH

D

p = 0.0004

p = 0.0002

BART mRNA normalized to GAPDH

Mock#5 G3BP-N#9 G3BP-C#7

E

IB: BART

IB: β-actin

F

p = 0.039

p = 0.069

c-Myc mRNA normalized to GAPDH

Mock#5 G3BP-N#9 G3BP-C#7
Figure 4

A

<table>
<thead>
<tr>
<th>DAPI</th>
<th>Myc</th>
<th>EB</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3BP-N#9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3BP-C#7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>DAPI</th>
<th>Myc</th>
<th>TIA-1</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA -</td>
<td>G3BP-N#9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G3BP-C#7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA +</td>
<td>G3BP-N#9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G3BP-C#7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

Z stack

a

b
Figure 5

A

IB: Phospho-eIF2α
IB: eIF2α
IB: β-actin

B

IB: G3BP

Mock#5  G3BP-N#9  G3BP-N#11  G3BP-C#7  G3BP-C#20  Mock#5 (+ Arsenite)

Mock#5  G3BP-N#9  G3BP-C#7
Figure 6

A

Mock#5  G3BP-N#9  G3BP-C#7

B

Mock#5  0 h  20 h
G3BP-N#9  G3BP-C#7

C

Number of cells

Mock#5  G3BP-N#9  G3BP-C#7

D

Number of cells

Mock#5  Mock#9  G3BP-N#9  G3BP-N#11  G3BP-C#7  G3BP-C#20

E

Number of cells

Mock#5  Mock#9  G3BP-N#9  G3BP-N#11  G3BP-C#7  G3BP-C#20

***

*
<table>
<thead>
<tr>
<th>GenBank accession number</th>
<th>Ratio: G3BP-N/Mock</th>
<th>Gene symbol and full name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell adhesion, invasion and ECM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_001831</td>
<td>2.6</td>
<td>Clusterin (CLU)</td>
</tr>
<tr>
<td>NM_001621</td>
<td>2.3</td>
<td>Aryl hydrocarbon receptor (AHR)</td>
</tr>
<tr>
<td>J03048</td>
<td>2.2</td>
<td>Hemopexin (HPX)</td>
</tr>
<tr>
<td>NM_004559</td>
<td>2.2</td>
<td>Nuclease sensitive element binding protein 1 (NSEP1)</td>
</tr>
<tr>
<td>M16006</td>
<td>2.1</td>
<td>Plasminogen activator inhibitor-1 (PAI1)</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_016606</td>
<td>2.4</td>
<td>Receptor accessory protein 2 (REEP2)</td>
</tr>
<tr>
<td>NM_003465</td>
<td>2.4</td>
<td>Chitinase 1 (CHIT1)</td>
</tr>
<tr>
<td>NM_006440</td>
<td>2.3</td>
<td>Thioredoxin reductase beta (TR)</td>
</tr>
<tr>
<td>NM_012266</td>
<td>2.3</td>
<td>Heat shock cognate 40 (HSC40)</td>
</tr>
<tr>
<td>AF289028</td>
<td>2.2</td>
<td>Inducible T-cell co-stimulator ligand (ICOSLG)</td>
</tr>
<tr>
<td>X56692</td>
<td>2.2</td>
<td>C-reactive protein (CRP)</td>
</tr>
<tr>
<td>NM_019618</td>
<td>2.1</td>
<td>Interleukin-1 homolog 1 (IL-1H1)</td>
</tr>
<tr>
<td>NM_005953</td>
<td>2 Metallothionein 2A (MT2A)</td>
<td></td>
</tr>
<tr>
<td>AF105067</td>
<td>2 Lipopolysaccharide-binding protein (LBP)</td>
<td></td>
</tr>
</tbody>
</table>

2. Genes downregulated by overexpression of N-terminal G3BP

<table>
<thead>
<tr>
<th>Ratio:Mock/ G3BP-N</th>
<th>Ratio:Mock/ G3BP-N</th>
<th>Gene symbol and full name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell adhesion, invasion and ECM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_005928</td>
<td>3.1</td>
<td>Milk fat globule-EGF factor 8 (MFG8)</td>
</tr>
<tr>
<td>NM_005529</td>
<td>2.7</td>
<td>Heparan sulfate proteoglycan 2 (perlecan) (HSPG2)</td>
</tr>
<tr>
<td>NM_000041</td>
<td>2.4</td>
<td>Apolipoprotein E (APOE)</td>
</tr>
<tr>
<td>NM_002305</td>
<td>2.1</td>
<td>Lectin, galactoside-binding, soluble, 1 (galectin 1) (LGALS1)</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_006026</td>
<td>2.3</td>
<td>H1 histone family, member X (H1FX)</td>
</tr>
<tr>
<td>NM_001823</td>
<td>2.3</td>
<td>Creatine kinase, brain (CKB)</td>
</tr>
</tbody>
</table>
The N-terminal domain of G3BP enhances cell motility and invasion by post-transcriptional regulation of BART

Keisuke Taniuchi, Isao Nishimori and Michael (Tony) A Hollingsworth

*Mol Cancer Res* Published OnlineFirst June 10, 2011.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-10-0574</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author Manuscript</td>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a>.</td>
</tr>
</tbody>
</table>