Murine guanylate-binding protein-2: an interferon-induced GTPase that inhibits cell adhesion, cell spreading and MMP-9 expression

Angela Mesmer-Blust
The University of Toledo

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A Dissertation

Entitled:

**Murine Guanylate-Binding Protein-2: An interferon-induced GTPase that inhibits cell adhesion, cell spreading and MMP-9 expression.**

By

Angela Messmer-Blust

Submitted as partial fulfillment of the requirement for the

Doctor of Philosophy Degree in Biology

Advisor: Dr. Deborah Vestal

College of Graduate Studies

The University of Toledo
December 2009
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An Abstract of

Murine Guanylate-binding Protein-2: An interferon-induced GTPase that inhibits cell adhesion, cell spreading, and MMP-9 expression.

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The University of Toledo

December 2009

Within the context of a living multicellular organism, rarely are individual cells exposed to only one growth factor and/or cytokine at a time. This is particularly true in situations where pro-inflammatory cytokines are present. While there is a growing understanding of how some growth factors, cytokines, and integrins crosstalk to modulate the responses observed by each as single agents, little is known about how the pro-inflammatory cytokine IFN-γ alters cell responses to growth factors and integrins. We show here that IFN-γ induction of the large GTPase mGBP-2 inhibits cell spreading initiated by both PDGF treatment and integrin engagement during adherence to fibronectin. This inhibition is accompanied by mGBP-2-mediated inhibition of Rac activation. Spreading can be rescued by the forced expression of activated Rac in the presence of mGBP-2. In addition, mGBP-2 also inhibits the ability of TNF-α to induce
the transcription of matrix metalloproteinase-9 (MMP-9). mGBP-2 inhibits TNF-α-induced transcription of MMP-9 by inhibiting the TNF-α-induced activation of NF-κB. This is the first report of an IFN-γ-induced protein that can modulate cellular responses to integrins, growth factors and cytokines.
ACKNOWLEDGEMENTS

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with strength and tenacity and although he has since passed, I know his support and love
is still with me through everything.
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<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned media</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42/Rac interactive binding domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GBP</td>
<td>Guanylate-binding protein</td>
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<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-Transferase</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamine</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffered saline solution</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-stimulated response element</td>
</tr>
<tr>
<td>IRF-1</td>
<td>Interferon regulatory transcription factor-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>ISG</td>
<td>Interferon-stimulated gene</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>LG</td>
<td>Large G Domain</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
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<tr>
<td>ON</td>
<td>Overnight</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
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<td>PBD</td>
<td>PAK binding domain</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulphonylfluoride</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>PV</td>
<td>Parasitophorous vacuole</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCL</td>
<td>Total cell lysate</td>
</tr>
<tr>
<td>Tiam1</td>
<td>T lymphocyte invasion and metastasis 1</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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CHAPTER I. OVERVIEW AND SIGNIFICANCE

Introduction to Interferons

The discovery of interferons (IFNs) over fifty years ago by Isaacs and Lindenmann led to numerous advances in the field of cell signaling [1]. First discovered as agents that interfere with virus replication, this family of pleiotropic cytokines also enhance innate and acquired immune responses, as well as modulate normal and tumor cell survival and death [1, 2]. In addition, IFNs are anti-proliferative, anti-tumorigenic, and pro-apoptotic in many cell types [3]. Other consequences of IFN exposure include changes in cell adhesion [4, 5], migration [6], cytoskeletal arrangement [7], and cellular morphology [8].

IFNs are subdivided into three groups: type I IFNs include the α, β, κ, ψ, and ε isoforms, type II IFN includes only the γ isoform [2], and the recently discovered type III IFNs include IFNs λ 1-3 (IL-28α, IL28β and IL-29) [9]. All IFNs signal through the JAK-STAT pathway. Each type of IFN binds to a distinct receptor, which causes the receptors to oligomerize and activates Janus kinases (JAKs). JAKs subsequently activate signal transducers and activator of transcription (STATs) by tyrosine phosphorylation, leading to their homo- or hetero-dimerization, nuclear translocation, and the transcription of IFN-stimulated genes (ISGs) (Fig. 1) [10]. Type I IFNs are important in anti-viral responses and IFN-α is used in the treatment of certain leukemias, Hodgkin’s lymphoma, Kaposi’s sarcoma, melanoma, and renal cell carcinoma [11, 12]. Discovered in 2002,
type III IFNs also exhibit anti-viral activity, yet are structurally and genetically distinct from type I IFNs. Interestingly, their intracellular signal transduction pathways and biological activities are surprisingly similar to type I IFNs. Type III IFNs exhibit antitumorigenic activities, similar to type I IFNs [9, 13].

Biological responses to type II IFN proceed through both STAT1-dependent and -independent pathways. In addition to the JAK-STAT pathway, IFN-γ activates other signal transduction molecules, including the mitogen-activated protein kinases (MAPKs), Pyk2, the Src-family kinase Fyn, the adaptor proteins c-Cbl, CrkL, CrkII and Vav, the G-protein-linked signaling molecules C3G, Ras GTPase-activating protein 1 (Rap-1), and the SH2-domain-containing protein tyrosine phosphatases SHP-1 and SHP-2S [14]. IFN-γ can inhibit tumor growth by increasing susceptibility to apoptosis initiated by Fas activators and cytotoxic chemotherapies in melanoma and colorectal carcinomas [3, 15, 16]. However, IFN-γ can function as a double-edged sword by promoting tumor growth in some cases and inhibiting growth in others. For example, exposure of H22 hepatomas to sustained high levels of IFN-γ have an antitumor effect, while low levels promote tumor development [17].

**Cell Adhesion and Cytoskeleton**

IFN-γ modulates cell adhesion, invasion/migration, and cytoskeletal rearrangement. Cell motility and membrane ruffling is markedly decreased in response to IFN-γ treatment of human fibroblasts [7]. IFN-γ also alters endothelial cell adhesion and migration [18]. IFN-γ inhibits chemotaxis in fibroblasts [6]. IFN-γ also induces macrophage activation and promotes adherence to laminin involving integrin-cytoskeletal
Figure 1. Interferon signaling by the JAK-STAT pathway. Interferons initiate signaling by binding to their cognate receptors. The multiple human type I IFN ligands act through the shared type I receptor complex of IFNAR1 and IFNAR2 chains. Type II IFN acts through a distinctly different receptor complex of IFNGR1 and IFNGR2 chains. The type III IFNs act through a receptor complex composed of the IL10R2 and IFNLR1 chains. Ligand binding by the cognate receptor mediates the activation of JAKs. In genes with an ISRE enhancer element, the STAT-dependent signal transduction activated by type I and III IFNs involves the activation and nuclear translocation of Stat1, Stat2, and IRF-9. For genes with a GAS enhancer element, the prototypical STAT-dependent signal transduction by type II IFN involves the activation and nuclear translocation of a Stat1 dimer, the GAF complex. (Reprinted by permission from The American Society for Biochemistry and Molecular Biology: [JOURNAL OF BIOLOGICAL CHEMISTRY] (Samuel, C., J. Biol. Chem. 282, 20045). copyright (2007).
interactions [4, 5]. However, little is known about the mechanism through which IFN-γ regulates cytoskeletal rearrangement. One way to analyze the ability of a cell to reorganize its actin cytoskeleton is to study the ability of cells to spread on a defined substrate. To achieve spreading, a cell must have a signal from the interaction of membrane-based integrins or growth factors with the extracellular matrix (ECM) to initiate a complex cascade of signaling events which subsequently triggers morphological changes and results in the generation of contractile forces [19-21]. Initially, fibroblasts adhere to fibronectin primarily by the α5β1 integrin, initiating a signaling cascade that regulates the cytoskeleton. Subsequently, a complex of 20-30 proteins are recruited to focal contacts (Fig. 2) [22]. Some of these cytoskeletal proteins include FAK, F-actin, tyrosine kinases and their substrates. Signal transduction molecules included in these focal contacts are phospholipase C (PLC)-γ, phosphoinositide-3-kinase (PI3-K), Rho family GTPases, Rho family guanine nucleotide exchange factors (GEFs), and the MAPK family [22].

**Signaling through Rho Family GTPases**

Small GTPases of the Rho family, Rho, Rac and Cdc42, are critical regulators of actin cytoskeleton dynamics, adhesion, cell cycle progression, and gene expression [23]. Members of the Rho family are well characterized for their role in regulating the types of F-actin changes required for cell spreading [24-26]. In fibroblasts, Rac1 is involved in the induction of lamellipodia, Cdc42 regulates filopodia formation, and RhoA activation leads to stress fiber formation [27, 28]. These GTPases act as molecular switches. The Rho family of GTPases cycle between an active, GTP-bound conformation to an inactive, GDP-bound conformation. Cycling between these states is regulated by more than 30
guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) [29].

Rac is a potent activator of actin polymerization, driving membrane protrusion at the leading edge of cells [30]. Rac mediates actin polymerization through activation of the WASP/WAVE family of Arp2/3 complex activators [30]. Signaling pathways that activate or inactivate Rac are complex. For example, both integrins and growth factor receptors use multiple cytoplasmic signaling pathways to activate Rac, resulting in cytoskeletal rearrangement. One of these pathways stimulates tyrosine phosphorylation of focal-adhesion kinase (FAK). Phosphorylation of FAK creates an SH2 binding site for PI3-K, Src family kinases, and the Grb2-Sos complex that subsequently activates Ras.

Another key molecule involved in Rac activation is Ras [31]. Considerable crosstalk and cooperation between Ras and Rho GTPases is required for cell spreading and migration [32]. Activation of Ras by growth factors and/or integrins can be achieved by PI3-K-dependent pathways, either directly by binding the p85 regulatory subunit or indirectly by forming a complex between the catalytic subunit and active Ras [33]. For example, the Rac GEF, T lymphoma induced activation and metastasis 1 (Tiam1), can be activated by Ras either indirectly through the Ras-GTP-p110 complex or directly by associating with Tiam1 through its Ras-binding domain [34]. PI3-K is downstream of integrins, IFNs and growth factor receptors and can activate Rac. In addition, inhibition of PI3-K blocks both Ras and Rac activation and migration [35].
Figure 2. The complexity of cytoskeletal and signaling complexes in focal adhesions.

Signal transduction proteins associated with, and/or activated by, integrins. Signaling molecules, such as FAK, bind to and recruit additional signaling molecules, creating a complex signaling network that is intimately connected to the cytoskeleton network. (Reprinted by permission from Macmillan Publishers Ltd: [NATURE CELL BIO] (Miranti, C and Brugge, J. Nature Cell Bio 4, 83). copyright (2002).)
Matrix Metalloproteinase Regulation

IFNs also alter cell adhesion and invasion through altering the interactions of cells with their extracellular environment. These changes can be accomplished by up or down-regulating the levels of cell adhesion molecules, but can also be facilitated by modulating the expression of enzymes that modify the ECM. Both IFN-α/β (type I IFNs) and IFN-γ (type II IFN) down-regulate the expression of at least three members of the matrix metalloproteinase (MMP) family [36-44]. MMPs are a family of Ca\(^{2+}\)- and Zn\(^{2+}\)-requiring endoproteases that collectively cleave most components of the ECM [45]. These proteins are important in such processes as migration, proliferation, wound healing, and angiogenesis. The greater than 20 MMPs can be divided into subgroups based on substrate specificity [46]. The gelatinases are those MMPs with elevated activity against denatured collagens and contain only two members, MMP-2 and MMP-9 [45], both of which can be down-regulated by IFNs. MMP-9, also called gelatinase B, can degrade collagens type IV, V, XIV, aggrecan, elastin, entactin, laminin, and vitronectin [47]. Since type IV collagen and laminin are common to all basement membranes, MMP-9 is important in metastasis, tumor growth, and angiogenesis [47-49]. Consistent with this, MMP-9 expression is frequently elevated in human tumors and correlates with increased metastasis [47, 50, 51]. MMP-9 is also associated with the angiogenic switch, which contributes to tumor progression [52].

The expression of MMP-9 can be modulated by a variety of cytokines and other agents in addition to IFNs [48, 53-58]. Cytokine-mediated transcriptional regulation of MMP-9 proceeds through a limited number of promoter elements and, presumably, transcription factors [48, 59]. The promoters of both human and murine MMP-9 genes
have an NF-κB site, two AP-1 sites, an Sp-1 site, and an Ets site [48]. These sites are involved in regulation by TNF-α [60], PMA, v-src, ras, and LPS [48, 58]. More recently, investigators have demonstrated the importance of chromatin remodeling, binding of coactivators, and histone acetylation for the expression of MMP-9 [59].

One of the early events following IFN binding to its receptor is the activation of STAT1 [10]. IFN-γ treatment of HeLa cells with PMA inhibits the induction of MMP-9 by activating STAT1α, which inhibits MMP-9 transcription by binding to the MMP-9 promoter and inhibiting the recruitment of the co-activators CBP/p300 [61]. This inhibition of CBP/p300 recruitment in turn inhibits the formation of the transcription complex required to initiate MMP-9 transcription. The inhibition by STAT1α is a relatively early event after IFN-γ exposure, requiring only 4 hr. In addition, activated STAT1α induces the expression of a variety of genes, including the transcription factor interferon responsive factor-1 (IRF-1). IRF-1 is a transcriptional activator and tumor suppressor [62, 63]. IRF-1 inhibits MMP-9 transcription in EW-1 cells by competing with the p65 subunit of NF-κB for binding to the NF-κB site of the MMP-9 promoter [64]. In both the mouse and human MMP-9 promoter, the NF-κB site contains a modified interferon-stimulated response element (ISRE), IRE. Binding of IRF-1 to the IRE of the NF-κB site blocks p65 binding and inhibits MMP-9 transcription [64]. In addition to IRF-1, another IFN-induced protein down-regulates TNF-α-induced MMP-9 expression in antigen presenting cells, the class II major histocompatibility complex transactivator (CIITA) [65]. CIITA expression is induced by IFN activated STAT1α, and like STAT1α, CIITA binds to CBP and inhibits both its recruitment to the MMP-9 promoter and histone acetylation. IFN-mediated inhibition of MMP-9 by STAT1α, IRF-
1, and CIITA are all early responses upon IFN induction.

Rho GTPases also play a role in MMP activation. Constitutively active Rac1 induces cell-associated activation of MMP-2 by collagen in fibrosarcoma cells [67]. Hsia, et. al. observed that Rac and FAK regulate cell invasion, MMP-2 expression and MMP-9 secretion [68]. Conversely, the FAK-Src signaling complex activates Rac and JNK, through DOCK180, leading to MMP-2 expression and MMP-9 secretion. In addition, Rac1 activity is necessary for H-Ras-induced activation of p38, and contributes to MMP-2/9 up-regulation in breast epithelial cells [69].

**Introduction to Guanylate-Binding Proteins**

In response to IFN-γ induction, one of the most robustly induced gene families is the guanylate-binding proteins (GBPs). The GBPs are a family of 65-67kDa GTPases identified only in vertebrates including humans, mice, rats, and chickens. The GBPs were originally identified as IFN response genes in the late 1970s [70-72]. We now know that they are also inducible by other pro-inflammatory cytokines, IFN-α, -β, TNF-α, IL-1α, and IL-β [73-75]. Murine family members 1-10 are up-regulated by IFN-γ [76] and mGBP-1, 2 and 5 are also up-regulated by LPS [75, 76]. In addition, TNF-α or IL-1β treatment modestly induces the GBPs, however, when these cytokines are paired with IFN-γ, synergistic induction is observed [75].

**Genomic Organization of the GBP gene family**

To date, *in silico* analysis of the GBP gene family has identified a cluster of 7 human GBPs and one GBP pseudogene on human chromosome 1 [77]. Murine chromosome 3 has two gene clusters of GBPs, one including mGBP-1, -2, and -5 and the second including mGBP-3 and mGBP-7 [77]. An additional gene cluster is found on
murine chromosome 5 containing mGBP-6, -7, -8, -9, -10, -11 and one pseudogene [76-78]. Based on the arrangement of family members within their respective gene clusters, mGBP-2 is believed to be the murine ortholog of hGBP-1. mGBP-1 and mGBP-2 are the most closely related of the murine GBPs (80% sequence identity, with the first 160 amino acids identical) [79].

**Structure and biochemistry of the GBPs**

Based on the crystal structure of full-length hGBP-1 in the absence of nucleotide [80] (Fig. 3), the N-terminus of hGBP-1 is a globular domain that contains the GTP binding region. The second main feature of hGBP-1 is a series of alpha helices extending toward the carboxy terminus, which makes the protein unusually long [80]. GBPs are more similar at their amino-termini and become progressively more divergent at their carboxy-termini [77]. Three of the human and murine family members contain a CaaX motif for isoprenylation, human and mouse GBP-1, -2, and -5 [76, 77]. mGBP-2 is isoprenylated by a C₂₀ geranygeranyl lipid and hGBP-1 is modified by a C₁₅ farnesyl lipid. The GTP binding motif of GBPs is novel compared to other GTPases; the third site for nucleotide contact differs from the consensus used by other GTPases [81]. Consequently, GBPs hydrolyze GTP to both GDP and GMP by two consecutive cleavage reactions [82, 83]. The GBPs are members of the dynamin superfamily of GTPases and the biochemical properties of the dynamin family shared by GBPs include: a high rate of GTP hydrolysis, low affinity for GTP, and oligomerization-dependent hydrolysis.

Biochemical and structural analyses of human GBP-1 show that it can form dimers and/or tetramers and that this multimerization of hGBP-1 is GTP-dependent [84]. Nucleotide binding and self-association of hGBP-1 subsequently stimulates hydrolytic
activity, as also observed for dynamin [81, 85]. In contrast to Ras-like and Gα GTPases, GBPs are stable in the absence of nucleotide. Additionally, Ras proteins have an intrinsic GTPase reaction rate in the order of 0.001 ± 0.1 min\(^{-1}\), whereas hGBP-1 has a hydrolysis rate of up to 80 min\(^{-1}\) [82]. The catalytic mechanisms for Ras and Gα proteins involve stabilization by a GTPase activating protein (GAP) [86]. A more recent report on hGBP-1 revealed that the α-6 helix of the intermediate region connecting the N-terminal globular domain to the C-terminal helical domain stabilizes dimer formation. The α-6 helix also plays a critical role in GMP formation by stimulating GTPase activity through allosteric interaction, and, thus, acts as an internal GAP [87]. In addition, analysis of steady-state kinetics showed that hGBP-1 had a higher catalytic efficiency for GMP formation than for GDP formation [87]. Generation of GMP by hydrolysis of GTP is unique to GBPs, however, the physiological relevance of this is still unclear [88].

Analyses of mutant GTP-binding proteins that are locked in specific nucleotide-bound states are useful for studying the cellular functions of the protein and/or their interactions with other proteins. For example, the Ras family of small GTPases and α-subunits of heterotrimeric G-proteins are commonly mutated in the last serine residue of the consensus sequence of the phosphate-binding loop (P-loop) to suppress GTPase function. GTP-binding proteins contain a P-loop, the primary structure responsible for binding of the γ-phosphate of GTP. In the Ras family, RasS17N, decreases the affinity for GTP and GDP. These mutant proteins remain nucleotide-free and interact very strongly with their guanine nucleotide exchange factors (GEFs) [89]. Biochemical analysis of human GBP-1 point mutants revealed hGBP-1 variants that are deficient in nucleotide binding, oligomerization, and/or GTP hydrolysis [81]. Highly conserved
**Figure 3. Crystal structure of hGBP-1.** A model of the tertiary structure of hGBP1 presented as a ribbon, where the LG domain is in purple, the connecting region in green, the helical domain in yellow and α12/α13 in cyan. Dashed lines indicate disordered regions in the molecule. The tentative nucleotide-binding area, identified by a Ras ± GBP overlay, is indicated by a sphere with radius 7Å (Reprinted by permission from Macmillan Publishers Ltd: [NATURE] (B. Prakash, G. J. K. Praefcke, L. Renault, A. Wittinghofer, C. Herrmann, *Nature* **403**, 567). copyright (2000).
residues of GTP-binding proteins in the GTP-binding motif of hGBP-1 were analyzed to determine which residues are important in nucleotide-dependent oligomerization and GTP hydrolysis. The Arg48 residue located in the P-loop, Ser52 located in the GTP-binding motif G1, and Asp184 in the third guanine nucleotide-binding motif were mutated to alanine, asparagine and asparagine, respectively. Based on dissociation constants, the R48A mutant is believed to be a permanently GTP-bound protein under physiological conditions. According to size-exclusion chromatography, the R48A hGBP-1 mutant is a dimer. In contrast, S52N hGBP-1 has such low affinity for guanine nucleotides that it is proposed to be a nucleotide-free monomer in cells. D184N hGBP-1 showed a 25-50 fold reduction in binding to all 3 nucleotides. D184N hGBP-1 can still bind and hydrolyze GTP, yet at a significantly lower rate with less GMP production. This suggests that D184N hGBP-1 can still bind GTP and form dimers under physiological conditions.

**Localization of the GBPs**

Murine family members 2-10 localize to discrete vesicle-like structures inside the cytoplasm [76, 79]. mGBP-2 is localized in punctate patterns in the cytoplasm and in heterogenous vesicles in an isoprenoid-dependent manner; in contrast, mGBP-1 fails to localize to intracellular vesicles. Despite having an identical CaaX to that of mGBP-2, little, if any, mGBP-1 is prenylated and this is the proposed reason for the failure to target to intracellular membranes [79, 90].

**Isoprenylation and its functional consequences.**

The CaaX motif is a four amino acid sequence at the extreme carboxy-terminus of the protein, which directs lipid modification. The cysteine residue of this motif is where
the isoprenoid is attached, the A signifies an aliphatic amino acid. The terminal amino acid of the protein, designated X, directs the addition of the prenyl group, X = S, M, A, or Q for a farnesyl lipid, C\textsubscript{15}, and X = L for a geranylgeranyl lipid, C\textsubscript{20}. Where studied, isoprenylation is critical for one or more of the functions of prenylated proteins [91]. For some isoprenylated proteins, their functions can be divided into those that require lipid modifications and those that do not. For example, the small GTPase, RhoA requires prenylation to mediate cytoskeletal rearrangement, but not for gene regulation [92]. The terminal amino acid for hGBP-2, hGBP-5, mGBP-1, and mGBP-2 is a leucine (L), which predicts the addition of the C\textsubscript{20} geranylgeranyl lipid moiety to the cysteine of the CaaX sequence. Only the hGBP-1 and mGBP-5 proteins have a terminal serine residue, suggesting modification by a C\textsubscript{15} farnesyl residue [77]. The ability to add isoprenoid \textit{in vivo} has been demonstrated for hGBP-1 [93], mGBP-2 [90], and rat GBP [94]. Despite the presence of an identical CaaX sequence compared to the closely related mGBP-2, mGBP-1 appears to be incompletely prenylated \textit{in vivo} [95].

\textbf{Functions of the GBPs}

Despite the discovery of guanylate-binding proteins over 25 years ago [71, 72], functional information for the family members has only recently become available. Human GBP-1 has modest anti-viral activity against vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV), with a 40-60% inhibition in viral release [96]. Anti-viral activity of hGBP-1 is further suggested by increased hepatitis C virus replicon replication after knock-down of hGBP-1 [97]. The murine ortholog of hGBP-1, mGBP-2, also exhibits modest anti-viral activity. VSV and EMCV release is inhibited in NIH 3T3 fibroblasts upon mGBP-2 expression [98]. Inhibition of EMCV by mGBP-2
requires wild-type GTP binding, as evidenced by the inability of cells expressing a mutant version to inhibit viral yield. S52N mGBP-2 contains a single amino acid substitution at position 52 of the GTP-binding region. S52N mGBP-2 was able to inhibit viral yield of VSV, which suggests that GTP binding is not required. Anti-viral activity observed for the GBPs is significantly lower than that of the Mx proteins [98], which suggests that anti-viral activity may not be the main function of GBPs.

mGBPs are highly up-regulated in mice upon infection with Lysteria monocytogenes or Toxoplasma gondii (T. gondii), suggesting a possible role in host defense against microbial infection [76, 78]. After IFN-γ-stimulation, murine family members mGBP-1, 2, 3, 6, 7 and 9 re-localize to the parasitophorous vacuole (PV) of intracellular T. gondii [76]. Although mGBP-5 does not re-localize to T. gondii vacuoles, it does localize to phagosomes induced by invading Salmonella [99]. Consistent with this, down-regulation of endogenous mGBP-5 mRNA by specific shRNA significantly decreases susceptibility of RAW 264.7 cells to Salmonella-induced pyroptosis under both conditions of basal expression of GBP-5 and IFN-γ-induction [99]. Pyroptosis is a form of programmed cell death associated with antimicrobial responses during inflammation [100]. Antimicrobial activity has not been directly measured for GBPs. However, hGBP-1 is secreted into the cerebrospinal fluid from patients with bacterial meningitis (71.9% compared with 5.4% of control patients have hGBP-1 secreted). This suggests that hGBP-1 secretion is associated with bacterial meningitis and future studies may correlate hGBP-1 with antimicrobial properties [101].

Two of the GBPs alter cell growth. mGBP-2 mediates the IFN-γ stimulation of fibroblast growth. However, S52N mGBP-2 is unable to promote proliferation, suggesting that nucleotide binding is important for growth promotion by mGBP-2 [102].
In contrast, hGBP-1 inhibits growth factor-stimulated proliferation of human endothelial cells [73]. Interestingly, IFN-γ promotes fibroblast proliferation but inhibits proliferation of endothelial cells [73, 102]. The C-terminal α-helices can inhibit growth, suggested by the ability of the truncated, C-terminal α-helical hGBP-1 mutant to inhibit endothelial proliferation [73]. The C-terminal helical mutant lacks the globular GTP binding region and contains only α-helices 6-13 [73]. D184N hGBP-1 is able to inhibit cell growth as efficiently as the wild-type. In addition, the hGBP-1 CaaX mutant also inhibits growth suggesting that isoprenylation is not required to inhibit growth. The CaaX mutant encodes a stop codon directly upstream of the isoprenylation motif at the carboxy terminus of GBP-1 [73]. In addition to altering cell growth, two of the GBPs inhibit cell death induced by the chemotherapeutic drug, paclitaxel. Paclitaxel is a microtubule-stabilizing agent used in the treatment of breast and ovarian cancers. mGBP-2 confers resistance to paclitaxel-induced cytotoxicity in NIH 3T3 fibroblasts [103]. Stably expressing hGBP-1 in a paclitaxel-sensitive ovarian cancer cell line, OVCAR8, was sufficient to confer resistance to paclitaxel [104].

hGBP-1 expression is considered a marker for endothelial cell activation during inflammatory diseases. Because hGBP-1 expression is induced by inflammatory cytokines, such as IFN-γ, TNF-α, IL-1α and IL-1β, hGBP-1 expression was examined in normal skin and different skin diseases with a high inflammatory component, such as adverse drug reaction, psoriasis, and Kaposi’s sarcoma [105]. All inflammatory skin diseases displayed high hGBP-1 expression, whereas it was undetectable in normal skin [74].

GBPs also alter the adhesive/invasive properties of cells. In human endothelial cells, hGBP-1 down-regulates matrix metalloproteinase-1 (MMP-1) expression, which inhibits endothelial cell invasion through collagen I and the ability of endothelial cells to
form tubes in a three-dimensional collagen matrix [106]. In contrast to hGBP-1-mediated growth inhibition, full-length hGBP-1 is required for MMP-1 down-regulation [106].

Multiple approaches to better understand the function of mGBP-2 were used in the studies below. First, the signaling mechanism involved in the IFN-γ-mediated inhibition of cell spreading was explored. Second, possible inhibition of NF-κB-mediated transcription of MMP-9 by both IFN-γ and mGBP-2 expression was examined. Lastly, mutagenesis of mGBP-2 was used to determine the properties for the inhibition of cell spreading.
HYPOTHESIS

IFN-γ can modulate the responses to integrins, growth factors and other cytokines. Some of these are involved in the regulation of cell adhesion, migration, cytoskeletal arrangement, and cellular morphology. We propose that one of the IFN-γ-induced proteins, mGBP-2, is involved in mediating some of these interferon responses. To more fully define the role of mGBP-2 in the modulation of cell adhesion, spreading, and MMP-9 levels, we have begun an extensive analysis of the mechanisms by which mGBP-2 functions. We hypothesize that the mGBP-2 inhibits a downstream molecule central to the downstream signals initiated by integrins and growth factors. We expect to understand the role of mGBP-2 in these changes by isolating the pathway in which mGBP-2 signals. In the subsequent chapters, I will present data that analyzes the mechanism by which mGBP-2 inhibits cell spreading and adhesion as well as MMP-9 inhibition.
CHAPTER II. mGBP-2 and IFN-γ inhibit Rac activation during cell spreading on fibronectin (FN) and PDGF treatment: possible role for phosphoinositide 3-kinase (PI3-K).

ABSTRACT

Interferon-γ inhibits the spreading of NIH 3T3 fibroblasts initiated by both integrin engagement and PDGF stimulation. Expression of the IFN-γ-induced GTPase, mGBP-2, is sufficient to retard cell spreading on fibronectin (FN) and after PDGF treatment. Knock-down of mGBP-2 by siRNA prevents IFN-γ-mediated inhibition of cell spreading. Constitutively active Rac1(G12V) rescues the inhibition of spreading by mGBP-2, but active RhoA(G14V) or Cdc42(G12V) do not. Consistent with this, mGBP-2 inhibits Rac activation during cell spreading and by PDGF treatment, demonstrating that mGBP-2 inhibits Rac activation by more than one stimulus. mGBP-2 inhibits the activation of Akt, a downstream target of PI3-K, during cell spreading. PI3-K is an activator of Rac. Inhibition of Akt activation is accompanied by the incorporation of mGBP-2 into a protein complex containing the catalytic subunit of PI3-K, p110. The association of mGBP-2 with p110 appears important for the inhibition of cell spreading because S52N mGBP-2, which does not incorporate into the protein complex with p110, is unable to inhibit cell spreading. This is the first report of a novel mechanism by which IFN-γ can alter how cells respond to subsequent extracellular signals, by the induction of mGBP-2.
INTRODUCTION

Among the consequences of IFN exposure are changes in adhesion [4, 5], migration [6], cytoskeletal arrangement [7], and cellular morphology [8]. While it is apparent that IFNs elicit these changes primarily through the transcriptional regulation of hundreds of genes [18, 107], it remains unclear which induced or repressed proteins are responsible for which phenotypic changes.

Cell adhesion and spreading are initiated by incoming signals from the interaction of membrane-based integrins or growth factors with the extracellular matrix (ECM) that activates a complex cascade of signaling events, which subsequently trigger morphological changes [20, 108]. Integrins transduce signals bidirectionally through outside-in and inside-out mechanisms. In addition, there is reciprocal crosstalk between integrins and growth factor receptors. Integrin engagement, in turn, can activate growth factor receptors, such as platelet-derived growth factor receptor (PDGFR), thus amplifying integrin signals and vice versa [109]. While our understanding of crosstalk between integrins and growth factors has increased tremendously, we know little about how IFNs modulate signals from either integrins or growth factors.

The guanylate-binding proteins (GBPs) are a family of 67 - 69 kDa GTPases that are induced by IFN-α/β and IFN-γ, as well as TNF-α and IL-1α/β [76, 77, 110]. The role of GBPs in IFN responses is unclear. In the studies presented here, we demonstrate that IFN-γ treatment inhibits the spreading of NIH 3T3 fibroblasts downstream of both integrin engagement and PDGF stimulation. mGBP-2 is the IFN-γ-induced protein responsible for this inhibition of cell spreading. mGBP-2 inhibits Rac activation as cells spread on fibronectin and after PDGF treatment, showing that mGBP-2 can inhibit Rac
activation by more than one stimulus. mGBP-2 also inhibits activation of Akt, a
downstream target of PI3-K. This inhibition is accompanied by the incorporation of
mGBP-2 into a protein complex containing the catalytic subunit of PI3-K, p110. This
study provides the first evidence that IFN-γ alters fibroblast cell spreading. It also identifies
mGBP-2 as the IFN-regulated protein responsible for the inhibition of cell spreading and
indicates that mGBP-2 functions to inhibit spreading by modulating signals that should
activate Rac, possibly by inhibiting PI3-K.

MATERIALS AND METHODS

I. Cells and Reagents

NIH 3T3 cells were obtained from American Type Culture Collection (ATCC,
Rockville, MD). The cell lines constitutively expressing Flag epitope-tagged wild-type
mGBP-2 clone 4.3 and clone 4.1, S52N mGBP-2, and control clones 20 and 16
containing empty vector were generated as previously described [102]. Unless stated
otherwise, cells were cultured in complete media (CM): Dulbecco’s modified Eagle’s
medium (DMEM) with 4.5 g/L glucose without glutamine (Glu), 10% fetal bovine serum
(FBS; Atlanta Biologicals, Lawrenceville, GA), 50 µg/ml penicillin/streptomycin (P/S;
Mediatech, Herndon, VA) and 2 mM L-glutamine (Mediatech) at 37°C in 5% CO₂. The
media for the stable transfectants was supplemented with G418 (400 µg/ml; Research
Products International, Mount Prospect, IL).
II. cDNA Constructs and Expression Plasmids

i. Generation of mGBP-2 mutants

The construction of Flag epitope-tagged mGBP-2, S52N mGBP-2 and STIL mGBP-2 were described previously [79, 102].

ii. Additional plasmids used.

The plasmids pRK5, pRK5 Myc-Rac1(G12V), pRK5 Myc-Cdc42(G12V), and pRK5 Myc-RhoA(G14V) were a gift from Dr. Amy Wilson-Delfosse (Case Western Reserve University, Cleveland, OH). The plasmid for the PBD of PAK1 as a GST fusion protein was obtained from Richard Cerione via Lawrence Quilliam. Myc epitope-tagged Tiam1 was obtained from Lawrence Quilliam (IUPUI, Indianapolis, IN).

III. Cytokines and Matrix Proteins

Purified human fibronectin and mouse laminin were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Mouse type IV collagen was purchased from Invitrogen. Murine PDGF was purchased from Calbiochem. Recombinant mouse interferon-γ (rmIFN-γ) was purchased from PBL Biomedical Laboratories (Piscataway, NJ).

VI. Generation of Cell Lysates, Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblot Analysis

Cells were lysed in RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 µl/ml Protease inhibitor cocktail (Sigma), 1 mM PMSF, 25 mM sodium fluoride, 10 mM sodium vanadate, 100 µM sodium molybdate,
and 50 mM sodium pyrophosphate), unless otherwise described. The protein concentrations of the lysates were analyzed using 2 µl of lysate and 198 µl of Protein Assay Dye Reagent Concentrate (Bio-Rad) diluted 1:5 in water in a 96 well plate. To determine the amount of protein in each lysates, the absorbance at 595 nm was compared to a standard curve generated using the absorbances obtained for 1, 2, 3, 4, 5, and 6 µg bovine serum albumin (BSA). Cell lysates were size fractionated on SDS-PAGE gels (8 – 12%) using Tris-Glycine-SDS TANK buffer (25 mM Tris, 0.192 M Glycine, 0.1% SDS) and transferred to Immobilon-PVDF transfer membrane (Millipore, Billerica MA). Wet transfer was performed using 1X transfer buffer (25 mM Tris, 0.192 M Glycine, 20% methanol) at 110 volts for 70 min or 30 V overnight (ON) for 1.5 mM gels.

Membranes were blocked in TBS-Tween (250 mM Tris, pH 8, 5 M NaCl, 0.05% Tween-20) plus 5% nonfat dry milk or 5% BSA for 1 hr at room temperature (RT) or ON at 4°C. Membranes were incubated for 1 hr or ON in the primary antibodies described below. Membranes were washed in TBS-Tween 3 times for 10 min each. The secondary antibodies, hydrogen peroxidase-conjugated goat anti-mouse immunoglobulin (1:2000) or goat anti-rabbit immunoglobulin (1:3000) (Jackson ImmunoResearch, West Grove, PA) were diluted in TBS-Tween containing 5% nonfat dry milk or BSA and incubated with the membranes for 1 hr at RT. Following incubation with secondary antibodies, membranes were washed 3 times for 10 min each in TBS-Tween and chemiluminescence was detected using either the Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) or the ECL Plus Western Blotting Detection System (Amersham Biosciences, Pittsburg PA) and exposure to X-ray film. Where necessary, the membranes were stripped using Restore Western Blot Stripping Reagent (Pierce, Rockford, IL) and
the complete removal of antibody was verified by re-exposure to X-ray film.

IV. Antibodies for Immunoblot Analysis

The following primary antibodies were diluted in TBS-Tween plus 5% nonfat dry milk: rabbit anti-mGBP-2 (1:800) [79], mouse anti-FLAG M2 (1:1000) (Sigma, Saint Louis, MO), affinity purified mouse anti-FLAG M2 (1:1000) (Sigma), rabbit anti-actin (1:3000) (Sigma), mouse anti-Myc clone 9E10 (1:200) (Santa Cruz, Santa Cruz, CA. The above antibodies were incubated with membrane for 1 hr at RT. The following primary antibodies were diluted in TBS-Tween plus 5% BSA and incubated with the membrane ON at 4°C: anti-α4 integrin (1:1000) (Santa Cruz, sc-6589-r), rabbit anti-PI3-Kp85 (1:500) (Millipore, Billerica, MA). The following antibodies were diluted in TBS-Tween plus 5% nonfat dry milk and incubated ON: mouse anti-Rac (1:500) (BD Transduction Laboratories, San Jose, CA), anti-Tiam1 (1:500) (Santa Cruz), rabbit anti-PI3-Kp85 (1:500) (BD Transduction Laboratories, San Jose, CA), rabbit anti-PI3-Kp110 (1:200) (Santa Cruz) rabbit anti-cyclophilin B (1:1500) (Abcam, Cambridge, MA), mouse anti-IGTP (1:500), and rabbit anti-mouse α4 integrin (Santa Cruz, sc-6589-r).

V. Spreading Assays and Measurement of Cell Surface Areas

NIH 3T3 cells were transfected with FuGENE 6 (Roche, Indianapolis, IN) or ExpressFECT (Denville Scientific, South Plainfield, NJ) per manufacturer’s instructions and cells were processed for spreading analysis 24 hrs later. Coverslips (12 mm circles) in 24-well dishes were ethanol sterilized and coated with 300 µl fibronectin (10 µg/ml) in PBS for 45 min. Cells were gently suspended by treatment with 0.05% trypsin/0.53 mM
EDTA (Invitrogen, Carlsbad, CA), diluted in serum-free media (SFM) and allowed to recover in suspension for 30 min at 37°C. Spreading assays done with the transfected siRNAs and for the co-immunoprecipitation assays were performed in 0.5% serum. Cells (1.5 x 10⁵) were plated per coverslip and allowed to adhere for 35 min. Unattached cells were removed by gentle washing with PBS. Attached cells were fixed for 40 min with 4% paraformaldehyde in PBS. After removal of paraformaldehyde and washing, coverslips were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Images were collected using a Leica DM IRB HC inverted microscope with a cooled Retiga EX Monochrome Digital camera and analyzed with ImagePro Plus software. For surface area determination, calibration was performed with a stage micrometer to provide the information of pixels/μm for the appropriate objective. Surface areas of 100 cells per coverslip were measured by using a mouse to circle the outside of the cells. The ImagePro software calculated the surface areas in μm² based on the pixels/μm. The attached control cells varied in surface area from 200 μm² to greater than 3000 μm². Surface areas were grouped into 14 increments of 200 μm² and expressed as the percent of the adherent population of cells. The smallest increment (increment 1) contained cells that had surface areas between 200 and 400 μm² and the largest (increment 14) contained cells with areas between 2800 and 3000 μm². Cells were classified as “spread” or “unspread” based on measured size cutoffs of ≤ 600 μm².

For the spreading assays with Tiam1, NIH 3T3 cells were transfected with FLAG-mGBP-2–containing vector and the Myc-Tiam1-containing vector (1:1 ratio of GBP to Tiam1 plasmid) together with pCMV-eGFP-C1 (3:1 ratio of GBP/Tiam1 to GFP). Previous experiments by Sujata Balasubramanian determined the ratios for the one
plasmid to GFP ratio to ensure each GFP-expressing cell contained the other co-
transfected plasmid. Twenty-four hrs after transfection cells were processed as described
above and surface areas of the GFP- expressing cells were measured as above.

The constitutively active Rho family spreading assays were performed by plating
control and mGBP-2 stably expressing cells (5 x 10⁵), allowing them to adhere ON.
Cells were transiently transfected with 6 µg Myc-tagged Rac(G12V), Rho(G14V),
Cdc42(G12V) or pRK5 using ExpressFECT (Denville Scientific), according to
manufacturer’s instructions. Following 24 hr, the cells were allowed to spread as
described previously. Following spreading, cells were fixed in 4% paraformaldehyde
(Tousimous, Rockville, MD) for 40 min, permeabilized in 0.2% Triton X-100 (Fisher) for
10min. Cells were washed 3 times in PBS, blocked in 10% Horse Serum, and incubated
in anti-Myc (1:200; Santa Cruz) and anti-mGBP-2 (1:200) for 1 hr at RT. Cells were
washed 3x in PBS and incubated in anti-rabbit Alexa Fluor 594-conjugated anti-rabbit
(1:500; Invitrogen) and Alexa Fluor 488-conjugated anti-mouse (1:500, Invitrogen)
antiseras. Coverslips were mounted onto slides with Vectashield plus DAPI (Vector
Labs) and analyzed by fluorescence microscopy. Surface areas of transfected cells were
measured by Image Pro software. Images for each field were taken in both green and red
excitation filters and the images were overlaid. For the cells transfected with mGBP-2 or
Rho family and control, only those expressing mGBP-2 or the Rho family were
measured. For the cells co-transfected with both mGBP-2 and the Rho family, only those
cells expressing both plasmids were measured.
VI. Statistics

The association between cell type or treatment and proportion of unspread cells was assessed using Cochran-Mantel-Haenszel tests of association, stratified by experiment repetition (CMH). The significance level of each test was 0.05; a Bonferroni correction was applied to the significance for a particular comparison when three tests were done (i.e., 0.05/3 = 0.017). Statistical analyses were performed using the SAS system (Cary, NC). Once it had been decided that an unspread cell would be defined as one with a surface area of 600 μm² or less, then the means, standard deviations, and p values were calculated using Microsoft Excel. While results from a single transfection and isolation are shown, they were confirmed using transfectants from a second transfection process and isolation.

VIII. Determination of levels of activated Rac

Control and mGBP-2-expressing cells (5 x 10⁶) were plated onto each of two 150-mm dishes and allowed to adhere ON. After serum starving for 2 hr, the cells were prepared for cell spreading as described above. Cells were allowed to spread for 30 min in serum-free media onto two 150-mm fibronectin-coated (10 µg/ml) plates. Attached cells were lysed in 250 µl of lysis buffer per dish (50 mM Tris, pH 7.5, 10 mM MgCl₂, 1% IGEPAL CA-630 (Sigma), 150 mM NaCl, 1 mM sodium vanadate, 1 µl/ml protease inhibitor cocktail (Sigma) and 1 mM PMSF [111]. Cell lysates were clarified by centrifugation at 13,000 x g for 3 min at 4°C. Cell lysates (500 µg) were added to 30 µg Pak1 PBD/GST beads in a final volume of 500 µl and the samples were rotated at 4°C for 45 min. The Pak1 PBD/GST fusion proteins were isolated from BL21 E. coli cells by growing in Luria Broth (LB; Fisher), inducing with 0.2 mM IPTG and isolating protein by sonication. The PBD/GST fusion proteins were incubated with glutathione sepharose 4B beads (Amersham), washed 10 times and quantified by both Bradford assay and
Coomassie-stained PAGE gel. The complexes were washed twice with 500 µl lysis buffer and then twice with 500 µl lysis buffer without IGEPAL. Complexes were dissociated in 5X Laemmli sample buffer by heating at 95°C for 4 min and size fractionated on 10% PAGE gels. Total cell lysates (20 µg) were also included. Rac levels were determined by immunoblot analysis as described above. Immunoblots were quantified using Kodak 1D Scientific Imaging software. Relative optical intensities for active Rac levels were performed by calculating the pixel intensity of each region of interest with an identical size and shape box for each band. The background was subtracted from the pixel intensities. The levels of active Rac from the pulldowns were normalized to total cellular Rac and set to 100% for the control cells for each blot. To analyze Rac activation by PDGF, control transfectants and mGBP-2-expressing cells (5 x 10^6) were plated, allowed to adhere ON, serum starved for 2 hr, and treated with 10 ng/ml PDGF (Chemicon International, Temecula CA) for 5 min. Cells were lysed and processed for analysis of levels of active Rac as described above.
VII. siRNA transfections and analyses

NIH 3T3 cells (3.25 x 10⁴) were plated into individual wells of a 24-well dish and co-transfected with 50 nM RISC-FREE transfection control and either 50 nM mGBP-2 SMART Pool siRNA (a pool of 5 siRNAs: 5'-GCUGUGUGGUGAAUUUGUAUU-3', 5'-GUUGAAACACUUCACAGAUU-3', 5'-AGACGAUUCGCUAACUUUUU-3', 5'-CAAAACAAAUCGUCUGGGAUU-3'), 50 nM cyclophilin B siRNA (5'-GGAAAGACUGUUCCAAAA-3') or 50 nM nontarget siRNA (5'-UAGCGACUAAACACAUAA-3') using Dharmafect #1 per manufacturer's instructions (Dharmacon, Lafayette, CO). Thirty H after transfection, cells were treated with IFN-γ (500 U/ml) for 18 hr. To analyze protein expression at the single cell level, cells were initially plated onto glass coverslips in the 24-well dishes and analyzed by indirect immunofluorescence as described below. For immunoblot analysis, cells from 6 wells of the 24-well dish were lysed, combined, and 20 µg of total cell lysates were analyzed as described for immunoblots. For spreading assays, multiple wells were transfected and treated as described. The cells were suspended as described for other spreading assays and cells of each type were plated (1.5 x 10⁵) onto FN-coated coverslips for 40 min. The surface areas of cells containing RISC-FREE reagent (red perinuclear staining) were determined as described previously.

IX. Indirect Immunofluorescence

For analysis of siRNA knockdown, cells were transfected with the siRNAs as described and processed for immunofluorescence using rabbit polyclonal anti-mGBP-2 (1851; 1:200), rabbit polyclonal anti-cyclophilin B (1:500; Santa Cruz), or mouse
monoclonal anti-IGTP (1:200; BD Transduction), and Alexa Fluor 488-conjugated
donkey anti-goat or anti-rabbit and Alexa Fluor 594-conjugated goat anti-rabbit or anti-
mouse antibodies (Molecular Probes).

The antibodies used for immunofluorescence were rabbit polyclonal anti-FLAG
(1:1000), anti-Myc 1:400 and Alexa 488-conjugated goat anti-mouse antibody and Alexa
594-conjugated goat anti-rabbit (Molecular Probes). Images were captured with a Leica
DM IRB HC inverted microscope with a cooled Retiga EX Monochrome Digital camera.

X. Actin Staining

Cells were plated onto coverslips in complete media for 4 hr, followed by incubation
in serum-free media for 16 hr. Cells were untreated or treated with 10 ng/ml platelet-
derived growth factor (PDGF; Chemicon) for 5 or 10 min on coverslips. Cells were
washed and actin filaments were labeled by fixing with 4% paraformaldehyde in PHEM
buffer (60 mM Pipes, 25 mM HEPES, 5 mM EGTA, 1 mM MgCl₂, 3% sucrose, and
0.1% Triton-X 100) containing 10 µl Alexa Fluor 594-conjugated Phalloidin (Invitrogen)
per 200 µl for 20 min at 37°C. Cells were washed 3 times with PBS and mounted in
Vectashield with DAPI. Images were captured with a Leica DM IRB HC inverted
microscope with a cooled Retiga EX Monochrome Digital camera. Surface areas were
measured as described for the spreading assays.

XI. Western Blots for Akt Activation

Control transfectants and mGBP-2-expressing NIH 3T3 cells were serum-starved for
1 hr and processed for cell spreading on FN. Adherent cells were lysed in 200 µl of cold
RIPA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium
deoxycholate, 0.1% SDS, 1 μl/ml Protease inhibitor cocktail (Sigma), 1 mM PMSF, 25 mM sodium fluoride, 10 mM sodium vanadate, 100 μM sodium molybdate, and 50 mM sodium pyrophosphate) per 10-mm FN-coated dish on ice. Samples of the cells were also taken: 1) after the 1 hr serum starvation and 2) before plating but after the recovery in suspension. Lysates were clarified by centrifugation at 10,000 x g for 5 min at 4°C and analyzed by immunoblot with anti-phospho-Akt antibodies as described below. The membranes were then stripped and immunodetected with antisera against total Akt. Films were quantified using Kodak 1D Scientific Imaging software. Relative optical intensities for active Akt levels were performed as described for active Rac and the results for levels of phospho-Akt were normalized to total Akt.

**XII. Co-immunoprecipitation Assays**

Control (C20/C16 clones), mGBP-2 (4.3/4.1 clones) stable clones were plated (5 x 10^6) in complete media per four 150-mm dishes each cell line and allowed to adhere ON. Four 150-mm dishes were coated with 6 ml of 10 μg/ml purified human fibronectin (Roche) for 45 min at RT. Control and mGBP-2-expressing cells were processed for cell spreading as previously described. Supernatants were removed and cells were resuspended in 12 ml DMEM with 0.5% FBS and kept in suspension for 20 min. Cells were plated onto two FN-coated 150-mm dishes each for both control and mGBP-2-expressing cells. Cells were allowed to adhere and spread for 35 min at 37°C, 5% CO₂. Unattached cells were removed by washing twice gently with 5ml HBSS (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 4.2 mM NaHCO₃, 1 mM sodium vanadate) on ice. Adherent cells were lysed with 250 μl lysis buffer A per dish (10 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1.0% Triton
X-100, 1 µl/ml Protease Inhibitor Cocktail (Sigma), 0.1 mM PMSF, 25 mM sodium fluoride, 10 mM sodium vanadate, 100 µM sodium molybdate, 50mM sodium pyrophosphate). Lysates were clarified by centrifugation at 10,000 x g for 5 min at 4°C. Cell lysates (500 µg) were added to 100 µl washed anti-FLAG M2 agarose (Sigma) beads, anti-PI3-Kp110 (Santa Cruz), or anti-PI3-Kp85 (Millipore or BD Transduction Laboratories) antibodies in a final volume of 500 µl and the samples were rotated at 4°C ON. Protein G agarose (45 µl of a 50% slurry) was added to the samples with anti-PI3-K and rotated at 4°C for 1 hr. The immune complexes were washed 4 times with 500 µl of lysis buffer A. Complexes were dissociated in 5X Laemmli sample buffer by heating at 95°C for 4 min, size fractionated on 8% PAGE gels and transferred to PVDF membrane. Total cell lysates (TCL; 20 µg) were also included. Membranes were probed for anti-mGBP-2 or anti-FLAG and anti-PI3-Kp110 antibodies. In addition, immunoprecipitations were repeated and the total cell lysates were pre-cleared with Pansorbin (Calbiochem, San Diego, CA) for 30 min and protein G agarose were incubated with 3% BSA for 1 hr.

RESULTS

i. mGBP-2 or IFN-γ retard cell spreading on fibronectin (FN).

mGBP-2 is undetectable in NIH 3T3 fibroblasts prior to IFN-γ treatment, and is only detectable after 4 hr of treatment [79]. After 8 hr of treatment, the level of mGBP-2 expression is high and remains high through 24 hr. Stable NIH 3T3 fibroblast cell lines expressing mGBP-2 were established to study the role of mGBP-2 in IFN responses. Expression levels of the stable mGBP-2-expressing cell lines are comparable to those after IFN-γ treatment for 24 hr [102]. mGBP-2-expressing cells are delayed in their
ability to re-attach and spread after trypsinization compared with the control transfectants.

During cell adhesion and spreading, cells undergo reorganization of the actin cytoskeleton to produce morphological changes. One way to analyze the ability of a cell to reorganize the actin cytoskeleton is to study the ability of cells to adhere and/or spread on a defined substrate. The effect of mGBP-2 expression on NIH 3T3 fibroblast adhesion to FN was examined. Stably transfected control and mGBP-2-expressing cells were suspended and plated in 24-well dishes for 20 min. Fewer cells expressing mGBP-2 adhered to all concentrations of FN examined compared to control cells (Fig. 4A). To confirm the inhibition of adhesion in mGBP-2-expressing cells, control and mGBP-2-expressing cells were labeled with 4 µM Calcein AM and plated on FN-coated dishes and allowed to adhere for 10 min as described above. Adhesion of mGBP-2-expressing cells was markedly decreased in mGBP-2-expressing cells (Fig. 4B).

Cell spreading is a highly complex process that follows cell attachment and involves the generation of new substrate adhesion sites, cytoskeletal reorganization, and lamellipodia formation [112]. To examine cell spreading, stably transfected control and mGBP-2-expressing cells were plated on FN-coated coverslips and allowed to attach. The unattached cells were gently removed by washing with PBS, fixed and mounted. The surface areas of 100 cells per each cell type were determined and graphically represented as the percentage of adherent cells with surface areas in increments of 200 µm². There were more mGBP-2-expressing cells in the smaller three size increments than control transfectants (Fig. 4C). Only 16 ± 5% of the control cells had surface areas ≤ 600 µm² (increments 1 + 2), while 42 ± 10% of the mGBP-2-expressing cells failed to spread to surface areas > 600 µm² (Fig. 4D). Using a less stringent measure of an
unspread cell (the first 3 increments, or \( \leq 800 \, \mu m^2 \)), still resulted in twice as many mGBP-2-expressing cells that failed to spread compared to control (Fig. 4D). Comparable results were obtained for other clones expressing mGBP-2 at similar levels (data not shown). To confirm that the inhibition of cell spreading was not an artifact due to clonal variation, additional control and mGBP-2 expressing clones were examined for cell spreading. Only 16 ± 1% of the control clone 16 cells were unspread, while 46 ± 6% of the mGBP-2 clone 1 cells were unspread.

The effect of IFN-\( \gamma \) treatment on NIH 3T3 fibroblast spreading on FN was examined. NIH 3T3 cells treated with IFN-\( \gamma \) for 8 and 18 hr were allowed to spread on FN and their surface areas were determined. By 8 hr of IFN-\( \gamma \) treatment, NIH 3T3 cells were defective in their ability to spread (70 ± 10% had surface areas \( \leq 600 \, \mu m^2 \) compared to 31 ± 12% for untreated cells) (Fig. 4G). After 18 hr of IFN-\( \gamma \) treatment, spreading was not further reduced (70 ± 15% had areas \( \leq 600 \, \mu m^2 \)). Both ectopic mGBP-2 expression and IFN-\( \gamma \) treatment significantly retarded cell spreading on FN.

**ii. S52N mGBP-2 does not inhibit cell spreading.**

S52N mGBP-2 stable cell lines were generated [102]. S52N mGBP-2 has a single amino acid substitution in the GTP-binding domain of mGBP-2, comparable to the S17N substitution in Ras (Fig. 4E). S52N mGBP-2-expressing NIH 3T3 cell lines were examined and are comparable to mGBP-2 levels in the stably expressing wild-type cell lines [102]. NIH 3T3 cells expressing S52N mGBP-2 were examined for their ability to spread on FN (Fig. 4C and D). No significant difference was observed between the spreading of S52N mGBP-2-expressing cells and the control transfectants (31 ± 10% or
27 ± 7% of the cells had surface areas < 600 \( \mu \text{m}^2 \) or < 800 \( \mu \text{m}^2 \), respectively) (Fig. 4D). Based on studies of S52N hGBP-1, S52N mGBP-2 is expected to have an extremely low affinity for guanine nucleotides and to be a nucleotide-free monomer in cells.

iii. Both control transfectants and mGBP-2-expressing cells are comparable in size.

To examine whether the smaller surface areas of spreading mGBP-2-expressing cells reflects just delayed cell spreading or whether these cells are actually smaller, control and mGBP-2-expressing cells were allowed to spread on FN-coated coverslips for 24 or 48 hr (Fig. 5). Following adhesion, the surface areas of 100 adherent cells per coverslip were determined using ImagePro Plus software. The surface areas were grouped into 14 increments of 200 \( \mu \text{m}^2 \) at both 24 (Fig. 5A) and 48 hr (Fig. 5B). Both control and mGBP-2-expressing cells were able to spread to surface areas above 2800 \( \mu \text{m}^2 \). These data suggest that mGBP-2 does not abolish cell spreading, but only slows it.
Figure 4

A

B

C

D

E

F

G

H
Figure 4. mGBP-2 and IFN-γ treatment inhibit NIH 3T3 cell adhesion and spreading on FN. (A) Control and mGBP-2-expressing cells were gently suspended in 0.05% trypsin/0.53 mM EDTA followed by DMEM containing 200 µg/ml of soybean trypsin inhibitor. After pelleting, cells were resuspended in DMEM containing 1% BSA and plated at 6 x 10⁵ cells per well in 24-well dishes. After 20 min, the cells were washed and fixed with 2.5% gluteraldehyde. Attached cells were stained with 0.5% crystal violet, washed, and extracted with 1% SDS. Results were measured by absorbance at 538 nm. Fewer cells expressing mGBP-2 adhered to all concentrations of FN examined compared to non-expressing cells. A representative figure from one of two experiments performed in triplicate is shown. (B) Equal numbers of control and mGBP-2-expressing cells were labeled with 4 µM Calcein AM (Molecular Probes) per manufacturer’s instructions before plating on FN (5 µg/ml) coated dishes and allowed to adhere for 10 min as described above. After removal of non-adherent cells, adherent cells were measured by absorbance at 530 nm. The adhesion of the control cells was set to 100% and the results are expressed as the mean percent of control adhesion ± standard deviation (n = 4). Prior to adhesion assays, dishes were coated with purified human FN per manufacturer’s instructions and blocked with 1% BSA in PBS ON at 4°C. (C) Cells were plated onto coverslips coated with 10 µg/ml FN and allowed to adhere for 20 min. The non-adherent cells were removed by washing with PBS and the adherent cells were fixed with 4% paraformaldehyde. The surface areas were measured of 100 cells from each type and grouped into 14 increments of 200 µm². A representative breakdown of the surface areas from one experiment is shown. More mGBP-2-expressing cells failed to spread beyond 800 µm² (increments 1 through 3). (D) The inhibition of cell spreading
by mGBP-2 is represented graphically as the number of cells with surface areas ≤ 600 or ≤ 800 µm². The results represent the mean ± standard deviation (n = 4). mGBP-2 expression significantly inhibited cell spreading (** = p < 0.001) when compared to control transfectants. In addition, the proportion of unspread S52N mGBP-2-expressing cells was not significantly different from the control cells. (E) Schematic of wild-type mGBP-2 and S52N mGBP-2. (F) Comparable expression levels were confirmed by immunoblot analysis with anti-flag antisera [102]. (G) The inhibition of cell spreading by another mGBP-2 clone is represented graphically as the number of cells with surface areas ≤ 1000 µm². The results represent the mean ± standard deviation (n = 2). (H) Retardation of cell spreading by IFN-γ is represented graphically as the number of cells with surface areas ≤ 600 µm². The results represent the mean ± standard (n = 3). Treatment with IFN-γ (500 U/ml) for either 8 or 18 hr was significantly associated with a larger proportion of unspread cells (* = p < 0.001). (Figure by D. Vestal, V. Gorbacheva)
Figure 5.

A  24 hours cell spreading

B  48 hours cell spreading
**Figure 5. mGBP-2-expressing cells are not smaller in size.** (A) Stably transfected control and mGBP-2 NIH 3T3 fibroblasts were plated onto coverslips coated with 10 µg/ml FN and allowed to spread for 24 hr or 48 hr. The non-adherent cells were removed by washing with PBS and the adherent cells were fixed. NIH 3T3 cells expressing mGBP-2 have similar size cells compared with control transfectants. Surface areas of cells spread on FN for 24 hr were determined and represented as percentage of adherent cells in increments of 200 µm². (B) Surface areas of cells spread on FN for 48 hr were determined and represented as percentage of adherent cells in increments of 200 µm².
iv. Knock-down of mGBP-2 by specific siRNAs rescues IFN-γ-mediated inhibition of cell spreading.

To confirm that mGBP-2 is required for IFN-γ-mediated inhibition of cell spreading, mGBP-2-specific siRNAs were used to knock-down mGBP-2 expression in IFN-γ-treated NIH 3T3 cells (Fig. 6 and Fig. 7). NIH 3T3 cells were co-transfected with RISC-free transfection control and mGBP-2 siRNAs, cyclophilin B siRNAs, or nontarget siRNAs. After 30 hrs, the cells were treated with IFN-γ for 18 hr and the specificity of the knock-downs were evaluated (Fig. 6A and Fig. 7). As expected, in the absence of IFN-γ, no mGBP-2 is detected. In the presence of the mGBP-2 siRNAs, the level of mGBP-2 is reduced by about 80%. Nontarget and cyclophilin B siRNAs have no effect on mGBP-2 expression. The mGBP-2 siRNAs have no effect on the expression of another IFN-γ-induced GTPase, Irgm3 (formerly IGTP) or on cyclophilin B expression (Fig. 6A and Fig. 7).

To examine whether IFN-γ treatment could still inhibit cell spreading when mGBP-2 expression is knocked-down, NIH 3T3 cells were transfected and treated with IFN-γ (500 U/ml) and analyzed for cell spreading (Fig. 6B). Non-target siRNAs did not rescue IFN-γ-mediated inhibition of cell spreading. However, knock-down of mGBP-2 completely rescued IFN-γ-induced inhibition of cell spreading (Fig. 6B). This indicates that mGBP-2 is necessary for the IFN-γ-induced inhibition of cell spreading.
Figure 6. Specific knockdown of mGBP-2 rescues cell spreading. (A) NIH 3T3 fibroblasts were transiently transfected with siRNAs as previously described and treated with or without IFN-γ (500 U/ml). Total cell lysates (TCL) were analyzed by immunoblot with antibodies listed. (B) NIH 3T3 fibroblasts were transiently transfected with siRNAs directed against mGBP-2 and nontarget controls with RISC-free transfection control, treated with IFN-γ and processed for cell spreading after 48 hr. The surface areas of the RISC-positive cells were measured and the results are expressed as mean percentage of unspread cells, (n = 3; ** = p < .001).
Figure 7. Specific knockdown of mGBP-2. NIH 3T3 fibroblasts were transiently transfected with 50nM GBP-2, 50nM Cyclophilin, 50nM Non-target siRNAs and RISC-FREE transfection control using Dharmafect lipid transfection reagent. Cells were treated with IFN-γ for 18 hr. After 48 hr of transfection, cells were stained with antibodies to mGBP-2, Cyclophilin B and lrgm3 for analysis by indirect immunofluorescence and analyzed by fluorescent microscopy. Bar = 50 µm
v. mGBP-2 is the major contributor to IFN-γ-mediated inhibition of cell spreading.

IFN-γ induces the expression of the entire murine GBP family [76]. mGBP-1 is the most closely related murine GBP to mGBP-2, with approximately 80% amino acid identity [77]. All other murine family members are significantly different from mGBP-2, with amino acid identities in the 50% range. No good commercial antibodies against mGBP-1 are available to determine whether the siRNAs against mGBP-2 have any effect on the expression of mGBP-1. To rule out a contribution of mGBP-1 to IFN-γ-initiated inhibition of cell spreading, NIH 3T3 cells were transiently transfected with mGBP-1 or mGBP-2 and their abilities to inhibit spreading on FN were evaluated. As shown in the stably transfected cells, mGBP-2 inhibits cell spreading (Fig. 8A), however, mGBP-1 does not (Fig. 8A). This indicates that mGBP-2 is necessary for the IFN-γ-induced inhibition of cell spreading.

vi. Neither IFN-γ treatment nor mGBP-2 expression reduced the levels of the FN receptor, α5β1, or promoted the expression of integrin α4

IFN-γ treatment of cells can result in the transcriptional regulation of hundreds of genes, including a number of integrins [107, 113]. Adhesion of NIH 3T3 cells to FN is mediated primarily by the integrin heterodimer, α5β1. IFN-γ treatment for up to 48 hr did not alter the amount of α5 or β1 expressed by NIH 3T3 cells (data not shown) nor did it reduce the steady state expression of the linkers, talin, vinculin, paxillin, or α-actinin (not shown).

A recent report showed that the putative human ortholog of mGBP-2, hGBP-1, inhibits human umbilical vein endothelial cells (HUVEC) cell spreading. Forced expression of
Figure 8. mGBP-1 does not contribute to IFN-γ-inhibition of cell spreading. (A) NIH 3T3 fibroblasts were transiently transfected with empty vector, FLAG-tagged mGBP-1- or mGBP-2-containing plasmids and GFP plasmid at a ratio of 3:1, respectively. Cell spreading was analyzed as described above and the surface areas of green fluorescent cells were measured. The results are expressed as mean percentage of unspread cells (≤ 600 μm²) ± standard deviation (n = 3; ** = p < 0.001 compared to empty vector control). (B) To confirm that transfected cells expressed comparable levels of the mGBP constructs, TCL were analyzed by immunoblot with anti-FLAG antisera.
hGBP-1 induced integrin α4 expression, an integrin previously shown to inhibit cell spreading [114]. The inhibition of cell spreading by hGBP-1 was correlated with the up-regulation of integrin α4 [114]. IL-1β, TNF-α and IFN-γ all induce hGBP-1, however, integrin α4 expression is only enhanced by IL-1β and TNF-α [114]. IFN-γ induces the highest levels of hGBP-1, yet according to the FACS analysis data published in the report, cells that were treated with IFN-γ had decreased surface expression of integrin α4 expression. In addition, IFN-γ-treated endothelial cells showed the greatest inhibition of cell spreading. To examine if mGBP-2 inhibits cell spreading by up-regulating integrin α4, immunoblot analysis with anti-α4 was performed on mGBP-2-expressing cells and IFN-γ-treated cells. Examination of NIH 3T3 cells showed that neither IFN-γ treatment nor mGBP-2 expression induced integrin α4 (Fig. 9). Previous literature confirmed that the anti-α4 integrin antisera does recognize α4 integrin expression in mice [115]. This suggests that inhibition of cell spreading by IFN-γ and mGBP-2 is facilitated by a different mechanism than the up-regulation of α4 integrin expression.

vii. mGBP-2 inhibits Rac activation during cell spreading on FN.

The Rho family of small GTPases are master regulators of the actin cytoskeleton and are well characterized for their role in regulating the types of F-actin changes required for cell spreading [24-26]. To determine if inhibition of one of the Rho family members was involved in mGBP-2-mediated inhibition of cell spreading, we first examined the ability of their constitutively active mutants to rescue cell spreading. NIH 3T3 fibroblasts were co-transfected with mGBP-2 and either Rac1(G12V), RhoA(G14V), Cdc42(G12V)-containing plasmids, or empty vector and spreading on FN was analyzed (Fig. 10). RhoA(G14V) alone inhibited cell spreading on FN, comparable to previous reports [116]. Cdc42(G12V) transfected cells also had a higher percentage of unspread
Figure 9. Integrin \( \alpha_4 \) expression was not induced in NIH 3T3 cells upon IFN-\( \gamma \) treatment or expression of mGBP-2. NIH 3T3 cells were treated with IFN-\( \gamma \) (500 U/ml) for 18 hr. The level of \( \alpha_4 \) integrin in lysates from control and mGBP-2-expressing cell lines or IFN-\( \gamma \)-treated cells was analyzed by immunoblotting with a rabbit anti-mouse \( \alpha_4 \) antibody. Cell lysate from MOLT 4 cells (Santa Cruz), a human acute lymphoblastic T-cell leukemia cell line, was used as a positive control for \( \alpha_4 \)-expression. Expression of \( \alpha_4 \) was undetectable in either mGBP-2-expressing cells or upon IFN-\( \gamma \) treatment. To confirm induction of mGBP-2 by IFN-\( \gamma \), blots were also immunodetected with anti-mGBP-2 antisera. A representative blot is shown (n = 2).
cells as compared with control. Rac1(G12V) was capable of restoring cell spreading in the presence of mGBP-2 (13 ± 1% unspread cells compared to 13 ± 1.5% unspread control cells). However, neither Cdc42(G12V) nor RhoA(G14V) could rescue mGBP-2-mediated inhibition of cell spreading (81 ± 6% unspread Cdc42 cells and 90 ± 6% unspread RhoA cells compared with 13 ± 1.5% unspread control cells). These data suggest that Rac1 is downstream of mGBP-2 but Cdc42 and RhoA are not. Statistical analysis of Rac, Rho, Cdc42 +/- mGBP-2 and controls resulted in a p-value of less than 0.001. The null hypothesis would be rejected as the results are at the 5% significance level, concluding that there is strong evidence that the expected values in these groups differ.

If mGBP-2 is upstream of Rac1, cell spreading in mGBP-2-expressing cells should result in lower levels of active Rac. To confirm that mGBP-2 inhibits Rac activation during cell spreading, active Rac was affinity precipitated using the Cdc42/Rac interactive binding domain (CRIB) of p21-activated kinase (PAK) fused to GST (Rac activity assay) [117]. Significantly less active Rac was detected in mGBP-2-expressing cells spreading on FN when compared with control cells (Fig. 11). Therefore, mGBP-2 inhibits Rac activation downstream of integrin engagement.

In some of the total cell lysates from mGBP-2-expressing cells during spreading, there was slightly less Rac. However, the inhibition of Rac activation was calculated directly from the TCL and did not affect the determination of active Rac. To confirm that Rac expression is not inhibited in mGBP-2-expressing cells, TCL from control and mGBP-2-expressing cells were examined by immunoblot (Fig. 11C). There is no difference in Rac expression in control and mGBP-2-expressing cell lines.
Figure 10. Constitutively active Rac rescues cell spreading in mGBP-2-expressing cells. NIH 3T3 cells were transfected with the epitope-tagged plasmids shown below the graph. After 48 hr, cells were processed for spreading on FN. Transfected cells were determined by indirect immunofluorescence with anti-FLAG and anti-Myc antisera as described. The surface areas of double stained cells were measured and the results are expressed as mean percentage of unspread cells ($\leq 600 \mu m^2$); $\pm$ standard deviation ($n = 3$; Student’s t-test: ** = $p < 0.001$; * = $p < 0.05$ compared to vector control).
**Figure 11.**

**Figure 11.** mGBP-2 inhibits Rac activation during cell spreading. (A) Control and mGBP-2-expressing NIH 3T3 cells were allowed to spread on FN-coated dishes (10 µg/ml) for 30 min, harvested, and analyzed for the levels of active Rac. The Rac-PAK PBD/GST complexes were denatured, size-fractionated by PAGE, and analyzed by immunoblot for Rac. (B) Immunoblots from PBD pulldowns were quantified and results for levels of active Rac were normalized to total cellular Rac and then set to 100% for control cells (* = p < 0.01; n = 4). (C) Control and mGBP-2-expressing cells were harvested and analyzed for Rac expression.
viii. IFN-γ-mediated cell spreading is rescued by constitutively active Rac.

Active Rac1 can rescue the inhibition of cell spreading by mGBP-2. We addressed the question of whether active Rac could also rescue the inhibition of cell spreading by IFN-γ. NIH 3T3 cells were transfected with control vector or Rac(G12V), treated with IFN-γ, and cell spreading was evaluated (Fig. 12A). In addition to restoring cell spreading in mGBP-2-expressing cells, active Rac restores cell spreading to IFN-γ-treated cells.

To confirm that IFN-γ inhibits Rac activation, active Rac was affinity precipitated with GST-PAK from total lysates from spread cells. These cells were treated or untreated with IFN-γ for 18 hr prior to cell spreading. In IFN-γ-treated cells, less active Rac was detected compared with untreated cells during cell spreading on FN (Fig. 13). IFN-γ also inhibits Rac activation downstream of integrin engagement.

ix. mGBP-2 inhibits spreading of NIH 3T3 cells initiated by PDGF.

PDGF can also induce spreading in many cell types, which is also Rac dependent [118-121]. Cell spreading by PDGF was examined in the presence or absence of mGBP-2. Control and mGBP-2-expressing cells were treated with PDGF for 0, 5, and 10 min and spreading was analyzed (Fig. 14). Prior to PDGF treatment, cells were serum starved for 2 hr and their surface areas decreased in size (compare to Fig. 5). Control and mGBP-2-expressing cells were comparable in size when left untreated. After 5 min PDGF treatment, the control cells increased in size, whereas the mGBP-2-expressing cells remained comparable in size to the untreated cells. After 10 min of PDGF treatment,
Figure 12. Rac(G12V) rescues cell spreading in IFN-γ-treated cells. (A) NIH 3T3 fibroblasts were transiently transfected with empty vector control or constitutively active Rac constructs for 48 hr and GFP at a ratio of 3:1, respectively. Cells were untreated or treated with IFN-γ for 18 hr. Cells were allowed to adhere and spread on FN. The surface areas of green fluorescent cells were measured and the results are expressed as mean percentage of unspread cells (≤ 600 μm²) ± standard deviation (n = 3; * = p < 0.05 compared to vector control).
Figure 13.  IFN-γ treatment inhibits Rac activation during cell spreading.  (A) NIH 3T3 cells were untreated or IFN-γ (500U/ml) treated for 18 hr allowed to spread on FN-coated dishes (10 µg/ml) for 35 min, harvested, and analyzed for the levels of active Rac. The Rac-PAK PBD/GST complexes were denatured, size-fractionated by PAGE, and analyzed by immunoblot for Rac.  (B) Immunoblots from PBD pulldowns were quantified and results for levels of active Rac were normalized to total cellular Rac and set to 100% for control cells (** = p < 0.001; n = 3).
the control transfectants continued to increase in size, and the difference between the control and mGBP-2 expressing cells became more striking. Together these data suggest that PDGF treatment induces cell spreading (increasing cell surface area) in NIH 3T3 fibroblasts and mGBP-2 inhibits this increase. Therefore, PDGF-induced spreading is inhibited by mGBP-2.

**x. mGBP-2 inhibits Rac activation by PDGF treatment.**

Because Rac is required for PDGF-induced cell spreading, Rac activation by PDGF was examined in the presence or absence of mGBP-2 (Fig. 15). As expected, PDGF treatment of control transfectants activated Rac (Fig. 15A). In the presence of mGBP-2, this activation was inhibited by approximately 50% (Fig. 15). Therefore, mGBP-2 inhibits Rac activation by both adhesion and PDGF treatment. Pre-treatment of NIH 3T3 cells with IFN-γ to induce mGBP-2 expression also inhibited PDGF-induced Rac activation by approximately 50% (Fig 16B). These data suggest that mGBP-2 acts downstream of integrin engagement, but upstream of Rac itself.

**xi. Rac-specific GEF, Tiam1, restores cell spreading on FN in mGBP-2-expressing cells.**

We expect that the protein directly inhibited by mGBP-2 would also bind, either directly or indirectly, to it. mGBP-2 inhibits Rac activation, however, we cannot detect an interaction between mGBP-2 and Rac. Activation of the Rho family of proteins involves the recruitment of guanine nucleotide exchange factors (GEFs), which activate these GTPases by catalyzing the exchange of GDP for GTP [122]. We examined if the Rac-specific GEF, T lymphoma invasion and metastasis 1 (Tiam1) could rescue cell
Figure 14.

A  Untreated

B  5 minutes PDGF

C  10 minutes PDGF
Figure 14. mGBP-2 inhibits cell spreading downstream of PDGF treatment. (A) Stably transfected control and mGBP-2 NIH 3T3 fibroblasts were plated onto coverslips coated with FN and allowed to adhere ON. Cells were serum starved for 2 hr and were left untreated for (A). The non-adherent cells were removed by washing with PBS and the adherent cells were fixed and stained for actin with Phalloidin 594. Surface areas of cells spread on FN for 24 hr were determined and represented as percentage of adherent cells in increments of 200 µm². NIH 3T3 cells expressing mGBP-2 have similar size cells compared with control transfectants on the untreated coverslips. (B) Cells were treated with PDGF (10 ng/ml) for 5 min. Surface areas of cells spread on FN for 48 hr were determined and represented as percentage of adherent cells in increments of 200 µm². (C) Cells were treated with PDGF (10 ng/ml) for 10 min. Surface areas of cells spread on FN for 48 hr were determined and represented as percentage of adherent cells in increments of 200 µm², n = 3.
Figure 15. mGBP-2 inhibits Rac activation by PDGF. (A) Control and mGBP-2-expressing NIH 3T3 cells were serum-starved for 2 hr and treated with PDGF (10 ng/ml) for 5 min, harvested, and analyzed for the levels of active Rac. The Rac-PAK PBD/GST complexes were analyzed by immunoblot for Rac. (B) Immunoblots were quantified and results were expressed as described in Fig. 7 (* = p < 0.05; n = 4).
Figure 16. IFN-γ treatment inhibits PDGF-induced Rac activation. (A) Control and IFN-γ treated NIH 3T3 cells were serum-starved and treated with 500U/ml IFN-γ ON, followed by 10 ng/ml PDGF, and analyzed for Rac activation. The Rac-PAK PBD/GST complexes were analyzed by immunoblot for Rac. (B) Immunoblots were quantified and the results for expressed as described (* = p < 0.05; n = 4).
spreading in the presence of mGBP-2. Forced over-expression of Tiam1 rescued the inhibition of cell spreading by mGBP-2 (Fig. 17A). However, the failure to observe Tiam1 in a protein complex containing mGBP-2 led us to conclude that it was not the protein directly inhibited by mGBP-2 (data not shown).

xii. mGBP-2 inhibits Akt activation during cell spreading on FN.

Signaling between growth factors and integrins can proceed through PI3-K to Tiam1, which subsequently activates Rac. mGBP-2-inhibition of cell spreading is rescued by forced overexpression of Tiam1 (Fig. 17). We cannot detect an interaction between Tiam1 and mGBP-2, suggesting that the inhibition could be of a molecule upstream of Tiam1. PI3-K is a major contributor to the Rac activation upon integrin engagement [123, 124]. To screen for a possible inhibition of PI3-K, differences in activated Akt were examined during cell spreading. Akt is activated by multi-site phosphorylation, which is dependent on PI3-K activity [125]. Akt activation was analyzed by monitoring the levels of active Akt (phospho-Akt) during cell spreading in the presence or absence of mGBP-2 (Figure 18A and B). Activated Akt was significantly reduced during cell spreading in mGBP-2-expressing cells, suggesting that mGBP-2 may inhibit PI3-K activity.

To explore the possibility that PI3-K is responsible for this inhibition of Akt, co-immunoprecipitation assays were performed. Control and mGBP-2-expressing cells were allowed to spread on FN for 35 min and mGBP-2 was isolated from cell lysates using anti-FLAG antibody. Immunoblot analysis of the immunoprecipitation with anti-FLAG
Figure 17. Tiam1 restores cell spreading in mGBP-2-expressing cells. (A) NIH 3T3 cells were transfected with pCMV-eGFP and the plasmids listed below the graph. After 48 hr, cells were processed for cell spreading on FN. Surface areas of green fluorescent cells were measured and results are expressed as mean percentage of unspread cells ($\leq 600 \mu m^2$) ± standard deviation (n = 3; $^* = p < 0.05$ compared to vector control). (B) To confirm that transfected cells expressed comparable levels of the Myc-tagged Tiam1 and FLAG-tagged mGBP-2, TCL were analyzed by immunoblotting with anti-Myc and anti-FLAG antisera.
showed that the p110 subunit of PI3-K is part of the protein complex containing mGBP-2 (Figure 18C). We confirmed this interaction in spreading cells by immunoprecipitation with anti-p110 antisera (Figure 18D). Immunoprecipitations with antisera against the p85 regulatory subunit demonstrated that p85 in these cells non-specifically bound to the protein G agarose beads used for the precipitation. The level of p85 in the protein complexes isolated in the presence of mGBP-2 was not above background (Figure 18C). We conclude from this that little, if any, p85 is in the same protein complex as mGBP-2 and p110.

To further characterize the molecular complex containing PI3-K and mGBP-2, cell lysates from spreading cells were immunoprecipitated with antisera against p85 (Fig. 19). Two different commercially available antibodies against p85 were used and both yielded the same results. Equal amounts of p85 were observed in both control and mGBP-2-expressing cells, however, in the absence of mGBP-2 less p85 was precipitated by both commercial antibodies. This suggests that in the absence of mGBP-2, most of
Figure 18.

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anti-phosphoAkt

anti-Akt

B

![Graph showing relative optical intensity](image)

C

IP-FLAG

Beads only

Control | Control | mGBP-2 | mGBP-2

anti-p85

anti-p110

anti-FLAG

TCL

D

IP-p110

WBlot:

anti-Flag

anti-PI3Kp110

TCL

anti-Flag

anti-PI3Kp110

62
Figure 18. mGBP-2 inhibits activation of AKT during cell spreading. (A) Control and mGBP-2-expressing NIH 3T3 cells were allowed to spread on FN-coated dishes (10 µg/ml) for 35 min, harvested and analyzed for the levels of activated AKT. Cells were size-fractionated by PAGE and analyzed by immunoblot with anti-phosphoAkt and anti-Akt. (B) Immunoblots were quantified and results for levels of pAkt were normalized to total cellular Akt, (n = 4, * = p < 0.05). (C) Control and FLAG-tagged-mGBP-2-expressing NIH3T3 cells were allowed to spread on FN-coated dishes (10 µg/ml) for 35 min. After removal of non-adherent cells by gentle washing, the adherent cells were lysed as described in Materials and Methods. TCL (500 µg) were prepared and immunoprecipitated with or without anti-FLAG antibody ON at 4° C. Protein G agarose was added to all samples for 1 hr. Immune complexes were washed, denatured and size-fractionated on 8% SDS-PAGE gels, transferred to PVDF membranes and immunodetected with anti-PI3Kp110. Membranes were stripped and incubated with rabbit anti-mGBP-2 antisera. TCL (20 µg) were also included. A representative blot is shown (n = 2). (D) Cells were allowed to spread on FN-coated dishes as described and cell lysates (500 µg) were incubated with anti-p110 antibody ON at 4° C, followed by the addition of Protein G agarose for 1 hr. The immune complexes were washed and denatured, size-fractionated by PAGE, and immunodetected with anti-FLAG and anti-p110 antibodies. TCL (20 µg) were also included. A representative blot is shown (n = 3).
the p85 is unavailable to these antibodies. We believe that this is most likely due to the binding of p85 with another molecule. The accessibility of p85 to both antisera in the presence of mGBP-2 suggests that mGBP-2 results in dissociation of p85 from this other molecule. As expected, the amount of p110 immunoprecipitated reflected the access of p85 to the antibodies. While mGBP-2 clearly influences the environment of p85, we were unable to detect mGBP-2 as part of the protein complex immunoprecipitated with p85.

**xiii. S52N mGBP-2 is not part of a protein complex containing the p110 subunit of PI3-K.**

The specificity of the interaction between p110 and mGBP-2 was further confirmed by determining if S52N mGBP-2, which is unable to inhibit spreading, interacts with p110 (Figure 19B). Immunoprecipitation of p110 from spreading cells demonstrated the expected interaction with mGBP-2 but S52N mGBP-2 did not interact with p110. Even at longer exposure times no interaction was detected between S52N mGBP-2 and p110. This demonstrates that PI3-K p110 specifically interacts with mGBP-2 during cell spreading.

What we have shown is a mechanism by which IFN-γ exposure inhibits signals from integrins and/or PDGF. These data suggest that mGBP-2 is necessary for the IFN-γ-mediated inhibition of cell spreading by inhibiting Rac activation downstream of both integrin engagement and PDGF. Inhibition of Rac activation would presumably slow the cytoskeletal rearrangements needed for cell spreading, consistent with our data. mGBP-2 may inhibit Rac activation by interaction with the catalytic subunit of PI3-K, p110, a well-described activator of Rac.
Figure 19.  

**Figure 19.**  **S52N mGBP-2 is not found in a protein complex with the PI3-K p110 catalytic subunit.** (A) Control and mGBP-2-expressing cells were allowed to adhere to FN-coated dishes. TCL (500 µg) were incubated with one of two different anti-p85 antibodies ON at 4°C. Protein G agarose was added to all samples for 1 hr. Immune complexes were separated on 8% PAGE gels, transferred to PVDF membranes and immunodetected with anti-p110 and anti-p85. TCL (20 µg) were also included. A representative blot is shown (n = 4).  

(B) Control, mGBP-2- and S52N mGBP-2-expressing cells were allowed to spread on FN-coated dishes as described. TCL (500 µg) were incubated with anti-p110 antibody ON at 4°C followed by the addition of Protein G agarose for 1 hr. The immune complexes were washed, denatured, size-fractionated by PAGE, and immunodetected with anti-FLAG antisera. Membranes were analyzed for p110. TCL (20 µg) were included to show the level of the respective proteins. A representative blot is shown (n = 2).
DISCUSSION

IFN-γ exposure modulates how cells respond to integrins, growth factors and other cytokines. While IFN-γ alters cell adhesion and migration, much remains unclear on the mechanism that accomplishes this. We demonstrate that IFN-γ treatment of NIH 3T3 fibroblasts results in retarded cell spreading initiated by binding to FN (Fig. 1). The expression of a single IFN-γ-induced protein, mGBP-2, is sufficient to mimic this IFN-γ-mediated inhibition of spreading (Fig. 1). Knock-down of mGBP-2 expression blocks IFN-γ-mediated inhibition of cell spreading (Fig. 2), demonstrating that mGBP-2 is necessary for this inhibition. Steady state levels of α5β1 and of some of the linker proteins normally associated with the cytoplasmic tail of β1 were unchanged (not shown), suggesting that the retardation of cell spreading is not the consequence of inhibition of a single integrin heterodimer. These data suggest that signaling components between integrins and the actin cytoskeleton are possibly inhibited by mGBP-2.

Cell spreading is inhibited by mGBP-2 on more than one matrix molecule (data not shown) and cell adhesion is reduced, suggesting that mGBP-2 inhibits a pathway to Rac activation that is common to multiple stimuli. Rac is also activated by PDGF [19, 126] and both IFN-γ-treated and mGBP-2-expressing cells inhibit this activation. Both integrins and growth factor receptors use multiple cytoplasmic signaling pathways to activate Rac.

PI-3K is activated by almost all integrins, growth factors and cytokine receptors, and some G-protein coupled receptors [123, 127, 128]. The best studied of the class I PI3-K enzymes is a heterodimer containing the regulatory subunit, p85, and a catalytic subunit, p110 [128]. Activation can be facilitated by the binding of the SH2 domain of
the p85 regulatory subunit to tyrosine-phosphorylated residues on receptors or associated molecules and recruitment of the p85/p110 dimer to the membrane [128, 129]. Members of the Rho family (Rac and Cdc42) can also bind to p85 and contribute to PI3-K activation [130, 131]. In addition, GTP-bound Ras can bind to the p110 catalytic subunit [128, 132]. PI3-K can activate Rac indirectly via production of the lipid second messenger, phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P3). In addition, PtdIns (3,4,5) P3 can also bind directly to Rac in vitro and facilitate the dissociation of GDP from Rac [123]. Furthermore, inhibition of PI3-K blocks both Ras and Rac activation and migration [35].

The finding that mGBP-2 inhibits Akt phosphorylation and is found within a protein complex containing the p110 subunit of PI3-K (Fig. 18) suggests that mGBP-2 may inhibit PI3-K activity as a consequence of this interaction. Consistent with this conclusion, S52N mGBP-2, which is not found within a complex containing p110, is unable to inhibit cell spreading (Fig. 19). Inhibiting PI3-K by binding to the p110 subunit has also been observed for activated Goq, which displaces Ras when it binds [127, 133]. However, we are not sure of the significance and if it is entirely responsible for the inhibition of Rac and subsequent cell spreading.

In the context of a whole vertebrate, rarely does a cell have only one growth factor, cytokine, or adhesive option to respond to at one time. In this study, we have identified a mechanism by which IFN-γ exposure can modulate or dampen subsequent signals arising from growth factors and/or integrins. Exposure to IFN-γ for greater than 4 to 8 hr results in the expression of mGBP-2, which inhibits subsequent Rac activation by adhesion/spreading on FN or treatment with PDGF.
CHAPTER III. mGBP-2 inhibits both basal and TNF-α-induced expression of matrix metalloproteinase-9 by inhibiting the transcription factor, NF-κB.

ABSTRACT
Matrix metalloproteinase-9 (MMP-9) is important in a wide variety of normal and pathological processes. In addition to having a significant role in the angiogenic switch during tumor development, MMP-9 is important in tumor metastasis. However, much remains unclear about how MMP-9 expression is regulated. TNF-α up-regulates MMP-9 expression and interferons (IFNs) inhibit MMP-9 up-regulation by TNF-α. In this study we demonstrate that the IFN-induced GTPase, mGBP-2, inhibits both basal and TNF-α-induced MMP-9 expression in NIH 3T3 fibroblasts. Both mGBP-2 and IFN-γ reduce the expression of MMP-9 at the level of transcription. mGBP-2 mediates this inhibition, in part, by inhibiting NF-κB DNA binding activity and subsequent NF-κB-mediated transcription. mGBP-2 does not inhibit the TNF-α-induced degradation of IκBα, responsible for the liberation of NF-κB. We propose that mGBP-2 mediates this inhibition of MMP-9 by reducing NF-κB DNA binding.
INTRODUCTION

Interferons (IFNs) are a family of cytokines that illicit a wide variety of cellular activities. They can alter cell adhesion and migration, in part through modulation of interactions of cells with their extracellular environment. This can occur by changing the expression levels of cell adhesion molecules, but also by modulating the expression of enzymes that alter the extracellular matrix (ECM). IFN-γ down-regulates the expression of at least three members of the matrix metalloproteinase (MMP) family: MMP-1, MMP-2, and MMP-9 [36-44]. MMP-9, also called gelatinase B, can degrade collagens type IV, V, XIV, aggrecan, elastin, entactin, laminin, and vitronectin [47]. Since type IV collagen and laminin are common to all basement membranes, MMP-9 is believed to be important in metastasis, tumor growth, and angiogenesis [47-49]. Consistent with this, MMP-9 expression is frequently elevated in human tumors and correlates with increased metastasis [47, 50, 51].

The expression of MMP-9 is inducible by pro-inflammatory cytokines including TNF-α and IL-1β [48, 53-58]. Cytokine-mediated transcriptional regulation of MMP-9 proceeds through a limited number of promoter elements and transcription factors, including an NF-κB site, two AP-1 sites, an Sp-1 site, and an Ets site [48, 59]. IFN-γ down-regulates MMP-9 expression, however, the mechanisms by which IFN-γ inhibits MMP-9 expression are still actively under investigation.

Upon IFN-γ binding, STAT1 is activated [10]. Treatment of HeLa cells with IFN-γ activates STAT1α, which inhibits MMP-9 transcription by binding to and inhibiting the recruitment of the co-activators CBP/p300 to the MMP-9 promoter [61]. This inhibition of CBP/p300 recruitment in turn inhibits the formation of the transcription
complex required to initiate MMP-9 transcription. The inhibition by STAT1α is a relatively early event after IFN-γ exposure, requiring only 4 hr. In addition, activated STAT1α can induce the expression of a variety of genes, including the transcription factor interferon responsive factor-1 (IRF-1). IRF-1 is a transcriptional activator and tumor suppressor [62, 63]. IRF-1 inhibits MMP-9 transcription in EW-1 cells by competing with the p65 subunit of NF-κB for binding to the NF-κB site of the MMP-9 promoter, which subsequently inhibits MMP-9 transcription [64]. In addition to IRF-1, another IFN-induced protein, the class II major histocompatibility complex transactivator (CIITA), down-regulates TNF-α-induced MMP-9 expression in antigen presenting cells [65]. CIITA expression is induced by IFN-activated STAT1α, and like STAT1α, CIITA binds to CBP and inhibits both its recruitment to the MMP-9 promoter and histone acetylation. IFN-mediated inhibition of MMP-9 by STAT1α, IRF-1, and CIITA are all early responses upon IFN induction. mGBP-2 is expected to inhibit MMP-9 as a late-response rather than the early STAT1 or IRF-1 response. A recent study showed that an IFN-induced large GTPase, hGBP-1, is responsible for the down-regulation of MMP-1 expression by pro-inflammatory cytokines in endothelial cells [106].

The guanylate-binding proteins (GBPs) are a family of large, unique GTPases induced by both type I and type II IFNs. hGBP-1 inhibits the expression of MMP-1 in growth factor-stimulated endothelial cells [106]. However, nothing is known about the mechanism by which hGBP-1 inhibits MMP expression. We asked whether the putative murine ortholog of hGBP-1, mGBP-2, also inhibited the expression of one or more MMPs.

In this report, we demonstrate that mGBP-2 inhibits both basal and TNF-α-
induced expression of MMP-9 in NIH 3T3 fibroblasts. The expression of MMP-2 is not altered in these cells by either TNF-α and/or mGBP-2, and MMP-1 is not detected (data not shown). The inhibition of MMP-9 is primarily at the level of transcription, as shown by luciferase assays. MMP-9 RNA levels are reduced in both IFN-γ-treated cells and mGBP-2 expressing cells. In addition, mGBP-2 reduces NF-κB-mediated transcription by inhibiting NF-κB DNA binding activity. This suggests a mechanism by which mGBP-2 attenuates TNF-α-induced expression of MMP-9 by inhibiting NF-κB-mediated transcription.

MATERIALS AND METHODS

I. Cells and Reagents

NIH 3T3 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). The cell lines constitutively expressing Flag epitope-tagged wild-type mGBP-2 clone 4.3 and clone 4.1, and control clones 20 and 16 containing empty vector were generated as previously described [102]. Unless stated otherwise, cells were cultured as described in Chapter II.

III. cDNA Constructs and Expression Plasmids

The construction of Flag epitope-tagged mGBP-2 and STIL mGBP-2 were described previously [79, 102].

The murine MMP-9 promoter in pGL3 (MMP-9 luc) was a gift from Dr. Yves St. Pierre (University of Quebec) [134]. The actin promoter driven β-galactosidase plasmid and NF-κB-Luciferase (NF-κB luc) were gifts from Dr. Brian Ashburner (University of
The NF-κB construct has 4 repeats of consensus p65/RelA binding sites that are identical to the NF-κB site of the murine MMP-9 promoter.

III. Generation of Cell Lysates, Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblot Analysis

Cells were lysed in RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 µl/ml Protease inhibitor cocktail (Sigma), 1 mM PMSF, 25 mM sodium flouride, 10 mM sodium vanadate, 100 µM sodium molybdate, and 50 mM sodium pyrophosphate), unless otherwise described. The protein concentrations of the lysates were determined by using the Bradford Assay as previously described. Cell lysates were size fractionated on SDS-PAGE gels, transferred to Immobilon-PVDF transfer membrane (Millipore, Billerica MA) and analyzed by Western blot as described in Chapter II.

Where indicated, cells were treated with 10 ng/ml cyclohexamide for 1 hr to inhibit protein synthesis (CHX, Acros Organics, Geel, Belgium).

IV. Antibodies for Immunoblot Analysis

The following primary antibodies were diluted in TBS-Tween plus 5% nonfat dry milk: rabbit anti-mGBP-2 (1:800) [79], mouse anti-Flag M2 (1:1000) (Sigma, Saint Louis, MO), affinity purified mouse anti-Flag M2 (1:1000) (Sigma), rabbit anti-actin (1:3000) (Sigma), mouse anti-Myc clone 9E10 (1:200) (Santa Cruz, Santa Cruz, CA), anti-IκBα (1:1000) (Santa Cruz). The above antibodies were incubated with membrane for 1 hr at RT. The following primary antibodies were diluted in TBS-Tween plus 5%
BSA: anti-phospho-JNK (1:500), rabbit anti-JNK (1:1000), anti-phospho-p38 (1:500), anti-p38 (1:500), anti-phospho-ERK (1:500), anti-ERK (1:1000) anti-phospho-c-jun (1:500) and anti-c-jun (1:1000) (Cell Signaling Technologies, Danvers, MA). The above antibodies were incubated with membrane ON at 4°C. The following antibodies were diluted in TBS-Tween plus 5% nonfat dry milk and incubated ON: rabbit polyclonal anti-p65 NF-κB IgG (1:500) (Santa Cruz) and rabbit anti-mouse MMP-9 antibody (Chemicon International, Temecula, CA). Secondary antibodies that were used include: horseradish peroxidase-conjugated goat anti-rabbit (1:3,000; Rockland), and anti-mouse (1:2000; Jackson Laboratories).

V. Luciferase Assays

NIH 3T3 cells (2 x 10^5/well) were plated in triplicate in 6-well plates in complete media and transfected with 0.5 µg mGBP-2-containing plasmid (or empty control vector), 0.5 µg dominant negative (DN) or constitutively active (CA) Rho family members 0.33 µg actin β-gal plasmid, and 0.33 µg NF-κB luc per well. Cells were transfected with the combination of plasmids using FuGene 6, as described previously (Roche Applied Science). After 18 hr, 10 ng/ml TNF-α was added and 6 hr later the cells were washed with PBS and lysed in 1X Cell Culture Lysis reagent (CCLR; 100 µl; 25mM Trisphosphate (pH 7.8) 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N’,N´-tetraacetic acid, 10% glycerol, 1% Triton® X-100; Promega, Madison, WI). Culture wells were scraped and the debris from the lysates was removed by centrifugation at 12,000 x g at 4°C for 2 min. Cleared lysates (20 µl) were added in triplicate into wells of a 96-well white plate (USA Scientific, Ocala, FL) for luciferase assays. White plates were used so
all light emitted by the samples would be reflected towards the detector. The Luciferase Assay Reagent was made prior to assay by mixing the Luciferase Assay Buffer and Luciferase Assay Substrate according to manufacturer instructions (Promega). The samples were read on a Lmax luminometer (Molecular Devices, Sunnyvale, CA) after the injection of 100 µl of Luciferase Assay Reagent (Promega) by the P injector. Luminescence was measured for 10 seconds after a 1.6 second delay and analyzed with the Softmax Pro for Lmax software (Version 1.1L, Molecular Devices). The values were recorded as relative light units (RLUs). To control for variations in transfection efficiencies, the cells were co-transfected with a β-galactosidase expression plasmid driven by β-actin. For the β-gal assay, lysates (5 µl) were added in triplicate into a clear 96-well plate, followed by the addition of 50 µl 1X CCLR (Promega) and 50 µl of 2X β-gal assay buffer (335.7 mM Na₂HPO₄, 154.4 mM NaH₂PO₄, 4.9 mM MgCl₂, 256.6 mM β-mercaptoethanol and 3.3 mg ONPG/ml) to each well. Mixtures are incubated at 37°C for 10-15 min until the solution goes from clear to yellow. The reaction was terminated by the addition of 150 µl 1M Tris. Absorbance was read at 420 nm using a Vmax Plate Reader (Molecular Devices, Sunnyvale, CA). The results are graphically represented as RLU/OD/µl where the RLU per µl was divided by the OD β-gal/µl. In some experiments the results are expressed as percentage of control where the RLU/OD/µl for each sample is compared to control values.
RESULTS

i. IFN-γ and mGBP-2 inhibit MMP-9 secretion.

Both IFNα/β and IFN-γ down-regulate the expression of MMP-2 and MMP-9 [40, 42, 64]. To determine if IFN-γ inhibits MMP-9 secretion in NIH 3T3 cells, the profile of the gelatinases secreted by NIH 3T3 cells was established by gelatin zymography of conditioned media (CM) (Fig. 23A, top panel). Enzymatic activities corresponding to molecular species of 105-107 kDa, 69-70 kDa, and 64-65 kDa were observed. Based on molecular size, the 105 kDa band is expected to be pro-MMP-9, while the lower two bands represent two different forms of MMP-2. After 24 hr of treatment with IFN-γ, only the activity of the 105 kDa molecular species was decreased. MMP-2 levels were unchanged. Immunoblot analyses of the CM confirmed that the 105 kDa band is pro-MMP-9 (Fig. 20A, bottom panel). To determine if the IFN-γ-induced mGBP-2 could be responsible for this inhibition of MMP-9 secretion, the CM from control and mGBP-2-expressing cells were examined by zymography (Fig. 20C). While there was some variability in the amounts of pro-MMP-9 secreted by the control transfectants and mGBP-2-expressing cells, the presence of mGBP-2 always correlated with reduced pro-MMP-9 secretion. Expression of mGBP-2 in the transfectants was verified by immunoblotting (not shown). Consistent with a role of mGBP-2 in this process, the NIH 3T3 cell line with lower mGBP-2 expression showed lesser inhibition of MMP-9.
ii. Both IFN-γ and mGBP-2 reduce steady state MMP-9 RNA levels.

MMPs are regulated at a variety of levels including transcription, RNA stability and secretion [136]. To determine if the reduction in secreted pro-MMP-9 protein reflected changes in the steady state levels of MMP-9 RNA, RNA was analyzed by real-time RT-PCR. In cells expressing mGBP-2 there is a significant reduction in the steady state expression of MMP-9 RNA compared to control transfectants (Fig. 21). As expected, treatment with IFN-γ also resulted in less MMP-9 RNA (Fig. 21). Lower steady state RNA levels suggest that inhibition of MMP-9 may be at the level of transcription.

iii. IFN-γ and mGBP-2 inhibit both basal and TNF-α-induced transcription of MMP-9.

IFNs α, β and γ inhibit TNF-α-induced MMP-9 expression [64]. To analyze the regulation of MMP-9 by mGBP-2, we obtained a plasmid containing the minimal murine MMP-9 promoter fused to the luciferase reporter gene [134]. After treatment with IFN-γ, activity of the MMP-9 minimal promoter was reduced to 45.9 ± 17.9% of the untreated control (Fig. 22A). TNF-α also induces MMP-9 promoter activity in NIH 3T3 cells to 346.4 ± 19.8% of the level observed in untreated cells (Fig. 22A). Pre-treatment with IFN-γ completely inhibited TNF-α activation of the MMP-9 transcription (48.2 ± 14.7% of control; Fig. 22A). NIH 3T3 cells were transiently co-transfected with control or mGBP-2-containing plasmid and MMP-9/luc to examine the consequences of mGBP-2 expression on MMP-9 transcription. mGBP-2 inhibits transcription of the MMP-9 minimal promoter (Fig. 22). The minimal MMP-9 promoter is sufficient both to respond
Figure 20

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pro-MMP-9

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pro-MMP-9
MMP-2
pro-MMP-9
**Figure 20. IFN-γ and mGBP-2 inhibit secretion of MMP-9.** (A) NIH 3T3 cells (5 x 10^5 cells per well/6-well dish) were plated, allowed to attach, and serum-starved for 24 hr. Cells were untreated or treated with IFN-γ (500 U/ml) for 18 hr and/or TNF-α (10 ng/ml) for the last 7 hr. CM were collected and cleared of debris by centrifugation at 17,000 x g for 5 min at 4°C. Top panel, CM (16 µl each) were resolved on 9% gels containing 1 mg/ml Bovine Skin Gelatin under non-reducing conditions. After electrophoretic separation, the gels were renatured for 1 hr in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 2.5% Triton-X 100, washed three times in distilled water, and incubated for 24 hr in developing buffer (renaturing buffer with 10 mM CaCl_2) [137, 138]. Gels were stained with Coomassie Blue R-250 to visualize enzymatically-cleared areas. Bottom panel, CM (70 µl each) were resolved by PAGE on 9% gels and analyzed by immunoblotting for MMP-9. The gels shown are representative of 3 experiments. (B) Cells from part A were lysed and cell lysates (20 µg) were separated by PAGE on 8% gels and analyzed for mGBP-2 expression by immunoblotting as described. The membranes were stripped and reprobed for actin to verify equal loading. A blot is shown at both short and long (LE) exposures and is representative of 3 experiments. (C) Two control and two mGBP-2-expressing NIH 3T3 cell lines were plated, serum-starved, and treated with TNF-α as above. CM were collected and analyzed by zymography (top panel) and Western analysis (bottom panel). Gels shown are representative of 3 experiments. (Figure by S. Balasubramanian)
Figure 21.
Figure 21. IFN-γ and mGBP-2 reduced steady state MMP-9 RNA. Total RNA was extracted from control transfectant and mGBP-2-expressing NIH 3T3 cells and untransfected NIH 3T3 cells with or without IFN-γ treatment (500 U/ml for 18 hr). SuperScript II First Strand synthesis kit (Invitrogen) was used to generate cDNA from 3 µg total RNA. Real Time PCR was carried out using 1 µl of the resulting cDNA, 12.5µl iQ™ Sybr Green (BioRad), and 200 nM each MMP-9 sense and antisense primers (sense-GAGGAAGCCCATACAGGGCCCTTC, antisense-CACGCCCTTGCTGAACAGCAGAG), or 50 nM murine GAPDH sense and antisense primers (sense-CCAGGTTCTCCTGCGACT, antisense-ATACCAGGAAATGAGCTTGACAAAGT). PCR was performed utilizing the BioRad iCycler iQ Real Time PCR detection system with an initial incubation of 95°C for 3.5 min, then 40 cycles of 95°C for 30 sec, 60°C for 30 sec, 74°C for 30 sec. Specificity of PCR amplification was determined using the melting curve from the cDNA samples, performed according to manufacturer’s instructions. Standard curves for MMP-9 and GAPDH were performed using serial dilutions of control cell cDNA in order to determine the efficiency of the PCR primers for MMP-9 and GAPDH (Efficiency=10^(-1/slope)). The relative quantity of MMP-9 mRNA was determined using the comparative threshold cycle (Ct) method in which the relative quantity of MMP-9 was normalized to GAPDH by the equation 1.5^-ΔΔCt, where ΔΔCt is derived from the mean Ct of the sample-mean Ct of GAPDH for that sample. The mean Ct is the average of the sample in triplicate. Values are expressed as % GAPDH, which is represented by 1.5^-ΔΔCT x 100 [139]. The analyses were performed 3 times and the graph shows a representative experiment. The bars represent mean percent GAPDH ± standard errors for the triplicate
samples calculated as described. (Figure by Jill Trendel)
to TNF-α in NIH 3T3 cells and for this induction to be inhibited by mGBP-2 (Fig. 22B). Treatment with TNF-α increased MMP-9 promoter activity to 280.5 ± 69.0% that of untreated controls. In addition, mGBP-2 expression significantly inhibited TNF-α activation of MMP-9 promoter activity (153.6 ± 53.3% of untreated control) (Fig. 22B).

iv. mGBP-2 inhibits NF-κB-mediated transcription.

TNF-α induction of MMP-9 proceeds, in part, through the activation of NF-κB. In addition, inhibition of NF-κB inhibits the production of MMP-9 [140-142]. Therefore, we asked if mGBP-2 inhibition of the MMP-9 promoter was the consequence of the inhibition of NF-κB. mGBP-2 inhibited NF-κB transcription under basal conditions by greater than 80% (Fig. 22C). As expected, TNF-α treatment enhanced NF-κB promoter activity, however, mGBP-2 blocked the TNF-α induction of NF-κB activity. To further confirm mGBP-2 inhibits NF-κB, electrophoretic mobility shift assays (EMSAs) were performed.

v. mGBP-2 inhibits NF-κB DNA binding activity

Our data suggests that mGBP-2 inhibits NF-κB-mediated transcription. To confirm, we asked if mGBP-2 inhibited binding of the p65 subunit of NF-κB to DNA. The consequences of mGBP-2-expression on p65 binding are shown. Nuclear extracts from control and mGBP-2-expressing cells with or without TNF-α treatment were analyzed for DNA binding by EMSA (Fig 23). While control cells showed strong inducible binding activity of NF-κB by TNF-α (10 ng/ml), no detectable binding of p65
Figure 22.
Figure 22. mGBP-2 inhibits both MMP-9 and NF-κB-mediated transcription. (A) NIH 3T3 cells were transfected with MMP-9 luc and actin β-gal. After 24 hr the cells were incubated in serum free media (SFM) for 24 hr with or without IFN-γ (500 U/ml). Where used, TNF-α (10 ng/ml) was added for the final 4 hr. Cells were lysed and processed for luciferase activity. Results are presented as percent of control activity after normalization for β-galactosidase activity (n = 4; * = p < 0.05; ** = p < 0.01 compared to control). (B) NIH 3T3 cells were transfected with actin β-gal, MMP-9 luc, and control or mGBP-2-containing vector. After 24 hr, cells were incubated in SFM for 1 hr before addition of TNF-α (10 ng/ml) for an additional 6 hr. MMP-9 promoter activity was analyzed by luciferase activity. Results are presented as percent of untreated control activity after normalization for β-galactosidase activity (n = 4; * = p < 0.05 compared to untreated control; ** = p < 0.05 compared to TNF-α-treated control). (C) NIH 3T3 cells (2 x 10^5/well) were plated ON and transfected with NF-κB luciferase, actin β-gal, and control or mGBP-2-containing vector. After 24 hr, cells were serum-starved for 24 hr. Where used, TNF-α (10 ng/ml) was added for 9 hr. Cells were lysed and processed for luciferase activity. Results presented as percent of untreated control activity normalized to β-galactosidase activity (n = 3; * = p < 0.05 compared to untreated control). (Figure parts A and B by S. Balasubramanian)
Figure 23. mGBP-2 inhibits p65 DNA binding. (A) Nuclear extracts were prepared from control and mGBP-2-expressing cells treated with TNF-α (10 ng/ml) for 0, 0.5, 1, 2 and 4 hr. Nuclei were extracted with nuclear lysis buffer (20mM Tris-HCl, pH 7.85, 250 mM sucrose, 0.4 M KCl, 1.1 mM MgCl₂, 5mM β-mercaptoethanol, 1 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 5 mg/ml soybean trypsin inhibitor, 5 mg/ml leupeptin and 1.75 mg/ml benzamine), and extracts were frozen and stored at -80°C [143]. To define the presence of specific NF-κB proteins, nuclear extracts were pre-incubated with a 1:50 dilution of anti-p65 antibody at 25°C for 0.5 hr and then subjected to electrophoretic mobility shift assays (EMSA). EMSAs were performed by incubating the nuclear extracts with a [³²P]-labeled κB probe [144]. Gels were visualized by PhosphorImage autoradiography. A representative experiment is shown (n = 2). (B) Nuclear extracts were prepared from control and mGBP-2-expressing cells treated with varying concentrations of TNF-α for 30min and subjected to EMSA as described above. A representative experiment is shown (n = 2). (Figure by the Pfeffer Lab, University of Tennessee Center for Cancer Research)
was observed in mGBP-2-expressing cells (Fig 23A). When TNF-α treatment exceeded 30 ng/ml, mGBP-2-expressing cells showed a significant reduction in NF-κB binding (Fig. 23B).

vii. **mGBP-2 does not inhibit TNF-α-induced NF-κB activation by inhibiting IκBα degradation.**

Our data show that mGBP-2 inhibits NF-κB DNA binding activity. NF-κB is normally latent in the cytoplasm bound to the IκB complex. TNF-α treatment activates the IKK complex, which results in the phosphorylation and degradation of IκBα [145]. Once released from IκBα, the NF-κB heterodimer, p50/p65, can translocate into the nucleus, bind to DNA, and initiate transcription. We did not detect any consistent differences in the phosphorylation of the inhibitor of κB (IκB) kinase (IKK), which is the major activator of NF-κB, in the presence of mGBP-2 (data not shown). In addition, no detectable differences in translocation of p65 to the nucleus by both subcellular fractionation and immunofluorescence assays were observed (data not shown). To determine how mGBP-2 inhibits TNF-α–induced NF-κB activity, we asked if mGBP-2 inhibited TNF-α-induced NF-κB-mediated transcription by altering the levels of the inhibitor IκBα (Fig. 24). mGBP-2 did not inhibit TNF-α-induced IκBα degradation (Fig. 24). mGBP-2 inhibits NF-κB DNA binding activity. However, NF-κB activity is not inhibited in mGBP-2-expressing cells by reducing IκBα degradation or p65 translocation.
**Figure 24.** mGBP-2 inhibition of NF-κB-mediated transcription is not due to decreased IκBα degradation. Control and mGBP-2-expressing NIH 3T3 cells (1 x 10^6) were plated in 60-mm dishes in complete media and allowed to adhere for 3 hr. Cells were serum-starved for 13 hr followed by treatment with CHX (10 ng/ml) for 1 hr to inhibit new protein synthesis. Cells were treated with TNF-α (10 ng/ml) for the indicated times. Cell lysates were separated on 10% SDS PAGE gels and analyzed for IκBα by immunoblotting. Membranes were stripped and reprobed with anti-actin to verify equal protein per lane. Results from a representative experiment (n = 2) are shown.
viii. MAPK signaling pathways are not inhibited in mGBP-2-expressing cells.

The regulation of NF-κB-mediated transcription occurs at a variety of levels, including IkBα degradation by IKK phosphorylation and cytoplasmic-nuclear shuttling of p65. However, we did not detect any consistent differences in IKK phosphorylation, or in the translocation of p65 to the nucleus in the presence or absence of mGBP-2 (data not shown). MMP-9 gene expression can be activated by multiple signal transduction pathways including ERK1/2, p38 MAPK, JNK, and PI3K/Akt, which are the upstream modulators of AP-1 or NF-κB [60, 146-148]. We investigated whether mGBP-2 inhibits NF-κB-mediated transcription of MMP-9 through inhibiting the activation of the ERK1/2, p38 MAPK, or JNK pathways by TNF-α. As expected, TNF-α induced activation of all of three members of the MAPKs as measured with phospho-specific antibodies. However, mGBP-2 did not inhibit activation of any of the MAPKs (Fig. 25A). These results suggest that mGBP-2 inhibits MMP-9 by a mechanism independent of MAPK regulation.

ix. Rac is required for TNF-α-induced NF-κB activity.

Rac is an important component of at least one signal transduction cascade initiated by TNF-α and is involved in NF-κB activation [142, 149-153]. To confirm Rac is involved in the TNF-α activation of NF-κB-mediated transcription in NIH 3T3 cells, we examined the effect of the dominant negative Rho family members on NF-κB-mediated transcription. NIH 3T3 cells were transfected with dominant negative versions of
Figure 25.

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**WBlot:**
- pERK
- Total ERK
- GAPDH

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**WBlot:**
- pJNK
- Total JNK
- Actin

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**WBlot:**
- pp38
- Total p38
- Actin
Figure 25. Effects of mGBP-2 on TNF-α-induced activation of MAPK signaling pathways. (A) Control transfectants and mGBP-2-expressing NIH 3T3 cells (1.2 x 10⁶) were plated in complete media and allowed to adhere for 3 hr. After attachment, cells were serum starved for 18 hr followed by TNF-α (10 ng/ml) treatment for 15, 30, or 45 min. Cell lysates were separated on 10% SDS PAGE gels and analyzed for phospho-p38, ERK or JNK by immunoblot analysis. Membranes were stripped and reprobed with anti-p38, ERK, or JNK. The results of a representative experiment (n = 2) are shown.
RhoA(T19N), Rac1(T17N), and Cdc42(T17N) and monitored for NF-κB activation by TNF-α by luciferase activity (Fig. 26A). As expected, TNF-α induction significantly increased NF-κB-mediated transcription in control, RhoA(T17N) and Cdc42(T17N). However, in cells expressing dominant negative Rac(T17N), TNF-α-induced NF-κB transcription was significantly inhibited. Consistent with the literature, Rac appears to be required for TNF-α induction of NF-κB-mediated transcription.

Data in the previous chapter suggest that mGBP-2 inhibits Rac activation by at least two other stimuli. To examine if mGBP-2 inhibition of Rac activation could be responsible for the inhibition of NF-κB-mediated gene transcription by TNF-α, NIH 3T3 cells were transfected with constitutively active versions of RhoA, Rac1, and Cdc42 in the presence or absence of mGBP-2 and monitored for NF-κB activation (Fig. 26B). As expected, mGBP-2 inhibited NF-κB-mediated transcription in control transfectants. Constitutively active Rho and Cdc42 had no significant effect on transcription. Rac(G12V) significantly increased NF-κB-mediated transcription compared with control. In the presence of both mGBP-2 and Rac(G12V), there are two possible interpretations, mGBP-2 inhibits Rac-induced NF-κB-mediated transcription and is downstream of Rac or Rac(G12V) restored NF-κB transcription to control levels in mGBP-2-transfectants, suggesting that mGBP-2 is upstream. Our results suggest that mGBP-2 impacts one or more signaling pathways between Rac and NF-κB and will be tested by Rac activity assays to determine if mGBP-2 inhibits Rac activation during TNF-α treatment.
Figure 26.

A

B

RLU/OD/ul

RLU/OD/ul

pRK5  Rho(T19N)  Rac(T17N)  CDC42(T17N)

pRK5  Rho(G14V)  Rac(G12V)  CDC42(G12V)

Untreated  TNF-α

CMV  mGBP-2

**
Figure 26. **Rac is required for TNF-α-induced NF-κB activity.** (A) NIH 3T3 cells (2 x 10⁵/well) were transfected with NF-κB luciferase, actin β-gal, and dominant negative Rac1, RhoA, Cdc42 or pRK5 plasmid. After 24 hr the cells were incubated in SFM for 24 hr, lysed and processed for luciferase activity as described. Results presented are the mean relative fluorescence after normalization for β-gal activity ± standard deviation (n = 3; ** = p < .01 compared to basal luciferase in control transfectants. (B) NIH 3T3 cells (2 x 10⁵/well) were transfected with NF-κB luciferase, actin β-gal, mGBP-2 or pCMV vector, and constitutively active Rac1, RhoA, or Cdc42 or pRK5 plasmid. After 24 hr the cells were incubated in SFM for 24 hr, lysed and processed for luciferase activity. Results presented are the mean relative fluorescence after normalization for β-gal activity ± standard deviation (n = 4; Student’s t-test: ** = p < 0.01 compared to basal luciferase in absence of mGBP-2 or Rho family member).
DISCUSSION

MMPs are important proteins in tissue remodeling during both normal and pathological conditions. They also modulate the availability and activity of growth factors. In particular, MMP-9 is credited with directing the “angiogenic switch” and its up-regulation in tumor cells correlates with progression and metastasis. Consistent with this, MMP-9 null mice show less angiogenesis and metastasis of LLC or B16-derived tumors from their primary sites [154]. Despite the biological importance of MMP-9, much remains unclear about its regulation. A variety of cytokines, growth factors, ras, and e-src can up-regulate MMP-9 but few repressors of MMP-9 have been identified. The metastasis suppressor, KiSS-I, inhibits MMP-9 expression by inhibiting NF-κB translocation to the nucleus subsequent to increased IκBα levels [155]. Interestingly, KiSS-I is unable to inhibit TNF-α-induced expression of MMP-9 [155].

The IFN-mediated inhibition of MMP-9 transcription by STAT1α, IRF-1, and CIITA are all early responses upon IFN induction [10, 61, 64, 65]. mGBP-2 is not expressed at detectable levels in NIH 3T3 cells prior to IFN exposure [90, 102], and requires about 8 - 12 hr of IFN-γ treatment before reaching maximal levels [90]. Therefore, mGBP-2 is expected to inhibit MMP-9 as a later response after IFN-γ treatment. As with STAT1 and IRF-1, the target of mGBP-2 is the NF-κB-mediated transcription of MMP-9. In the presence of mGBP-2, TNF-α-induced IκBα degradation and release of NF-κB is not inhibited. However, in mGBP-2-expressing cells, NF-κB DNA binding activity is decreased.

NF-κB activation by a variety of stimuli requires Rac activity. TNF-α is no exception (Fig. 30). Rac activation induces the nuclear translocation of the transcription
factor NF-κB [151]. In addition, there is speculation is that Rac-induced reactive oxygen species (ROS) are involved in NF-κB activation [156-159]. Preliminary data suggest that mGBP-2 inhibits steady state ROS levels in NIH 3T3 cells (S. Balasubramanian; data not shown). However, it is currently unclear if Rac is involved in mGBP-2-mediated inhibition of NF-κB activity. Our results suggest that mGBP-2 impacts one or more signaling pathways between Rac and NF-κB and will be tested by Rac activity assays.

We have identified a novel mechanism for IFN-induced repression of MMP-9 induction by TNF-α. We speculate that it is possible that the mechanism by which IFN-γ sustains MMP-9 inhibition following the rapid STAT1α and IRF-1 responses is by induction of mGBP-2. Understanding how mGBP-2 facilitates this inhibition will significantly increase our understanding of ways in which MMP-9 expression can be repressed.
CHAPTER IV. mGBP-2 requires GTP-binding, dimerization, and isoprenylation to inhibit cell spreading.

ABSTRACT

Murine Guanylate-Binding Protein-2 (mGBP-2) is responsible for the inhibition of cell spreading by IFN-γ. Analysis of the ability of a series of truncations and point mutations of mGBP-2 to inhibit cell spreading suggests that for mGBP-2 to inhibit cell spreading GTP-binding, dimerization, and isoprenylation are all required. In addition, the mGBP-2 C-terminal α-helices 6 through 13 can inhibit cell spreading in the absence of the N-terminal globular GTP-binding portion. The ability of the C-terminal α-helices 6 – 13 to inhibit spreading suggests that the interactions of mGBP-2 with putative downstream proteins/effectors proceeds through the α-helices and not the globular domain to facilitate the inhibition of cell spreading. Our data suggest that in the context of the full-length protein, mGBP-2 requires GTP-binding and dimerization to release the C-terminal α-helices for subsequent protein-protein interactions that inhibit cell spreading.
INTRODUCTION

Interferons regulate hundreds of genes, some of which are responsible for changes in adhesion [4, 5], migration [6], cytoskeletal arrangement [7], and cellular morphology [8]. One of the most robustly induced gene families by IFN-γ is the guanylate-binding proteins (GBPs).

The GBPs are a family of 67 - 69 kDa GTPases that are induced by both Type I (IFN-α/β) and Type II (IFN-γ) IFNs, as well as TNF-α and IL-1α/β [110]. The primary amino acid sequences of GBPs contain two recognizable motifs: a GTP binding motif and a CaaX motif, the site of isoprenoid addition [80]. Unique among GTPases, GBPs hydrolyze GTP to both GDP and GMP [81, 83, 160]. In addition, GBPs have other properties that differ from those of the Ras-like and other GTP-binding proteins. For example, they are stable in the absence of guanine nucleotides and bind nucleotides with a much lower affinity than other GTPases (micromolar range) [82, 83]. In addition, GBPs exhibit cooperative GTPase activity, GTP-induced homodimerization, and form GMP as the main product after hydrolysis.

Crystal structures of the full-length hGBP-1 (hGBP1FL) were solved in the nucleotide-free state and in complex with the GTP analogue, GppNHp [80, 161]. hGBP-1 has a 36-kDa N-terminal large G (LG) domain and a 32-kDa carboxy-terminal elongated α-helical domain. Recently, three additional hGBP-1 crystal structures were solved, each at progressive steps during nucleotide-binding and hydrolysis, GDP•AlF₃-, GMP•AlF₄- and GMP-bound hGBP1LG. These structures and previous data from the Hermann lab confirm that hGBP-1 homodimerization is nucleotide-dependent and is regulated by structural changes in the switch regions [80, 161]. As predicted previously
by size-exclusion chromatography [81], GDP•AlF$\text{3}^{-}$, GMP•AlF$\text{4}^{-}$ and GMP-bound hGBP1$^{LG}$ are dimers (Fig. 27). Mutation of the residues involved in dimerization of hGBP-1 decreases nucleotide binding and GTPase activity. During dimerization, it is proposed that the backbone of helix $\alpha4$ shifts upward towards the C-terminal $\alpha12$ and $\alpha13$ helices. This shift is mandatory to avoid steric clash with the switch II region of its dimer counterpart and is not observed in the monomeric GMP-bound hGBP1$^{LG}$ structure. Therefore, dimer formation causes substantial changes in the switch regions (Fig. 27).

Classical GTPases, such as the Ras superfamily, function as molecular switches, cycling between an active GTP- and an inactive GDP-bound conformation. Relatively large conformational changes occur between the two states in two regions, designated switch I and switch II. These switch regions interact with downstream targets to transduce signals [162-164]. The switch regions of the GBPs are also flexible regions that contribute to the structural changes initiated by guanine nucleotide binding. However, the switch regions in hGBP1 are involved in dimerization and stay buried until the hydrolysis of GTP to GMP is complete, and are inaccessible for effectors [80, 161].

Analyses of mutant GTPases that are locked in specific nucleotide-bound states are useful for studying the cellular functions of the protein and/or its interactions with other proteins. Mutation of highly conserved residues for nucleotide binding and hydrolysis were made based on the crystal structures of nucleotide-free and -bound hGBP-1 [81]. The GTPase domain of hGBP-1 is identical to that of mGBP-2 as indicated by sequence alignments between the two proteins (Fig. 28). Therefore, we made the same mutations in mGBP-2 to analyze their effects on cell spreading.
Figure 27. View of the hGBP1 dimer and its interface. (A) “The two-fold symmetry related hGBP1\textsubscript{LG}\textbullet GppNHp dimer buries most of the regions that have nucleotide-dependent conformation. The P-loop is represented in yellow, switch I in green, switch II in red, RD184 in cyan and the guanine cap in lilac. (B) Model of the hGBP1\textsubscript{FL} \textbullet GppNHp dimer obtained by superimposing monomeric hGBP1\textsubscript{FL} on dimeric hGBP1\textsubscript{LG}. Localization of the prenylation CaaX motifs at hGBP1\textsubscript{FL} C termini is indicated.” (C) (Reprinted by permission from Macmillan Publishers Ltd: [NATURE] (B. Prakash, G. J. K. Praefcke, L. Renault, A. Wittinghofer, C. Herrmann, Nature 403, 567). copyright (2000).
We have previously demonstrated that IFN-γ treatment inhibits cell spreading upon integrin engagement and PDGF treatment and that mGBP-2 is sufficient to inhibit spreading in the absence of other IFN-induced proteins. In addition, both mGBP-2 and IFN-γ inhibit Rac activation subsequent to PDGF treatment and cell spreading on fibronectin (FN). Analysis of S52N, D184N, and R48A mGBP-2 suggest that the inhibition of cell spreading by mGBP-2 is dependent on GTP-binding and dimerization. In addition, the isoprenylated carboxy-terminal α-helices 6 - 13 of mGBP-2, C290 mGBP-2, can inhibit cell spreading. Together these data suggest that in the context of the full-length protein, mGBP-2 requires GTP-binding and dimerization to relieve the restrictions of the N-terminal globular region on the C-terminal α-helices 6 - 13 for subsequent protein-protein interactions that inhibit cell spreading.

MATERIALS AND METHODS

I. Cells and Reagents

NIH 3T3 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). The cell lines constitutively expressing Flag epitope-tagged wild-type mGBP-2 clone 4.3 and clone 4.1, and control clones 20 and 16 containing empty vector were generated as previously described [102]. Unless stated otherwise, cells were cultured as described in Chapter II.

II. cDNA Constructs and Expression Plasmids

i. Generation of mGBP-2 mutants

The construction of Flag epitope-tagged mGBP-2 and STIL mGBP-2 were described
previously [79, 102]. The generation of the plasmid c-Myc-pcDNA3.1hygro(-) was also previously described [102]. The plasmid c-Myc-mGBP-2-pcDNA3.1 was generated by excising mGBP-2 from FLAG-mGBP-2-pCMV2(NH) (Vestal et al., 1998) using Notl/Kpn1 and ligation into Notl/Kpn1 cut c-Myc-pcDNA3.1hygro(-). mGBP-1 was cloned using RT-PCR from RAW 264.7 cells treated with 500 U/ml IFN-γ for 18 hr using 5’-ATATGCAGGACGCTCACAGATCCCATGTCG-3’ and 5’-GGTACCTTAAATATGCAGATCCCATGTCG-3’ as forward and reverse primers, respectively.

The Asp184 residue is in the third GTP-binding site of mGBP-2. The D184N mGBP-2 was generated by two-step PCR by first amplifying the 5’ region of mGBP-2 using 5’-ATATGCAGGACGCTCACAGATCCCATGTCG-3’ as forward and 5’-CGAGGGAGAAAGCTTCAGAGTATCCCTCAGAGTACGATCCACAAAGGTT-3’ as reverse primer. The 3’ region was amplified using 5’-AACCTTTGGTGGACTCTGACAAAACCTCCTCCTCCTCG-3’ as forward and 5’-ACACTCAGAGGAGCTTCAGAGTATAGTCACCTCC-3’ as reverse. The two PCR fragments were combined and amplified using the forward primer from the 5’ amplification and the reverse primer from the 3’ amplification. The fragment was digested and inserted into NotI and BglII cut pCMV(NH) Flag. The Arg48 residue is located in the first GTP binding motif, G1. The R48A mGBP-2 was generated by two-step PCR by first amplifying the 5’ region of mGBP-2 using 5’-ATATGCAGGACGCTCACAGATCCCATGTCG-3’ as forward and 5’-AGGATTTGCCTGCTCGTAGGAGGAGGAGGAGACAATCC-3’ as reverse primer. The 3’ end was amplified using 5’-TGGGCCTCTACGCGACAGGCAAATCCT-3’ as forward and 5’-
TGTGAGATCTGGCCTTCAGAGTATAGTGCACTTCC-3’ as reverse primers. The two PCR fragments were combined and amplified using the forward primer from the 5’ amplification and the reverse primer from the 3’ amplification. The fragment was digested and inserted into NotI and BglII cut pCMV(NH) Flag. C290 mGBP-2 mutant contains only α-helices 6 - 13. C290 mGBP-2 was generated by PCR amplification using 5’-ATATGCGGCCGCGCTCTAAAGAGCCTGGGTGCAG-3’ as forward primer and the reverse primer used for D184N mGBP-2. The fragment was cut with NotI and BglII and inserted into pCMV(NH) Flag. C473 mGBP-2 contains α-helices 12 and 13. The C473 mGBP-2 was amplified with 5’-ATATGCGGCCGCTCAGAGAGGCAGCAAAGGAG-3’ as forward and the same reverse primer, cut with NotI and BglII and inserted into the same vector. mGBP-2 has a CTIL CaaX motif that receives a C20 geranylgeranyl lipid. To change the CTIL of mGBP-2 to the CTIS motif of the C15 farnesylated hGBP-1, Flag epitope-tagged CTIS was generated. This mutant was generated by PCR using 5’-ATATGCGGCCGCGCTCAGAGATCCACATGTCG-3’ as the forward primer and 5’-TATCTAGAGGCTTCAGAGATAGTGCACTTCCCC-3’ as the reverse primer. The amplified fragment was cut and ligated into NotI/XbaI cut pCMV(NH) Flag. C290 STIL mGBP-2 and C473 STIL mGBP-2 contain a single point mutation in the isoprenylation motif, CaaX, rendering these mutants unable to be isoprenylated. C290 STIL mGBP-2 was generated by PCR amplification using 5’-ATATGCGGCCGCTCAGAGATCCACATGTCG-3’ and the reverse primer for STIL mGBP-2. C473 STIL mGBP-2 was amplified with 5’-ATATGCGGCCGCTCAGAGGCGAGCAAAGGAG-3’ as the forward primer and
the same reverse primer as the CTIS mutant. All of the constructs were sequenced to verify fidelity. Dr. Deborah Vestal, Victoria Gorbacheva and Dr. Sujata Balasubramanian generated the above mutants.

II. Generation of Cell Lysates, Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblot Analysis

Cells were lysed in RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 µl/ml Protease inhibitor cocktail (Sigma), 1 mM PMSF, 25 mM sodium fluoride, 10 mM sodium vanadate, 100 µM sodium molybdate, and 50 mM sodium pyrophosphate), unless otherwise described. The protein concentrations of the lysates were determined by using the Bradford Assay as previously described. Cell lysates were size fractionated on SDS-PAGE gels, transferred to Immobilon-PVDF transfer membrane (Millipore, Billerica MA) and analyzed by Western blot as described in Chapter II.

Where described, cells were treated with 50 µM compactin (gift of Dr. Akira Endo, Tokyo) for 24 hr before immunoblot analysis for Rac1.

III. Antibodies for Immunoblot Analysis

The following primary antibodies were diluted in TBS-Tween plus 5% nonfat dry milk: rabbit anti-mGBP-2 (1:800) [79], affinity purified mouse anti-Flag M2 (1:1000) (Sigma), rabbit anti-actin (1:3000) (Sigma), mouse anti-Myc clone 9E10 (1:200) (Santa Cruz, Santa Cruz, CA). The above antibodies were incubated with membrane ON at 4°C. Mouse anti-Rac antibody (1:500; BD Transduction Laboratories, San Jose, CA) was
diluted in TBS-Tween plus 5% nonfat dry milk and incubated ON. Secondary antibodies that were used include: horseradish peroxidase-conjugated goat anti-rabbit (1:3,000; Rockland) and anti-mouse (1:2000; Jackson Laboratories) antisera.

IV. Spreading Assays and Measurement of Cell Surface Areas

NIH 3T3 cells were co-transfected with pCMV-eGFP-C1 (Clontech, Mountain View, CA) and D184N, R48A, C290, 473, C290 STIL, 473 STIL mGBP-2, and mGBP-2/1 chimera constructs (at a ratio of 3:1 GBP:GFP) by FuGENE 6 (Roche, Indianapolis, IN) or ExpressFECT (Denville Scientific, South Plainfield, NJ) per manufacturer’s instructions and 24 hr later cells were processed for spreading analysis as described in Chapter II. Previous immunofluorescence experiments by Sujata Balasubramanian determined the ratio of GBP plasmid to GFP ratio (3:1) to ensure each GFP-expressing cell contained GBP. For the spreading assay with constitutively active Rac with or without CTIS mGBP-2, NIH 3T3 cells were transfected with mGBP-2 and the active Ras plasmid (1:1 ratio of GBP to Rac plasmid) together with pCMV-eGFP-C1 (3:1 ratio of GBP/Ras to GFP). Twenty-four H after transfection cells were processed as for spreading and surface areas of the GFP-expressing cells were measured.

V. Indirect Immunofluorescence

To determine the localization of FLAG/mGBP-2 or FLAG/STIL mGBP-2 during cell spreading, NIH 3T3 cells were transfected as described and processed for cell spreading. After 30 min the cells were fixed and stained as described previously (Vestal et al., 2000). The antibodies used were anti- FLAG (M2; 1:1000) and Alexa 488-conjugated goat anti-mouse antibody (1:1000; Molecular Probes). Images were captured with a
Leica DM IRB HC inverted microscope with a cooled Retiga EX Monochrome Digital camera.

RESULTS

i. Analysis of the effects of GTP-binding site mutations, removal of the GTP binding sites, and abolition of prenylation on cell spreading.

The crystal structures of hGBP-1 demonstrate that the protein has an N-terminal globular portion followed by an elongated series of α-helices (Fig. 3). The sequence alignment between hGBP-1, mGBP-1, and -2 shows that the arrangement of α-helices, β-sheets, and turns between all three proteins are similar (Fig. 28). In addition, the guanine nucleotide binding sites between the proteins are identical [77]. In order to identify the structural and/or functional component(s) of mGBP-2 responsible for the inhibition of cell spreading, various mutants of mGBP-2 were generated (Fig. 29A). The appropriate expression of these mutants was confirmed by Western analysis (Fig. 29B). D184N mGBP-2 contains a single amino acid change from aspartate to asparagine at position 184. Asp184 is located in the unusual third guanine nucleotide binding region within the β5 sheet of mGBP-2 (Fig. 29). The Asp184 residue is involved in binding to the guanine base and not in catalysis, D184N hGBP-1 has a 25 – 50 fold decrease in nucleotide binding [81]. D184N hGBP-1 can still perform GTP hydrolysis under physiological conditions. However, D184N hGBP-1 hydrolysis is much slower and produces less GMP than wild-type hGBP-1 [81]. D184N hGBP-1 also dimerizes when GTP-bound [81]. This suggests that D184N mGBP-2 is GTP-bound and possibly dimerized. D184N mGBP-2 inhibits cell spreading on FN as well as wild type mGBP-2 (54 ± 7% unspread
cells for mGBP-2 versus 51 ± 15% for D184N) (Fig. 29C). These data suggest that mGBP-2 needs to be GTP-bound and possibly dimerized to inhibit cell spreading. In Chapter II, we showed that S52N mGBP-2 was unable to inhibit cell spreading. S52N mGBP-2 is expected to be a nucleotide-free monomer in cells, unable to dimerize. mGBP-2 has a CTIL CaaX motif, which directs isoprenylation. C290 mGBP-2 lacks the entire globular GTP binding domain and expresses only α-helices 6 through 13 (Fig. 28). C290 mGBP-2 is able to inhibit cell spreading (56 ± 10% unspread cells) as efficiently as wild type mGBP-2. This suggests that removing the N-terminal globular portion also removes the steric hindrance placed on the α-helices. We believe that the α-helices are the binding region for the protein-protein interactions that inhibit cell spreading. The data from D184N, S52N, C290 and C473 mGBP-2 suggest that some of the conformations of the GTP-binding domain constrain the ability of the C-terminus to function. The results from C473 mGBP-2 that contains only α-helices 12 and 13 are equivocal (Fig. 29C). While this protein shows some inhibition of spreading, the inhibition does not reach statistical significance. This suggests that while the α-helices 12 and 13 are available for protein-protein interactions, these alone may not be enough to facilitate full binding of the protein(s) that are required for mGBP-2 to inhibit cell spreading. STIL mGBP-2 has an amino acid substitution in the CaaX motif that changes from a cysteine to a serine and prevents isoprenoid addition. STIL mGBP-2 is unable to inhibit spreading (23 ± 7% unspread cells), demonstrating that lipid attachment is necessary for the inhibition of cell spreading.
Figure 28. Sequence alignment of hGBP-1 with murine family members 1 and 2.

The secondary structures are denoted above the hGBP-1 sequence and conserved amino acids are denoted in red. (Figure by Louis Renault, Laboratoire d'Enzymologie et Biochimie Structurales, CNRS, Gif-sur-Yvette, France)
Figure 29. mGBP-2 requires GTP-binding, dimerization and isoprenylation to inhibit cell spreading. (A) Representations of WT and mutant mGBP-2s are shown. Both C290 and C473 have unaltered CaaX sequences. (B) NIH 3T3 cells were transfected with the constructs shown above. After 24 hr, cells were harvested and TCL (20 µg) were examined for mGBP-2 expression by immunoblot analysis with anti-FLAG antisera. (C) NIH 3T3 cells were co-transfected with pCMV-eGFP and WT or mutant mGBP-2 constructs. After 48 hr, cells were processed for spreading on FN as described. Surface areas of green fluorescent cells were measured and expressed as mean percentage of unspread cells (≤ 600 µm²) ± standard deviation (n = 3; ** = p <0.01; * = p < 0.05). (Figure by S. Balasubramanian).
While D184N mGBP-2 has decreased GTP-binding, it is able to bind and hydrolyze GTP at a slower rate. Previous experiments indicate that D184N mGBP-2 can form dimers. However, it is expected that the S52N mGBP-2 is a nucleotide-free monomer in cells, unable to dimerize. Analyses of these mutations of mGBP-2 to inhibit cell spreading suggest that for mGBP-2 to inhibit cell spreading, GTP-binding and dimerization are required. The ability of the C-terminal α-helices 6 – 13 to inhibit spreading suggests that the interactions of mGBP-2 with putative downstream proteins/effectors proceeds to facilitate the inhibition of cell spreading through the α-helices and not the globular domain.

**ii. The putative constitutively active mGBP-2 is able to inhibit cell spreading.**

R48A mGBP-2 was generated and its expression was verified to further evaluate the role of GTPase activity on the inhibition of cell spreading (Fig. 30A and B). Analysis of the R48A substitution in hGBP-1 showed that the protein has a higher affinity for the GTP analog (mant-GppNHp) and lower affinities for GDP and GMP than the wild type protein [81]. R48A hGBP-1 GTPase activity is reduced by two orders of magnitude compared to wild type [81]. Together, this information suggests that R48A mGBP-2 would bind to but not hydrolyze GTP. In other words, it would function as a constitutively active GTP binding protein. R48A mGBP-2-mediated inhibition of cell spreading was not significantly different from wild type (Fig. 30C). This is consistent with GTP-binding and dimerization being necessary for the inhibition of cell spreading.
Figure 30. R48A mutant inhibits cell spreading as efficiently as wild-type mGBP-2.

(A) Schematic of R48A mGBP-2. (B) NIH 3T3 cells were transfected with control plasmid, mGBP-2, or R48A mGBP-2 and GFP as described previously. TCL (20 µg) were examined for mGBP-2 expression by immunoblot analysis with anti-FLAG antisera. (C) NIH 3T3 cells were co-transfected with pCMV-eGFP and one of the constructs shown above at a ratio of 3:1. After 48 hr, cells were processed for cell spreading on FN. The surface areas of green fluorescent cells were measured and expressed as mean percentage of unspread cells ($\leq 600 \mu m^2$) ± standard deviation (n = 3; * = p < 0.05 compared to control plasmid). (D) NIH 3T3 were transfected with mGBP-2 or STIL mGBP-2 and after 24 hr were allowed to spread on fibronectin for 35 min. The localization of each protein in the spreading cells was determined by indirect immunofluorescence with anti-FLAG antisera as described (100X magnification).
iii. Intracellular localizations of the wild-type mGBP-2 vs. STIL mGBP-2 during cell spreading.

To determine whether differences in the intracellular locations of mGBP-2 and STIL mGBP-2 could account for their differences in function, the intracellular localization of both proteins was examined in cells spreading on FN. In cells expressing mGBP-2 the distribution was similar to that of fully spread, “resting” cells [102]. The protein is found in a punctate distribution throughout the cytoplasm. In many cells, mGBP-2 localized to a population of heterogeneous vesicles (Fig. 30D). A very similar distribution was observed for STIL mGBP-2, except it was not found in intracellular vesicles during cell spreading [79]. There are not large differences between the localization of both proteins, other than mGBP-2 goes to vesicles. However, we are not sure what the vesicles are and therefore are not sure of the significance of these findings.

iv. Confirmation that the isoprenylated carboxy-terminal α-helices of mGBP-2 are necessary to inhibit cell spreading.

To further evaluate the role of isoprenylation on the ability of mGBP-2 to inhibit cell spreading, additional mutants were generated (Fig. 31A). The C290 mGBP-2 lacks the entire globular GTP binding domain and has only α-helices 6 – 13, as previously described. C290 mGBP-2 is able to inhibit cell spreading as efficiently as wild type mGBP-2. The CaaX motif of C290 STIL mGBP-2 has a single point mutation from a cysteine to a serine. C290 mGBP-2 is able to inhibit cell spreading. However, C290 STIL mGBP-2 is unable to be prenylated and cannot inhibit cell spreading. C473 mGBP-2 slightly inhibits spreading. However, C473 STIL mGBP-2 cell spreading is not
Figure 31. The isoprenylated α-helical region of mGBP-2 can inhibit cell spreading.

(A) Schematic of CaaX substitutions. (B) NIH 3T3 cells were co-transfected with pCMV-eGFP and one of the constructs shown above at a ratio of 3:1. After 48 hr, cells were processed for cell spreading on FN. The surface areas of green fluorescent cells were measured and expressed as mean percentage of unspread cells (≤ 600 µm²) ± standard deviation (n = 3; * = p < 0.05 compared to control plasmid) as previously described.
inhibited at all without lipid modification. These data further demonstrate that although the α-helical region can inhibit cell spreading, it must be lipid modified to do so (Fig. 31B).

v. Farnesylated mGBP-2 can inhibit cell spreading as efficiently as geranylgeranylated mGBP-2.

To determine whether the inhibition of cell spreading is dependent on the type of prenyl group attached to mGBP-2, the mGBP-2 CTIS was generated. The mGBP-2 prenylation sequence (CTIL) was replaced with that of hGBP-1 (CTIS), which directs the addition of C_{15} farnesyl [93]. These data suggest that the substitution results in a farnesylated mGBP-2 that inhibits spreading as efficiently as the geranylgeranylated mGBP-2 (Fig 32B). Lipid attachment is necessary for mGBP-2 to inhibit cell spreading, however, it does not matter if it is modified by a farnesyl or a geranylgeranyl lipid (Fig. 32B).

vii. mGBP-2 does not inhibit Rac activation by inhibiting its prenylation.

Prenylation of Rac1 is required for its activities [165, 166]. Robust expression of a prenylated protein, like the geranylgeranylated mGBP-2, could inhibit Rac activity by competing with Rac for the available geranylgeranyltransferase I (GGT I) and inhibiting its prenylation. Several lines of evidence suggest that this is not the case. First, rescue of cell spreading by forced over-expression of active Rac, which must be lipid modified to function, indicate that mGBP-2 does not inhibit Rac prenylation (Fig. 10). While
Figure 32. Farnesylated mGBP-2 can inhibit cell spreading as efficiently as geranylgeranylated mGBP-2. (A) Schematic of wild type mGBP-2 and CTIS mGBP-2. (B) NIH 3T3 cells were co-transfected with pCMV-eGFP and one of the constructs shown above at a ratio of 3:1. After 48 hr, cells were processed for cell spreading on FN. The surface areas of green fluorescent cells were measured and expressed as mean percentage of unspread cells ($\leq 600 \, \mu m^2$) ± standard deviation (n = 3; * = p < 0.05 compared to control plasmid). (C) NIH 3T3 cells were transfected with control, mGBP-2, STIL or CTIS and GFP as described previously. Cells lysates (20 µg) were examined for mGBP-2 expression by immunoblot analysis with anti-Myc antisera.
unprenylated Rac is observed in cells treated with the HMG-CoA reductase inhibitor, compactin, for 24 hr, no Rac having an electrophoretic mobility consistent with that of unprenylated Rac is detected in mGBP-2-expressing NIH 3T3 cells (Fig. 33A). Inhibition of Rac activity could also occur through competition for a Rac-specific GDI. Rac GDIs specifically bind to the geranylgeranyl moiety of Rac. However, replacing the mGBP-2 prenylation sequence with that of farnesylated hGBP-1 (CTIS), results in a protein that inhibits spreading as efficiently as the wild type C20-modified mGBP-2 (Fig 33C). This suggests that mGBP-2 does not inhibit Rac activation by competing for GGTI or for a Rac-specific GDI to inhibit cell spreading.
Figure 33.
Figure 33. mGBP-2 does not compete with Rac for prenylation or for its GDI. (A) To determine the correct molecular size for Rac when unprenylated, mGBP-2-expressing cells were treated with 50 µM compactin for 24 hr (mGBP-2.2 + comp). Control transfectants and mGBP-2-expressing cells were analyzed by immunoblot with anti-Rac antisera. (B) Schematic of isoprenylation. Farnesyltransferase and geranylgeranyltransferase I recognize the CaaX box at the C-terminus of the target protein. C is the cysteine that is prenylated, a is any aliphatic amino acid, and the identity of X determines which enzyme acts on the protein. Modified from Casey, P., J Lipid Res. (33) 2002. (C) NIH 3T3 fibroblasts were transfected with GFP and controls, RacG12V with or without mGBP-2 CTIS constructs. After 48 hr, cells were processed for cell spreading on FN. The surface areas of green fluorescent cells were measured and expressed as mean percentage of unspread cells (≤ 600 µm²) ± standard deviation (n = 3; * = p < 0.05). (D) To confirm similar expression levels of Rac and mGBP-2 CTIS, TCLs immunodetected with anti-Rac and anti-mGBP-2 antisera.
DISCUSSION

Previously, we demonstrated that mGBP-2 mediates the inhibition of cell spreading by inhibiting Rac activation. However, it is unclear which region(s) of mGBP-2 are necessary to inhibit cell spreading. Structural hallmarks of the ortholog, hGBP-1 include a large, globular N-terminal domain that binds GTP, and an elongated C-terminal α-helical region with a CaaX motif at the C-terminus that specifies geranylgeranyl lipid modification of mGBP-2 [80, 93]. In order to identify the structural and/or functional component(s) of mGBP-2 responsible for the inhibition of cell spreading, various mGBP-2 mutants were generated. Previous studies of hGBP-1 suggest that the inhibition of endothelial cell proliferation is mediated by the carboxyl terminus of hGBP-1 (equivalent to our C290 mGBP-2) [73]. In addition, the D184N version of hGBP-1 can rescue hGBP-1-mediated growth inhibition in endothelial cells. When interpreted, in light of the solution biochemistry for this protein, it suggests that GTP binding and dimerization are not necessary for the inhibition of endothelial cell growth [73]. However, in the studies presented here, D184N mGBP-2 inhibits cell spreading as efficiently as wild-type mGBP-2. These data suggest that mGBP-2 needs to be GTP-bound and possibly dimerized to inhibit cell spreading. The constitutively GTP-bound, dimerized R48A mGBP-2 also inhibits cell spreading (Fig. 30C) while the S52N mGBP-2 is unable to inhibit cell spreading (Fig. 4C and D). S52N mGBP-2 is nucleotide-free and unable to dimerize. Both mutants further confirm the hypothesis that mGBP-2 requires GTP-binding and dimerization to inhibit cell spreading. The C-terminal α-helices of C290 mGBP-2 (amino acids 290 to 589) inhibit cell spreading, provided they have a competent CaaX sequence (Fig. 29C and 31B). This indicates that protein-protein interaction(s)
occur in the C-terminal α-helical region. We believe that C290 mGBP-2 is able to inhibit cell spreading as efficiently as the wild-type protein resulting from the removal of the N-terminal globular portion, thereby liberating the α-helices for protein-protein interactions.

Where studied, isoprenylation is critical for one or more of the functions of other proteins [91]. Lipid modifications increase hydrophobicity, which increases both protein-protein interactions and/or membrane associations [91]. For example, small GTPases, such as Ras, require farnesylation for targeting to the plasma membrane. Although the CaaX motif is necessary for isoprenylation, it is not sufficient. Regions other than the CaaX sequence designate prenylation [167]. Another small GTPase, Rab, requires an effector domain at the N-terminus for proper isoprenylation [168, 169]. For a variety of isoprenylated proteins, their functions can be divided into those that require lipid modifications and those that do not. For example, the small GTPase, RhoA requires prenylation for cytoskeletal rearrangement, but not for gene regulation [92]. Although mGBP-2 requires prenylation to inhibit cell spreading, it does not matter which isoprenoid (farnesyl or geranylgeranyl) group is attached. The predicted farnesylated (C_{15}-modified) mGBP-2, CTIS mGBP-2, was able to inhibit cell spreading as efficiently as geryanylgeranylated (C_{20}-modified) wild-type mGBP-2 (Fig. 3B).

Having demonstrated that mGBP-2 is responsible for IFN-γ-mediated inhibition of cell spreading in NIH 3T3 cells, we now show which properties of mGBP-2 are responsible for this inhibition. Our data suggest that in the context of the full-length protein, mGBP-2 requires isoprenylation, GTP-binding and dimerization to inhibit cell spreading. Binding of GTP and dimerization releases the C-terminal α-helices for subsequent protein-protein interactions that inhibit cell spreading.
REFERENCES


is selectively induced by inflammatory cytokines and is an activation marker of endothelial cells during inflammatory diseases. *Am J Pathol* **161**, 1749-1759.


