The Interferon-γ-induced GTPase, mGBP-2, Inhibits Tumor Necrosis Factor α (TNF-α) Induction of Matrix Metalloproteinase-9 (MMP-9) by Inhibiting NF-κB and Rac Protein*

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Matrix metalloproteinase-9 (MMP-9) is important in numerous normal and pathological processes, including the angiogenic switch during tumor development and tumor metastasis. Whereas TNF-α and other cytokines up-regulate MMP-9 expression, interferons (IFNs) inhibit MMP-9 expression. We found that IFN-γ treatment or forced expression of the IFN-induced GTPase, mGBP-2, inhibit TNF-α-induced MMP-9 expression in NIH 3T3 fibroblasts, by inhibiting MMP-9 transcription. The NF-κB transcription factor is required for full induction of MMP-9 by TNF-α. Both IFN-γ and mGBP-2 inhibit the transcription of a NF-κB-dependent reporter construct, suggesting that mGBP-2 inhibits MMP-9 induction via inhibition of NF-κB-mediated transcription. Interestingly, mGBP-2 does not inhibit TNF-α-induced degradation of IκBα or p65/RelA translocation into the nucleus. However, mGBP-2 inhibits p65 binding to a κB oligonucleotide probe in gel shift assays and to the MMP-9 promoter in chromatin immunoprecipitation assays. In addition, TNF-α activation of NF-κB in NIH 3T3 cells is dependent on Rac activation, as evidenced by the inhibition of TNF-α induction of NF-κB-mediated transcription by a dominant inhibitory form of Rac1. A role for Rac in the inhibitory action of mGBP-2 on NF-κB is further shown by the findings that mGBP-2 inhibits TNF-α activation of endogenous Rac and constitutively active Rac can restore NF-κB transcription in the presence of mGBP-2. This is a novel mechanism by which IFNs can inhibit the cytokine induction of MMP-9 expression.

Interferons (IFNs) are a family of cytokines that elicit a wide variety of cellular activities (reviewed in Refs. 1, 2). Best known for their antiviral activities, IFNs also possess anti-proliferative, immunomodulatory, and anti-angiogenic activities. IFNs can also alter cell adhesion and migration, in part by modulating the interactions of cells with their extracellular environment. This can occur by changing the expression levels of cell adhesion molecules, but can also be facilitated by modulating the expression of enzymes that alter the extracellular matrix. 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 (3–8). MMPs are a family of Ca2+- and Zn2+-requiring endoproteases that can cleave most of the components of the ECM (9). These proteins are important in such processes as cell migration, proliferation, wound healing, and angiogenesis. The greater than 20 MMPs can be divided into subgroups based on substrate specificity (10). The gelatinases are MMPs with elevated activity against denatured collagens and contain only two members, MMP-2 and MMP-9 (9), 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 (11). Because type IV collagen and laminin are common to all basement membranes, MMP-9 is important in metastasis, tumor growth, and angiogenesis (11–13). MMP-9 expression is frequently elevated in human tumors and correlates with increased metastasis (11, 14, 15). MMP-9 is also associated with the angiogenic switch, which contributes to tumor progression (16). The expression of MMP-9 can be modulated by a variety of cytokines and other agents, in addition to IFNs (12, 17–22).

Cytokines regulate MMP-9 transcription by activating a number of transcription factors that bind to specific promoter elements (12, 23). The promoters of both the human and murine MMP-9 genes have an NF-κB site, two AP-1 sites, an Sp-1 site, and an Ets site (12). These sites are involved in regulation by TNF-α (24), PMA, v-src, ras, and LPS (12, 22). More recently, investigators have demonstrated the importance of chromatin remodeling pathways, including binding of coactivators and histone acetylation, in regulating the expression of MMP-9 (23). The mechanisms by which IFNs inhibit MMP-9 expression are still actively under investigation.

The guanylate-binding proteins (GBPs) are a family of large, unique GTPases induced by both type I and type II IFNs (for review see Ref. 25). Human guanylate-binding protein-1 (hGBP-1) inhibits the expression of MMP-1 in growth factor-stimulated endothelial cells (26). We examined whether the
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Cell Lines and Tissue Culture—NIH 3T3 cells (American Type Culture Collection) and control transfectant and mGBP-2-expressing NIH 3T3 cells were generated and cultured as described (27).

Eukaryotic Expression Plasmids—The generation of FLAG-mGBP-2 in pCMV+/FLAG(NH) was described (31). The murine MMP-9 promoter in pGL3 (MMP-9 luc) was a kind gift from Dr. Yves St. Pierre (University of Quebec) (32). The plasmids pRK5, pRK5 Rac1(G12V), pRK5 Rac1(T17N), pRK5 Cdc42(G12V), pRK5 Cdc42(T12N), pRK5 RhoA(G14V), and pRK5 RhoA(T19N) were a gift from Dr. Amy Wilson-Delfosse (Case Western Reserve University, Cleveland, OH). The actin promoter driven β-galactosidase (actin-β-gal) construct (Lonza, Basel, Switzerland) was used as a positive control (not shown). Western blots were also performed with anti-IκBα (1:1000), anti-p65 NF-κB (1:400), anti-mGBP-2 (1851; 1:800), and anti-actin (1:3000).

Small Interfering RNA (siRNA) Transfections and Analyses—NIH 3T3 cells (2 × 10^6) were suspended in Amaxa Cell Line Nucleofector Kit R solution per manufacturer’s instructions (Lonza, Basel, Switzerland). Cells were added to 350 nM nontarget siRNA or mGBP-2 SMART pool siRNA (Dharmacon RNA Technologies, Lafayette, CO) in manufacturer supplied cuvettes. Transfections were performed using program U-030 on the Amaxa Nucleofector. The cells were equally divided between four 6-cm dishes. After adhering for at least 3 h, cells were treated with 500 units/ml IFN-γ for 24 h. TNF-α (5 ng/ml) was added for the final 7 h of the IFN-γ treatment, where appropriate. Cells were then lysed and processed for Western blot as described.

Luciferase Assays—Cells (2 × 10^5/well) were plated overnight in 6-well dishes in complete media. For analysis of IFN-γ and TNF-α effects on the murine MMP-9 minimal promoter, cells were transfected with 0.5 μg each of MMP-9 luc and actin-β-gal per well using FuGene 6 (Roche Applied Science, Indianapolis, IN). After 24 h, the cells were shifted to SFM ± IFN-γ (500 units/ml) for 24 h. After 20 h, 10 ng/ml TNF-α was added, and 4 h later the cells were lysed and processed. To analyze the effects of mGBP-2 and TNF-α on MMP-9 promoter activity, cells were transfected with 0.5 μg of mGBP-2 plasmid (or empty control vector), 0.33 μg actin-β-gal plasmid, and 0.33 μg MPP-9 luc per well. To analyze the role of Rho family members on NF-κB transcription, NIH 3T3 cells were transfected with 0.5 μg of mGBP-2-containing plasmid (or empty control vector), 0.5 μg of dominant negative (DN) or constitutively active (CA) Rho family members, 0.33 μg of actin-β-gal plasmid, and 0.33 μg of NF-κB luc. After 18 h, 10 ng/ml TNF-α was added for 6 h (DN and CA Rho family) or for 9 h (± mGBP-2 with NF-κB luc). The cells were washed with PBS and lysed in CCLR (25 mM Tris-phosphate (pH 7.8) 2 mM DTT, 2 mM 1,2-diaminocyclo-
hexane-NN,N,N'-tetraacetic acid, 10% glycerol, 1% Triton X-100; Promega, Madison, WI). Cleared lysates (20 μl) 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 s after a 1.6 s 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 transcription efficiencies, the cells were co-transfected with a β-galactosidase expression plasmid driven by the β-actin promoter. The lysates (5 μl) were added to 50 μl of 1× CCLR and 50 μl of 2× 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). Mixtures were incubated at 37 °C for 10–15 min. The reaction was terminated by the addition of 150 μl of 1 M 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 with control values.

Quantitative Real Time PCR—Cells (1 × 106/well) were plated in 6-cm dishes and allowed to adhere for 3 h before incubation ± 500 units/ml of IFNγ for 18 h. Total RNA was extracted using 500 μl Trizol reagent (Invitrogen, Carlsbad, CA). SuperScript II First Strand synthesis kit (Invitrogen) was used to generate cDNA and real Time PCR was carried out using 1 μl of the resulting cDNA, 12.5 μl of iQ™ SYBR Green (Bio-Rad), and 200 nm each MPP-9 sense and antisense primers (sense-GAGGAAGCCTCAGGCGCCCTC, antisense-CACGCCCCTTGCTGAACAGCAGAG), or 50 nM murine GAPDH sense and antisense primers (sense-CCAGGGTGACAGCAGTCTT, antisense-TTGGTCCTTAGGTCCTTTCC, antisense-TTGGTGCCTTAGGTCCTTTCC). PCR was performed utilizing a Bio-Rad 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 s, 60 °C for 30 s, 74 °C for 30 s. The relative quantity of MPP-9 mRNA was determined using the comparative threshold cycle (Ct) method in which the relative quantity of MPP-9 was normalized to GAPDH by the equation 1.5^△Ct, where △Ct results 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^△Ct × 100 (36).

For the time course analysis, cells were treated with 10 ng/ml TNF-α for the time points indicated. RNA was isolated using RNeasy kit (Qiagen) and qRT-PCR was performed using iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad). Primers used were: β-actin (sense-5’-AGTGTACGTGATCACCTTGTA-3’, antisense-5’-GCCAGAGCAGTATCTCCTTCTT-3’); MMP-9 (sense-CTGGAACAGCCACAGACTAAG, antisense-CTCCGCGCAGCAGTTCACAGAGG, antisense-GCGCCGACAGCCGATCTCC), IL-6 (sense-TAGTCCCTCTATCCCCATTTCC, antisense-CTGGTCTCCTAGCCACTCTTCC) CXCL11 (sense-GGCTTCTCATATGTGTACTTC, antisense-GCGCCGACAGCCGATCTCC), or 50 nM protein G magnetic beads overnight at 4 °C. Immunoprecipitated DNA was purified with the MinElute PCR Purification kit (Qiagen) and qPCR was performed using iQ™ SYBR® Green supermix (Bio-Rad) with the following parameters (enzyme activation: 95 °C 5 min; 40 cycles: 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s; and extension: 72 °C for 10 min).

RT-PCR Analysis for MPP-1—In mice there are two MPP-1-related genes, MPP1A and MPP-1B (37). To determine whether NIH 3T3 cells express MPP-1A or MPP-1B, PCR was performed. cDNA from control and mGBP-2-expressing cells were synthesized as described above. Genomic DNA was extracted as described (38). PCR primers were designed to span an intron to allow detection of genomic DNA. For MPP-1A, the primers were sense-GGATTCGGTAGGATATATCCAGGTTAC and antisense-CTGGGTTAACCTGGATCCATGG and would amplify a genomic fragment that spanned the 640 bp intron between exons 8 and 9. The GAPDH primers were described above.

NF-κB DNA Binding Activity Assays—Nuclei were extracted with 20 mM Tris-HCl, pH 7.85, 250 mM sucrose, 0.4 mM KCl, 11 mM MgCl2, 5 mM β-mercaptoethanol, 1 mM NaF, 1 mM Na3VO4, 1 mM PMSE, 5 mg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 1.75 mg/ml benzamidin and extracts were frozen and stored at −80 °C (39). For electrophoretic mobility shift assays (EMSA), the nuclear extracts were incubated with a [32P]-labeled κB probe (5′-AGTTGACGGGACTTTCCCAGG-3′) derived from an NF-κB binding sequence in the immunoglobulin gene promoter (40). To define the presence of specific NF-κB proteins, nuclear extracts were pre-incubated with a 1:50 dilution of anti-p65/RelA antibodies at 25 °C for 0.5 h and then subjected to EMSA. Gels were subjected to PhosphorImage autoradiography.

Chromosome Immunoprecipitation Assays for the p65 NF-κB subunit—Cells (~5 × 106) were treated with 10 ng/ml mouse TNF-α for 30 min. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT™ Express kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. In brief, chromatin from cells was cross-linked with 1% formaldehyde (10 min at 22 °C), and sheared to an average size of ~500 bp by sonication (Branson Sonifier 250, 20 s × pulse × 20 pulses at ~20% power). Sheared chromatin was incubated with 3 μg of control IgG or anti-p65 (Santa Cruz Biotechnology) and 50 μl of protein G magnetic beads overnight at 4 °C. Immunoprecipitated DNA was purified with the MiniElute PCR Purification kit (Qiagen) and qPCR was performed using iQ™ SYBR® Green supermix (Bio-Rad) with the following parameters (enzyme activation: 95 °C 5 min; 40 cycles: 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s; and extension: 72 °C for 10 min). The following sense and antisense primers for the putative NF-κB site present within the Mmp-9 promoter were designed using Primer 3 plus program: Sense-TCTCTATCCACAGGACAGTG and Antisense-CCATCCACACTGACATAG. The iif203 promoter which does not have an NF-κB site was chosen as our control sequence. The primers used for this site were: forward ATTCCCCATTGCTGCTG and reverse CCTCAGAGTCTGCCTG (38). The PCR products of the endogenous MMP-9 and Iif203 promoter regions are 167 bp and 128 bp, respectively. The levels of anti-p65 bound DNA fragment was normalized to that bound by control IgG for each sample and expressed as average ± S.E. (n = 3).
**RESULTS**

**IFN-γ and mGBP-2 Inhibit the Basal Expression of MMP-9**—IFNs regulate the expression of the gelatinase MMPs, MMP-2, and MMP-9 (3, 4, 6). To characterize the gelatinases produced by NIH 3T3 cells, gelatin zymography was performed on conditioned media (CM). Enzymatic activities corresponding to pro-MMP-9 (105 kDa) and two different forms of MMP-2 (70 and 65 kDa) were observed (Fig. 1A, top panel). After 24 h of IFN-γ treatment, only the activity of the 105 kDa molecular species was reduced. Western blot analyses of the CM confirmed that the 105-kDa band is MMP-9 and that IFN-γ treatment reduced the level of secreted pro-MMP-9 (Fig. 1A, bottom panel). The amount of pro-MMP-9 secreted by cells is inversely correlated with the amount of mGBP-2 expressed (Fig. 1A and B). No mGBP-2 was detected in untreated NIH 3T3 cells but mGBP-2 was robustly induced by 24 h IFN-γ treatment (Fig. 1B). To determine whether IFN-γ-induced mGBP-2 expression was responsible for the inhibition of MMP-9, the CM from control and mGBP-2-expressing cells was examined by zymography (Fig. 1C, top panel) and immunoblotting (Fig. 1C, lower panel). While there was some variation in the amounts of pro-MMP-9 secreted by the control transfectants and mGBP-2-expressing cells, mGBP-2 expression correlated with reduced pro-MMP-9 secretion (Fig. 1D). The intracellular levels of MMP-9 were also reduced by IFN-γ treatment or by expression of mGBP-2 (Fig. 2A).

Whether the reduction in secreted and intracellular pro-MMP-9 protein reflected changes in the steady state levels of MMP-9 RNA was determined by real-time RT-PCR. In cells expressing mGBP-2 there is a significant reduction in MMP-9 RNA compared with control transfectants (Fig. 2B). As expected, treatment with IFN-γ also resulted in reduced levels of MMP-9 RNA (Fig. 2C).

**mGBP-2 Inhibits MMP-9 Expression by Inhibiting NF-κB**

**Rac Activity Assays**—Cells (5 × 10⁶) were serum starved for 2 h and treated with 10 ng/ml TNF-α (Chemicon International, Temecula CA) for 5 min. Cells were lysed in 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 mixture (Sigma), and 1 mM PMSF. 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. Rac levels were determined by immunoblot analysis using Kodak one-dimensional 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. Total cell lysates (20 μg) were also included. 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.
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confirm a role for mGBP-2 in IFN-γ-mediated inhibition of MMP-9 induction by TNF-α.

**mGBP-2 Inhibits Basal and TNF-α-induced Transcription Driven by the Murine MMP-9 Promoter** —Many normal cells do not express detectable MMP-9, so little is known about the regulation of basal MMP-9 expression. To determine whether IFN-γ and/or mGBP-2 could regulate the murine MMP-9 minimal promoter when expressed in NIH 3T3 cells, luciferase assays were performed. After treatment with IFN-γ for 24 h, the activity of the MMP-9 minimal promoter was reduced to 46 ± 18% of untreated control (Fig. 3A). In similar experiments, mGBP-2 reduced the luciferase expression driven by the MMP-9 promoter to 71 ± 13% of control values (Fig. 3B).

TNF-α induction of the murine MMP-9 promoter has been previously demonstrated in rat C6 cells (32). We found that TNF-α could also induce MMP-9 promoter activity in NIH 3T3 cells to 34.6 ± 19.8% of the level of activity in untreated cells (Fig. 3A). Pretreatment with IFN-γ completely inhibited the ability of TNF-α to induce the MMP-9 promoter (48.2 ± 14.7%...
of control; Fig. 3A). mGBP-2 expression was also able to significantly inhibit the induction of MMP-9 promoter activity by TNF-α (Fig. 3B).

**mGBP-2 Inhibits NF-κB-mediated Transcription**—TNF-α induction of MMP-9 proceeds in part through NF-κB activation (42, 44, 45), and inhibiting NF-κB reduces MMP-9 production (45). Therefore, a NF-κB-dependent reporter construct was used to determine the role of NF-κB in the inhibition of the MMP-9 by mGBP-2. mGBP-2 expression inhibited NF-κB mediated transcription under basal conditions by about 85% (Fig. 3C). As expected, TNF-α increased NF-κB mediated transcription greater than 3.5-fold in the absence of mGBP-2 (Fig. 3C). The presence of mGBP-2 inhibited the ability of TNF-α to promote NF-κB-mediated transcription by greater than 2-fold (Fig. 3C).

**mGBP-2 Inhibits p65/RelA binding in Vitro and in Vivo**—To further explore the inhibition of NF-κB by mGBP-2, electrophoretic mobility shift assays (EMSA) were used to examine the ability of nuclear extracts of mGBP-2-expressing cells to bind to a synthetic NF-κB oligonucleotide probe (Fig. 4, A–C). In the absence of mGBP-2, NIH3T3 cells treated with TNF-α showed dose-dependent binding of p65 with maximum binding at 30 ng/ml (Fig. 4A). Supershift analysis with anti-p65 demonstrated the presence of p65 in TNF-treated nuclear extracts, consistent with our previous studies (40). Treatment of control transfectants with 10 ng/ml of TNF-α induced NF-κB binding within 30 min of addition, and the level of NF-κB binding peaked at about 1 h and then declined through 4 h (Fig. 4C). In contrast, in cells expressing mGBP-2, no binding to the oligonucleotide probe was observed (Fig. 4C).

To determine whether mGBP-2 expression inhibits p65/RelA binding to the MMP-9 promoter in vivo, ChIP assays were performed on chromatin prepared from control transfectants and mGBP-2-expressing cells treated with TNF-α. As shown in Fig. 4D, treatment of control transfectants with TNF-α (30 min) induced the binding of p65/RelA to the MMP-9 promoter, while there was no detectable p65/RelA binding to the promoter in untreated control cells. In contrast, in cells expressing mGBP-2, only low levels of TNF-induced p65 binding was observed.

**IkBα Degradation after TNF-α Treatment Is Not Inhibited by mGBP-2**—NF-κB is normally latent in the cytoplasm bound to the IκB complex. Various stimuli, including TNF-α treatment, activate the IKK complex, which results in the phosphorylation and degradation of IκBα. We asked whether mGBP-2 inhibited NF-κB-mediated transcription by inhibiting the degradation of the inhibitor IκBα, thereby inhibiting NF-κB release (Fig. 5A). Treatment with TNF-α resulted in a rapid loss of IκBα, becoming undetectable within 10 min. Expression of mGBP-2 did not affect TNF-induced levels of IκBα. Therefore, inhibition of p65 binding to DNA in mGBP-2-expressing cells was not a consequence of inhibiting IκBα degradation.

**mGBP-2 Does Not Block Movement of p65/RelA into the Nucleus upon TNF-α Treatment**—Once released from IκBα, NF-κB translocates from the cytoplasm into the nucleus to bind DNA and activate transcription. To examine the movement of p65 into the nucleus of cells after TNF-α treatment, control transfectants and mGBP-2-expressing cells were treated with

![FIGURE 4](image-url)
TNF-α and the intracellular distribution of p65 was examined by indirect immunofluorescence (Fig. 5B). Within 15 min, p65 translocated into the nuclei of all TNF- treated cells. mGBP-2 expression did not inhibit p65 translocation into the nucleus.

**mGBP-2 Inhibits the TNF-α Activation of NF-κB-regulated Genes**—To determine whether mGBP-2 inhibited TNF-α induction of other genes that were regulated by NF-κB, control transfectants and mGBP-2-expressing cells were treated with TNF-α for the times indicated, total RNA prepared, and gene expression was determined by qPCR. As predicted, TNF-α treatment induced the transcription of MMP-9 in control

![Image](49x304 to 299x733)
transfectants (Fig. 6A) and induction was severely inhibited by mGBP-2 expression. The cytokine IL-6 is also induced by TNF-α treatment and the primary transcription factor involved in this induction is NF-κB (46, 47). IL-6 transcription is induced by TNF-α in control cells but the induction is significantly inhibited in the presence of mGBP-2 (Fig. 6B). The chemokine CXCL11 (also known as beta-R1, I-TAC, or H-174) is a TNF-α responsive gene that contains an NF-κB site in its promoter (48, 49). In control transfectants, CXCL11 gene expression is induced by TNF-α but its expression is unaffected by mGBP-2 expression (Fig. 6C). This suggests that mGBP-2 expression inhibits the TNF-α induction of a subset of NF-κB responsive genes.

Rac Is Required for TNF-α Induction of NF-κB-mediated Transcription in NIH 3T3 Cells—The small GTPase Rac is a component of a TNF-α signal transduction cascade (42, 50). In fact, Rac is required for the activation of NF-κB by a variety of stimuli (51–54). To determine whether Rac is required for TNF-α activation of NF-κB-mediated transcription in NIH 3T3 cells, cells were co-transfected with the NF-κB-driven luciferase reporter construct and dominant-negative (DN) constructs of Rac (T17N), Rho (T19N), and Cdc42 (T17N). While DN-Rho and DN-Cdc42 did not significantly inhibit TNF-α-induced NF-κB-dependent transcription, DN-Rac1 markedly inhibited TNF-induced NF-κB activity (Fig. 6A). This demonstrates a requirement for Rac1 in NF-κB activation by TNF-α.

mGBP-2 Inhibits Rac Activation of NF-κB—We asked whether mGBP-2 inhibits NF-κB activation in NIH 3T3 cells by inhibiting Rac activity. NIH 3T3 cells were transfected with constitutively active forms of RhoA, Rac1, and Cdc42 in the presence or absence of mGBP-2 and monitored for NF-κB activation (Fig. 7B). Only Rac1(G12V) activated NF-κB above control levels and was able to restore NF-κB transcription to control levels in the presence of mGBP-2 (Fig. 7B). This suggests that mGBP-2 inhibits NF-κB activity in part by acting at the level of Rac. To confirm that mGBP-2 inhibits TNF-α activation of Rac, the relative levels of active Rac were measured in control transfectants and mGBP-2-expressing cells following TNF-α treatment. mGBP-2 lowered the level of active Rac in TNF-α treated cells by about 80% (Fig. 7, C and D).

This is the first report of a novel mechanism for IFN-γ-mediated inhibition of MMP-9 expression, the induction of mGBP-2. mGBP-2 inhibits MMP-9 transcription, at least in part, by inhibition of NF-κB p65/RelA binding to the MMP-9 promoter. This inhibition is not the consequence of failure to transport p65 into the nucleus. The coincident inhibition of Rac1 by mGBP-2 suggests that post-translational modifications of p65 necessary for optimal DNA binding and transcription are inhibited.

DISCUSSION

MMPs are important proteins in tissue remodeling during both normal and pathological processes. They also modulate the availability and activity of growth factors. In particular, MMP-9 is believed to direct the “angiogenic switch,” and its up-regulation in tumor cells correlates with progression and metastasis (reviewed in Refs. 9, 10, 12). Consistent with this, MMP-9 null mice show less angiogenesis and metastasis of LLC- or B16-derived tumors from their primary sites (55). Despite the biological importance of MMP-9, much remains unclear about its regulation. A variety of cytokines, growth factors, ras, and c-src can up-regulate MMP-9 expression, but few repressors of MMP-9 expression have been identified. The metastasis suppressor, KiSS-1, inhibits MMP-9 expression by inhibiting NF-κB translocation to the nucleus subsequent to increasing IκBα levels (56). Interestingly, KiSS-1 is unable to inhibit TNF-α-induced expression of MMP-9 (56). Recently, transgelin (SM22) was identified as a MMP-9 inhibitor (57). Transgelin is a small actin-binding protein that inhibits AP-1-dependent transcription of MMP-9 (57). In addition, type I IFNs (IFN-α/β) and type II IFN (IFN-γ) can inhibit cytokine-induced MMP-9 expression. This repression appears to be complicated, with multiple IFN-stimulated proteins involved.

An early event following IFN binding to its receptor is the activation of STAT1 (1). Treatment of HeLa cells with IFN-γ suppresses PMA induction of MMP-9 by activating STAT1α, which inhibits MMP-9 transcription by binding to and inhibiting the recruitment of the CBP/p300 co-activator to the MMP-9 promoter (58), which in turn inhibits the formation of the transcription complex required to initiate MMP-9 transcription. Sp-1, NF-κB, and AP-1 binding to the MMP-9 promoter are not inhibited by IFN-γ but recruitment of CBP/p300 and histone acetylation was (58). This ultimately resulted in reduced recruitment of RNA polymerase II. The inhibition by STAT1α is a relatively early event after IFN-γ exposure, requiring less than 4 h. 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 (59, 60). IRF-1 inhibits MMP-9 transcription in EW-1 cells by competing with the p65 subunit of NF-κB for binding to the MMP-9 promoter (41). In both the mouse and human MMP-9 promoter, the NF-κB site contains a modified interferon-stimulated response element (ISRE), called an CRE. 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) (61). CIITA expression is induced by IFN-activated STAT1α, and like STAT1α, CIITA binds to CRE and inhibits both its recruitment to the MMP-9 promoter and histone acetylation.

Little is known about the functions of mGBP-2. Whereas mGBP-2 is not expressed in NIH 3T3 cells prior to IFN exposure (27, 62), mGBP-2 is induced to detectable levels by about 4 h of IFN-γ treatment (62). mGBP-2 is therefore expected to be important in inhibiting MMP-9 at later times after IFN exposure than either STAT1 or IRF-1.

However, as with STAT1 and IRF-1, the target of mGBP-2 is NF-κB-mediated transcription of MMP-9. In the presence of mGBP-2, TNF-α induced IκBα degradation, release of NF-κB and translocation into the nucleus are not inhibited. However, mGBP-2 expression does inhibit the binding of p65 to the NF-κB element in the MMP-9 promoter. This inhibition of NF-κB is accompanied by inhibition of Rac activation.

In the present study we show that TNF-induced NF-κB activation requires Rac activity, which has been shown for a variety of other stimuli (63, 64). However, the precise molec-
ular mechanism whereby Rac activates NF-κB remains unclear. Other studies suggest that Rac activation of NF-κB may be the consequence of NIK-mediated IKK activation (65), PAK activation (66, 67), and activation of the PI3-K/Akt pathway (68). Other studies suggest that TLRs transactivation of NF-κB requires Rac but is independent of IκBα degradation (54). Rac-induced reactive oxygen species (ROS) may be involved in NF-κB activation (52, 69), as well as Rac-activated MAPKs (70), but the pathway involved in NF-κB activation remains unclear.

Taken together our results identify a novel mechanism for IFN-induced repression of MMP-9 expression. The induction of mGBP-2 by IFN-γ results in the inhibition of Rac activation by TNF-α, which inhibits the full activation of NF-κB. Not unexpectedly the inhibition of NF-κB resulted in the inhibition of other NF-κB-dependent, TNF-α induced genes, besides MMP-9. Recently IFN-γ-induction of mGBP-2 was also shown to inhibit Rac activation downstream of both integrin engagement during cell spreading and after PDGF treatment (30). This suggests that the IFN-γ-mediated inhibition of Rac by mGBP-2

FIGURE 7. mGBP-2 expression inhibits Rac activation by TNF-α. A, NIH 3T3 cells were transfected with NF-κB luc, actin-β-gal, and the vectors listed. After 18 h the cells were treated with 10 ng/ml TNF-α for 6 h and cell lysates were analyzed for luciferase activity. Results presented are the mean relative fluorescence after normalization for β-gal activity ± S.D. (n = 3; **, p < 0.01 compared with TNF-α-treated cells with empty vector). B, NIH 3T3 cells were transfected with NF-κB luciferase, actin-β-gal, mGBP-2, or pCMV vector, and Rac1 (G12V), RhoA (G14V), Cdc42 (G14V), or pRK5 plasmid. After 24 h the cells were incubated in SFM for 24 h, lysed, and processed for luciferase activity as described. Results presented are the mean relative fluorescence after normalization for β-gal activity ± S.D. (n = 4; **, p < 0.01 compared with basal luciferase in absence of mGBP-2 or Rho family member). C, control and mGBP-2-expressing cells were serum-starved for 2 h followed by treatment with 10 ng/ml TNF-α for 5 min. Cell lysates were analyzed for relative levels of active Rac as described under “Experimental Procedures.” A representative blot from three experiments is shown. D, relative optical intensities were determined, and the results are presented as means ± S.D. (n = 3).
is important in inhibiting the induction of MMP-9 and may constitute a more universal mechanism for IFN-γ to inhibit/ dampen responses to subsequent exposures to cytokines, growth factors, and integrins.

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