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Transcription factor ZBP-89 is required for STAT1 constitutive expression

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ABSTRACT

IFNγ is a pro-inflammatory cytokine that potentiates p53-independent apoptosis in a variety of cell types. STAT1 is the primary mediator of IFNγ action. ZBP-89 is a transcription factor that binds to the G/C-rich elements and mediates p53-independent apoptosis. In this study, site-directed mutagenesis revealed that a G-rich element from +171 to +179 within the first intron of the STAT1 gene is critical for optimal STAT1 promoter activity. Electrophoretic mobility shift assays and promoter analysis revealed that ZBP-89 binds directly to this STAT1 G-rich element along with Sp1 and Sp3. Reduction of ZBP-89 with siRNA attenuated both basal and IFNγ-induced STAT1 expression and subsequently diminished the activation of apoptotic markers, e.g. caspase-3 and PARP. Taken together, we conclude that ZBP-89 is required for constitutive STAT1 expression and in this way contributes to the ability of cells to be activated by IFNγ.

INTRODUCTION

ZBP-89 (ZNF148, Zfp148) is a Krüppel-type zinc finger protein that is ubiquitously expressed (1). Recent studies have revealed that ZBP-89 possesses multiple functions, including transcriptional regulation of a variety of genes (2), cell growth arrest (3,4) and cell death (3). Recently a haploinsufficient mouse model of ZBP-89 has underscored the importance of the protein in cell growth. Zfp148+/– embryonic stem (ES) cells are resistant to the growth-inhibitory effects of serum starvation and continue to proliferate (5). In contrast, wild-type ES cells that are serum starved arrest their cell growth. Phosphorylation of p53 at Ser15 is reduced in haploinsufficient (Zfp148+/–) ES cells. Thus reduced ZBP-89 protein levels render ES cells susceptible to unregulated cell growth through a p53-dependent mechanism. Moreover, this mouse study confirms the prior biochemical observation that ZBP-89 inhibition of cell growth requires p53 stabilization (3).

In addition to its role in cell growth, elevated levels of ZBP-89 initiate programmed cell death (3). Using a p53 null cell line, we showed that p53 is not required for ZBP-89-mediated apoptosis (3). This result raised the possibility that ZBP-89 might be a key mediator of pro-apoptotic signals that do not require p53. The molecular mechanisms of p53-independent apoptosis are poorly defined. Extracellular signals that trigger p53-independent apoptotic pathways generally include withdrawal of growth factors and pro-inflammatory cytokines. For example, the pro-inflammatory cytokine IFNγ induces apoptosis through mechanisms independent of p53 (6). In addition to induction of apoptosis, IFNγ also sensitizes cancer cells to death signals, e.g. Fas ligand and TRAIL, that also function independently of p53 status (7,8). In some cells, the synergistic actions of IFNγ and death-inducing agents has been attributed to STAT1 activation by IFNγ and subsequent increased expression of caspase-8, –9 and –3 and inhibition of Bcl-2 expression (8,9). This may have relevance in breast cancer treatment since BRCA-1 confers IFNγ-, but not IFN-α- or IFN-β-mediated apoptosis (10). Interestingly, a recent microarray analysis of MCF-7 cells revealed that overexpression of BRCA-1 increases ZBP-89 expression 2-fold, suggesting that ZBP-89 lies downstream of this tumor suppressor gene product.

Among the various STAT proteins, STAT1 is a key mediator of IFNγ action. STAT1 mediates IFNγ action by directly regulating the expression of a variety of genes involved in cell growth arrest and apoptosis (11). STAT1 expression in tumors has been shown to be important for the elimination of malignant cells by immune surveillance (12). Change in its phosphorylation status is generally the accepted mechanism for STAT1 activation and translocation to the nucleus (11). However, the regulation of STAT1 transcription remains undefined. Recently, an enhancer region conferring IFNγ activation to the STAT1 gene was characterized (8,9). The enhancer is located in the first intron at +1 to +495 and is G/C-rich. Here we show that ZBP-89 binds to a G-rich element within the first intron of the human STAT1 gene and is required for constitutive expression.

MATERIALS AND METHODS

Antibodies and chemicals

Rabbit ZBP-89 antibody has been previously described (1). Monoclonal antibodies against caspase-8 and polyclonal antibodies against JNK, cleaved PARP, caspase-3 and cleaved caspase-3 were obtained from Cell Signaling (Beverly, MA). The monoclonal Flag M2 antibody was purchased from Sigma (St Louis, MO). Rabbit polyclonal antibodies against Sp1 and

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Sp3, monoclonal anti-STAT1α and goat polyclonal anti-phospho-STAT1α were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The STAT Sampler Antibody Kit was from BD Transduction Laboratories (San Diego, CA).

Cell culture

The A549 (human non-small-cell lung cancer cell line), MCF-7 (human breast cancer cell line), HeLa (human cervical cancer cell line), U2OS (human ostrosarcoma cell line) and 293T cell lines were purchased from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). HCT116 p53 (+/+) and p53 (–/–) cells were gifts from Dr Bert Vogelstein (Johns Hopkins University) and were cultured in McCoy's 5A medium with 10% FBS.

Adenovirus

Replication-deficient recombinant Ad-ZBP-89 expressing full-length Flag-tagged rat ZBP-89 cDNA and control Ad-β-galactosidase adenovirus have been previously described (13).

Construction of STAT1 reporter and luciferase assays

The BAC clone RP11–629B4 that contains human STAT1 genomic DNA was purchased from Children's Hospital Oakland Research Institute (Oakland, CA). The following primers were used to amplify three STAT1 5′ genomic segments between –972 and +884. Forward from –972: 5′-GAGGTACCTGTCATGGGAGGAAACTGGTGG-3′. Forward from –4: 5′-GAGGTACCATTCGGGAATCTACTGC-AGAAGG-3′. Forward from +584: 5′-GAGGTACCACAGA-TCTTGATGATCCAAATTGGG-3′.

All constructs used the same backward primer at +884 that was: 5′-GAAGATCTCCTTATCTATACAAACAACATTC-3′. The PCR fragments were inserted into the pGL3-basic vector (Stratagene) and verified by sequencing. Mutations introduced into the STAT1 promoter were created by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and the following primers. To generate the G-rich mutation the forward primer was 5′-CGTGAGGTTCCGGGTGCCGGCCATGGGACGCGCAG-GGACAGAG-3′ and the reverse primer was 5′-CTCTGTC-CCTGCGCGTCCCATGGCCGGCACCCGGAACCTCACG-3′. To generate the C-rich STAT1 mutation the forward primer was 5′-GACTATATTATTTTGGGGTACCGGGTCTGCAA-AAACTAAACATC-3′ and the reverse primer was 5′-GATGTTTAGTTTTTGCAGACCCGGTACCCCAAAATA-ATATAGTC-3′. Cells were cultured in 24-well plates and transfected using FUGENE 6 (Roche). The transfection efficiency was normalized to β-galactosidase activity expressed from pCMV-β-gal.

Electrophoretic mobility shift assay

Nuclear protein was prepared from HCT116 cells and DNA binding was performed as described previously (13). A ^{32}P-labeled double-strand G-rich element, 5′-GTGCCGGG-GGTGGGACGCG-3′, was used as the probe. Five micrograms of protein extracted from nuclei (nuclear extract) were first incubated on ice with the indicated antibodies for 30 min and then the probe was added and incubated for another 15 min at 4°C. The gel was run at 250 V for 3.5 h in the cold room to separate the ZBP-89-DNA complex from the Sp1/Sp3-DNA complexes.

Immunoblot analyses

Whole-cell extracts were prepared in the lysis buffer [20 mM Tris–HCl pH 7.4, 0.2% Nonidet P-40, 0.5 mM EDTA, 1 mM dithiothreitol (DTT)] and analyzed by immunoblotting (13).

RNase protection assay

Total RNA was isolated from MCF-7 cells using TRIZOL reagent (Invitrogen). The hSTAT Multi-Probe Template set (BD Pharmingen, San Diego, CA) was used to generate riboprobes using MAXIscript *In Vitro* Transcription Kit (Ambion, Austin, TX). The RNase protection assay was performed as described previously (13).

RNA interference

RNA interference (RNAi) experiments with small interfering RNA (siRNA) were carried out using the method of Elbashir *et al.* (14). The region of ZBP-89 cDNA targeted for siRNA was: +144 5′-AAGATCGAAGTATGCCTCACCTT-3′. A mutated ZBP-89 siRNA (5′-AAGATCGAACGTGTCCTCA-CCTT-3′) and a siRNA targeted to pGL2 luciferase cDNA (5′-AACGTACGCGGAATACTTCGATT-3′) were used as controls. Synthetic 21mer sense and antisense oligonucleotides (Xeragon, Germantown, MD) were annealed before use. siRNAs were transfected into cells with Oligofectamine (Invitrogen). In the 12-well plates, each well was transfected with 5 µl of 20 µM stock siRNA oligonucleotides.

RESULTS

ZBP-89 deficiency reduces STAT1 expression

IFNγ is a pro-inflammatory cytokine that induces or predisposes cancer cells to apoptosis in a p53-independent manner. STAT1 is the key mediator of IFNγ action and the IFNγ-regulatory element in the STAT1 gene fragment is G/C rich. Since ZBP-89 binds to G/C-rich elements and promotes p53-independent apoptosis, we tested whether ZBP-89 regulates STAT1 expression and contributes to IFNγ-induced apoptosis. To test whether ZBP-89 regulates STAT1 expression, Flag-tagged ZBP-89 was overexpressed in HCT116 cells using an adenoviral vector. We found that elevated levels of ZBP-89 had no significant effect on STAT1 protein levels in the cell (Fig. 1A). Protein levels of the stress-regulated kinase JNK were used as the loading control. Since elevated levels of ZBP-89 did not increase STAT1 protein, we examined whether reduced levels of ZBP-89 affected both STAT1 protein levels and phosphorylation status. A ZBP-89-specific siRNA targeting the ZBP-89 cDNA from +144 to +164 was tested in the cell. A point mutation within the ZBP-89 siRNA abolished the ability of siRNA to reduce protein levels (Fig. 1B). We found that reduced levels of ZBP-89 reduced the basal levels of STAT1 protein but not STAT2, 3, 5 and 6 (Fig. 1B). IFNγ had a minimal effect on the endogenous levels of ZBP-89 and STAT2, 3, 5 and 6, but significantly induced the protein levels and phosphorylation state of STAT1. Consistent with its ability to reduce STAT1 protein levels, lower levels of ZBP-89 also attenuated IFNγ-induced STAT1 phosphorylation. The reduction of STAT1 expression and

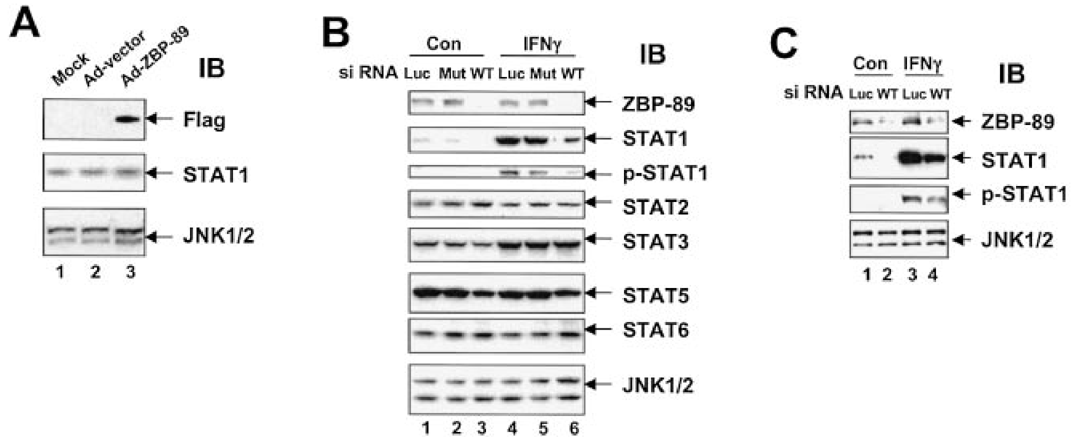

Figure 1. Silencing ZBP-89 gene expression reduces STAT1 expression. (**A**) HCT116 cells were infected with 100 MOI of Ad-ZBP-89 or Ad-β-gal. ZBP-89 is epitope tagged with FLAG. Two days later, the cells were collected and immunoblots were performed. (**B**) HCT116 cells were transfected with siRNAs targeted to pGL2 luciferase (Luc), ZBP-89 cDNA from +144 to +162 (WT) or a mutant form of ZBP-89 siRNA (Mut) for 48 h and then treated with 500 U/ml of IFNγ for 16 h. Whole-cell extracts were prepared for immunoblots. (**C**) HCT116 p53 null cells were transfected with siRNAs targeting ZBP-89 (WT) or luciferase (Luc) for 48 h and then treated with IFNγ at 500 U/ml for 16 h. Whole-cell extracts were prepared for immunoblots.

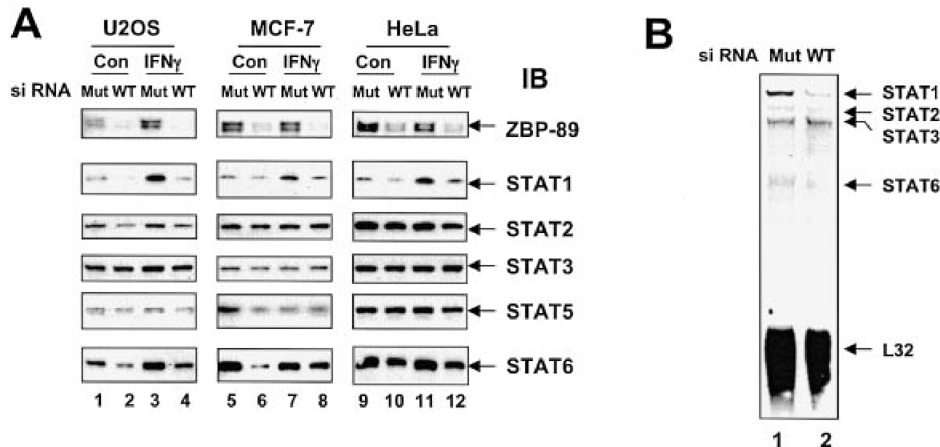

Figure 2. Reduction of ZBP-89 expression selectively reduces STAT1 and STAT6 expression. (**A**) U2OS, MCF-7 and HeLa cells were transfected with ZBP-89 mutant siRNAs (Mut) or ZBP-89 siRNA (WT) for 48 h and then treated with 500 U/ml of IFNγ for another 20 h. Whole-cell extracts were prepared and the profiles of STAT proteins were detected using their specific antibodies. (**B**) Total RNA was isolated from MCF-7 cells and was used for RNase protection assay as described in Materials and Methods.

phosphorylation was independent of p53 status (Fig. 1B and C). Therefore we concluded that there is a threshold level of ZBP-89 that is required for basal STAT1 expression.

To examine the effect of ZBP-89 siRNA on basal and IFNγ induction of STAT1 as well as expression of other STATs, three additional cell lines were used. We found that IFNγ induction of ZBP-89 was modest in the U2OS osteosarcoma cell line and coincided with the strong induction of STAT1 protein. Despite no significant induction of ZBP-89 by IFNγ in the MCF-7 and HeLa cell lines, reduced levels of ZBP-89 diminished basal and inducible STAT1 protein. In U2OS and MCF-7 cells, there was also a significant reduction in the basal levels of STAT6 (Fig. 2A). To further examine whether ZBP-89 siRNA affects STAT1 expression at the transcriptional level, an RNase protection assay was performed. As shown in Figure 2B, ZBP-89 siRNA significantly and specifically reduced STAT1 and STAT6 mRNA levels in MCF-7 cells. Therefore reduction of ZBP-89 selectively affects the levels of STAT1 and sometimes STAT6 in cells, but not STAT2, 3 and 5. This result indicates that ZBP-89 regulation is most specific for STAT1 in a number of cell types.

ZBP-89 is required for STAT1 constitutive expression

To understand how ZBP-89 regulates STAT1 expression, three different segments of the human STAT1 gene were subcloned into the pGL3–basic luciferase reporter vector. Two of the contructs contained either 972 or 4 bp of 5′ flanking sequence upstream from the start site of transcripiton, and included up to 884 bp of downstream sequence that contained the first and second exons separated by the first intron (Fig. 3). The IFNγ-responsive element reported previously is contained within the DNA segment from +1 to +498 (15). The basal promoter activity of these three STAT1 constructs was tested by transfecting them into HCT116 cells. Deletion of the region from –972 to –5 slightly reduced the promoter activity, while the most dramatic reduction in the promoter activity occurred

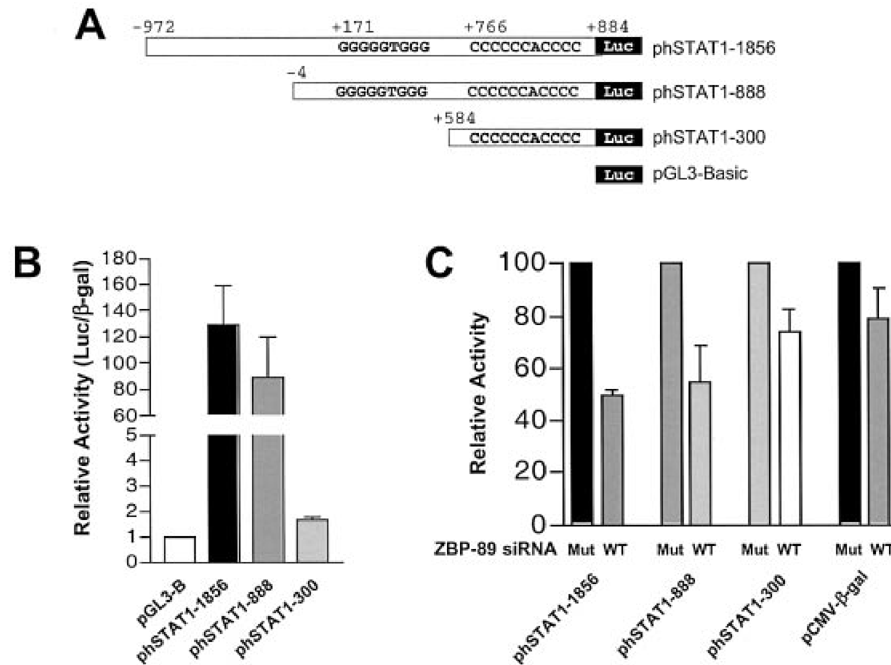

Figure 3. Reduction of ZBP-89 expression reduces the basal promoter activities of human STAT1. (**A**) Schematic diagram of the human STAT1 5′-genomic region. (**B**) HCT116 cells were each transfected with 100 ng/well of the indicated plasmid for 48 h. The relative luciferase activity is shown as the mean ± SEM for three independent experiments. (**C**) HCT116 cells were first transfected with ZBP-89 mutant siRNAs (Mut) or wild-type ZBP-89 siRNA (WT) for 36 h, and then transfected with the human STAT1 reporter phSTAT1-1856, phSTAT1-888, phSTAT1-300 or pCMV-β-gal and incubated for another 36 h. The relative luciferase and β-galactosidase activities were assayed. To directly compare the effects of ZBP-89 siRNA on each reporter, the basal activity of each reporter was set to 100%. The relative luciferase activity is shown as the mean ± SEM for three independent experiments.

by eliminating the region from –4 to +583. Removal of this segment and loss of the basal promoter activity overlapped the region from +1 to +498 that directs IFNγ induction of STAT1. Within the region from +1 to +498, the IFNγ response element has been reported to be specifically at +466 to +495 (15). Reduction of ZBP-89 protein levels with siRNA decreased the activity of phSTAT1-1856 and phSTAT1-888 reporter constructs by ~50% in HCT116 cells (Fig. 3C). These two STAT1 reporter constructs contained the intact G-rich element. In contrast, the effect of ZBP-89 siRNA was minimal on phSTAT1-300 element in which the G-rich element was deleted. Moreover the effect on an unrelated reporter, e.g. pCMV-β-gal, was also minimal. These data indicated that the ZBP-89-responsive element resides within the region from –4 to +584 and is essential for basal STAT1 transcription.

The G-rich element is essential for basal STAT1 transcription

Transcription factor binding analysis revealed that there are two putative ZBP-89 binding sites within the region from –4 to +888: a G-rich element from +171 to +179 and a C-rich element from +766 to +776 (Fig. 4A). Site-directed mutagenesis was employed to disrupt the G- or C-rich elements. As shown in Figure 4B, disruption of the G-rich element attenuated STAT1 promoter activity by ~50–80% in the different cell lines examined, while mutation of the C-rich element had no significant effect on STAT1 promoter activity. To further examine whether ZBP-89 was required for the residual promoter activity of phSTAT1-1856/M2 construct containing the mutated G-rich element, HCT116 cells were first transfected with ZBP-89 siRNA followed by transfection of the phSTAT1-1856WT and mutant reporters. siRNA treatment of cells transfected with phSTAT1-1856WT or M1 constructs exhibited ~35–44% lower promoter activity. In contrast, siRNA treatment had no significant effect on expression of the phSTAT1-1856M2 construct that contained the mutated putative ZBP-89 G-rich site (Fig. 4C). Thus ZBP-89 regulates STAT1 basal promoter activity via the G-rich element at +171 to +179.

ZBP-89 binds to the G-rich element on STAT1 promoter

To further test whether ZBP-89 binds to this G-rich element, electrophoretic mobility shift assays were performed. As shown in Figure 5A, five major DNA–protein complexes were observed with a ^{32}P-labeled double-stranded G-rich element. One hundred times the molar excess of unlabeled G-rich element abolished all five of the DNA–protein complexes. The rabbit IgG slightly reduced the intensities of all the DNA–protein complexes but did not disrupt or shift any of the DNA–protein complexes. In the presence of ZBP-89 specific antibody, the third DNA–protein complex was supershifted into the Sp1 complex (note increased intensity) and also generated a very slowly migrating complex. To further explore the relationship between ZBP-89–DNA and Sp1/Sp3–DNA binding, ZBP-89 siRNA was used to silence ZBP-89 expression in HCT116 cells and the nuclear protein was used for gel shift assays. Reduction of ZBP-89 increased the binding of Sp1 but had a minimal effect on Sp1 protein levels (Fig. 5B and C). Taken together, these results demonstrated

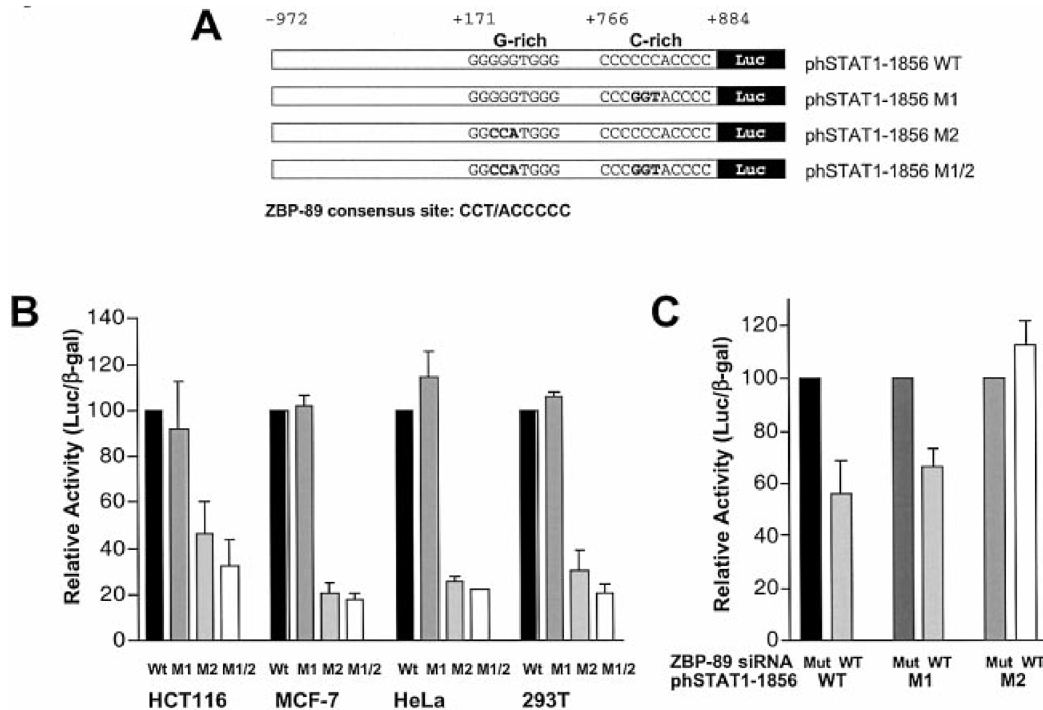

Figure 4. A G-rich element on STAT1 promoter is required for its constitutive activity. (**A**) The putative ZBP-89 binding sites located in the STAT1 promoter. (**B**) Cells were transfected with 100 ng/well of each indicated plasmid for 48 h. The relative luciferase activity is shown as the mean ± SEM for three independent experiments. (**C**) HCT116 cells were first transfected with ZBP-89 mutant siRNAs (Mut) or wild-type ZBP-89 siRNA (WT) for 36 h, and then transfected with the human STAT1 reporter phSTAT1-1856WT, M1 or M2 and incubated for another 36 h. The relative luciferase activity was detected as described above. To directly compare the effects of ZBP-89 siRNA on each reporter, the basal activity of each reporter was set to 100%. The relative luciferase activity is shown as the mean ± SEM for three independent experiments.

that ZBP-89 binds to a STAT1 regulatory sequence and is a critical transcription factor that mediates constitutive STAT1 transcription.

Reduced ZBP-89 levels attenuate IFNγ-induced apoptosis

IFNγ induces apoptosis or sensitizes cancer cells to apoptotic stimuli by increasing STAT1-mediated caspase-9, –8 and/or –3 expression in a variety of cancer cells. Since ZBP-89 is required for STAT1 constitutive expression, we examined whether ZBP-89 is required for IFNγ-mediated apoptosis. siRNA was used to reduce cellular ZBP-89 expression prior to IFNγ treatment (Fig. 6). We found that reduced ZBP-89 levels attenuated IFNγ-induced caspase-8 and –3 activation and downstream PARP cleavage. Thus physiological levels of ZBP-89 also contribute to IFNγ-mediated apoptotic action.

DISCUSSION

In this study, we identified a G-rich element in the STAT1 promoter that confers ZBP-89 regulation. ZBP-89 was found to regulate basal rather than inducible expression of the STAT1 gene. The results indicate that reduced levels of ZBP-89 correlated with reduced levels of STAT1 expression. These lower levels also diminished induction of STAT1 expression by IFNγ. Only one of two candidate G/C-rich ZBP-89 elements resides within the IFNγ-inducible fragment of the STAT1 gene. Site-directed mutagenesis of the gene and EMSAs revealed that regulation by ZBP-89 occurred at the G-rich element in the first intron. As observed in other promoters, the element recognized by ZBP-89 in the STAT1 gene was also bound by Sp1 and Sp3. Sp1, Sp3 and ZBP-89 have similar G/C-rich target regulatory elements. For example, the ZBP-89 consensus binding site is CCT/ACCCCC, while the core binding site for Sp1/Sp3 is GGGCGGG (16,17). Sp1, Sp3 and other Sp-like transcription factors are important components of the eukaryotic transcriptional machinery and regulate the expression of a number of genes with G/C-rich promoters. Generally, Sp1 functions as a transcriptional activator while Sp3 functions as either an activator or repressor, depending on the promoter. Our result does not exclude the role of Sp1 and Sp3 in the basal transcription of STAT1, but would need further analysis.

Reducing ZBP-89 levels or mutation of the G-rich element decreased STAT1 promoter activity by 40–80% (Figs 3 and 4), which suggests that other transcription factors, e.g. Sp1 and Sp3, are also required for STAT1 basal transcription. Although few studies have investigated the combined role of STAT1 and Sp1 in the regulation of gene expression, there is a precedent for the involvement of binding sites for these two transcription factors in the IFNγ induction of the interleukin 6 promoter (18). Moreover, there is also evidence that Sp1 and STAT1 form protein–protein complexes (19). Likewise, ZBP-89 may either cooperate or compete with Sp1-dependent promoter activation (16,17,20). Interestingly, the ability of ZBP-89 to form a complex with Sp1 may explain the presence of the slowest migrating DNA–protein–antibody complex supershifted with ZBP-89 antibody

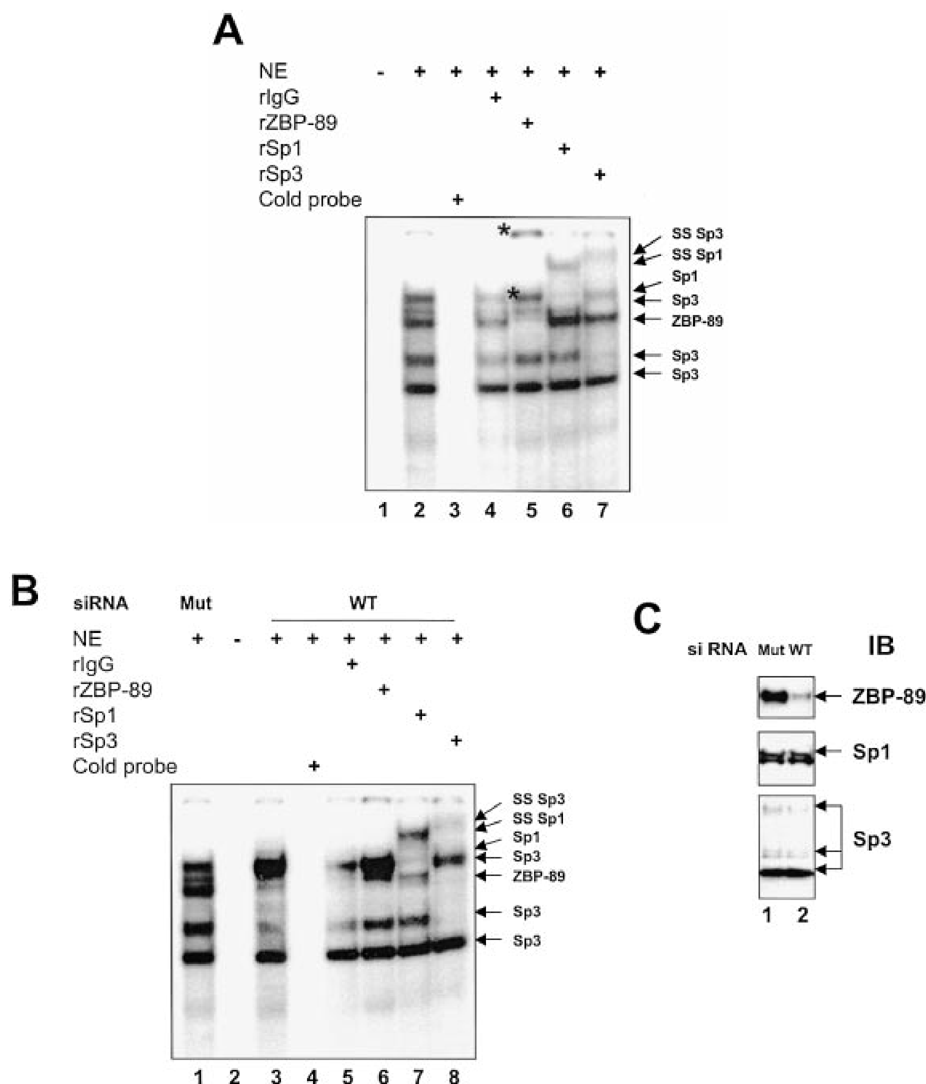

Figure 5. ZBP-89 binds to the G-rich element on STAT1 promoter. (**A**) Nuclear protein was prepared from HCT116 cells and a ^{32}P-labeled human STAT1 G-rich element was used for EMSA. One hundred times the molar excess of unlabeled G-rich element was used in lane 3. SS indicates the supershifted DNA–protein–antibody complexes and the asterisks indicate the supershifted DNA–ZBP-89–antibody complexes. (**B**) HCT116 cells were transfected with ZBP-89-specific siRNA for 48 h and nuclear protein was prepared for gel shift assay. (**C**) Five micrograms of nuclear protein was prepared from ZBP-89 mutant siRNAs (Mut) or wild-type ZBP-89 siRNA (WT) transfected HCT116 cells and used for immunoblot. The arrows indicate ZBP-89, Sp1 and Sp3 proteins, respectively.

(Fig. 5A, lane 5). Moreover, reduced ZBP-89 expression increased Sp1 binding to the G-rich element (Fig. 5B), suggesting possible competition between ZBP-89 and Sp1 for the same element.

Multiple extracellular signals initiate apoptosis in the absence of functional p53. Moreover, these mechanisms have been the focus of intense investigation owing to the frequent occurrence of mutated p53 in cancers rendering the cell essentially null for p53 and resistant to chemotherapy (21). Inflammation appears to produce prime candidates in the form of cytokines, which trigger apoptosis independent of p53 (5). This may explain why they are effective adjuncts to anticancer therapy despite non-functional p53. IFNγ treatment stimulates STAT1 activation and subsequently downstream promoters, e.g. caspase-8, –9 and –3 (15). Although IFNγ increases ZBP-89 levels in some cell lines, e.g. U2OS (Fig. 2A), the predominant effect of ZBP-89 on IFNγ induction is on sustaining basal levels of STAT1 gene expression available for inducible phosphorylation. In this way, ZBP-89 contributes to effective IFNγ-mediated apoptosis. Similar to our findings here, BRCA1, a tumor suppressor gene implicated in transcriptional regulation, also regulates IFNγ-mediated apoptotic cell death (21). In our study using a BRCA1 mutant cell line MCF-7, silencing ZBP-89 expression with siRNA reduced both STAT1 promoter activity and protein levels (Figs 2 and 3B). In fact, microarray analysis of the MCF-7 cell line has revealed that ZBP-89 increases 2-fold when wild-type BRCA-1 is overexpressed in this cell line. This result suggests that ZBP-89 lies downstream of this tumor suppressor gene product (22). Thus there may be synergy between BRCA1 and ZBP-89 proteins to mediate IFNγ-mediated apoptosis in breast cancer cell lines.

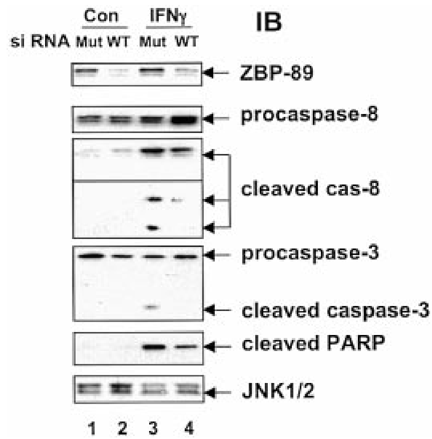

Figure 6. ZBP-89 contributes to IFNγ-induced caspase and PARP cleavage. A549 cells were transfected with ZBP-89 mutant siRNAs (Mut) or wild-type siRNA (WT) for 48 h and then treated with 500 U/ml IFNγ for another 20 h. Whole-cell extracts were prepared for immunoblots.

Besides STAT1, silencing ZBP-89 also reduced STAT6 protein levels in both MCF-7 and U2OS cells. Whether the effect of ZBP-89 is due to direct binding to this promoter has not been determined. STAT6 mediates multiple cytokine actions. STAT6 null mice show reduced IL-4-mediated Th2 differentiation (23). Furthermore, *in vitro* studies performed in MCF-7 cells show that STAT6 not only inhibits cellular proliferation and induces apoptosis but also mediates IL-4-induced growth inhibition (24). Whether ZBP-89 plays a role in IL-4 cytokine activation is not yet known, but certainly warrants further investigation.

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