STAT-1 Interacts with p53 to Enhance DNA Damage-induced Apoptosis*

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The STAT-1 transcription factor has been implicated as a tumor suppressor by virtue of its ability to inhibit cell growth and promoting apoptosis. However, the mechanisms by which STAT-1 mediates these effects remain unclear. Using human and mouse STAT-1-deficient cells, we show here that STAT-1 is required for optimal DNA damage-induced apoptosis. The basal level of the p53 inhibitor Mdm2 is increased in STAT-1(−/−) cells, suggesting that STAT-1 is a negative regulator of Mdm2 expression. Correspondingly, both basal p53 levels, and those induced by DNA damage were lower in STAT-1(−/−) cells. In agreement with this lower p53 response to DNA damage in cells lacking STAT-1, the induction of p53 responsive genes, such as Bax, Noxa, and Fas, was reduced in STAT-1-deficient cells. Conversely, STAT-1 overexpression enhances transcription of these genes, an effect that is abolished if the p53 response element in their promoters is mutated. Moreover, STAT-1 interacts directly with p53, an association, which is enhanced following DNA damage. Therefore, in addition to negatively regulating Mdm2, STAT-1 also acts as a coactivator for p53. Hence STAT-1 is another member of a growing family of protein partners able to modulate the p53-activated apoptotic pathway.

The signal transducer and activator of transcription 1 (STAT-1) protein is essential for signaling by interferons (IFNs) (1), which, in addition to their role in innate immunity, serve as potent inhibitors of growth and promoters of apoptosis. The C-terminal domain of STAT-1 includes a transcriptional transactivation domain, plus two phosphorylation sites, a tyrosine at position 701, which is targeted by Janus kinases (JAKs), and a serine at position 727, which is mitogen-activated protein kinases (MAPKs). STAT-1 dimerization and nuclear relocation depends on tyrosine 701 phosphorylation and that serine 727 phosphorylation is essential for maximal STAT-1 function (1). Although STAT-1-deficient mice develop no spontaneous tumors, they are highly susceptible to chemical carcinogen-induced tumorigenesis (2). Crossing the STAT-1 knockout into a p53-deficient background yields animals that develop tumors more rapidly, and with a broader spectrum of tumor types, than is seen with p53 single mutants (2).

Recently STAT-1 has been directly implicated in apoptotic cell death. For example, STAT-1-deficient human U3A fibrosarcoma cells are less susceptible to tumor necrosis factor α-induced cell death than parental cells containing STAT-1 (3). We have also demonstrated that the U3A STAT-1-deficient cells are resistant to hypoxia-induced cell death (4). STAT-1 also promotes apoptosis in cardiac myocytes exposed to ischemia/reperfusion injury (5–7). We showed that STAT-1 serine 727 but not tyrosine 701 phosphorylation is required for the effects of STAT-1 on apoptosis (4–5,7). The requirement for STAT-1 in apoptosis and growth arrest of some cell types may be explained by its ability to up-regulate caspases, Fas, FasL, and the cdk inhibitors p21Waf1 and p27Kip1 (8–10). Interestingly, p21Waf1 up-regulation by STAT-1 in mammary cells appears to involve BRCA1, which is often lost in familial and other forms of breast cancer (10).

p53 transcriptional activity is stimulated by a variety of genotoxic stimuli. Thus, stabilization of p53 protein levels is regulated by the Mdm2 protein, which interacts with p53 and promotes its degradation by ubiquitination (11). The interaction between p53 and Mdm2 is also negatively regulated upstream by p14ARF (human) and p19ARF (mouse) proteins, which bind to Mdm2 and inhibit p53-Mdm2 interaction (12). In response to DNA-damaging agents, p53 can either mediate cell cycle arrest or apoptosis. However, the mechanisms determining which of these is induced remains to be fully elucidated.

Recently, several proteins, that interact with p53 have been shown to promote the apoptotic function of p53, but not its ability to cause cell cycle arrest. For example ASPP1, a protein homologue of p53BP2, enhances the DNA binding and transactivation function of p53 and promotes its apoptotic role (13). In addition, the BRCA1-associated protein BARD1 also interacts with p53 to enhance genotoxic stress-induced apoptosis (14). In contrast, the POU family transcription factor Brn-3a interacts with p53 and inhibits transactivation of the pro-apoptotic Bax gene promoter, while promoting activation of the growth arrest p21 gene promoter (15). Thus, p53 interacts with specific protein partners following different stressful stimuli, which may result in p53-dependent transactivation of a number of genes involved in p53-induced apoptosis or growth arrest.

There is circumstantial evidence to link p53 and STAT-1 in modulating similar genes. For example, both p53 and STAT-1 activate the p21 gene promoter (16–17). STAT-1, like p53, also interacts with the transcriptional co-activators p300/CREB-binding protein (CBP) at different sites (18–19). p53-CBP in-
teraction results in acetylation of p53, which enhances sequence-specific p53-DNA binding (20). Hence, STAT-1 and p53 may form a complex with CBP and with other proteins to modulate transcriptional activity of genes in p53-dependent apoptotic signaling pathways. Thus, STAT-1 may regulate the tumor suppression function of p53.

Therefore in the present study we have utilized both human cell lines and mouse embryonic fibroblasts deficient in STAT-1 to determine their response to DNA-damaging agents. We show that STAT-1 is required for maximal induction of apoptosis in response to DNA-damaging agents. Interestingly, STAT-1-deficient cells have reduced levels of p53 and enhanced levels of Mdm2, and we show that the in wild-type cells Mdm2 gene promoter is negatively modulated by STAT-1 in response to DNA damage. More significantly, p53 was found to be physically associated with STAT-1 and this protein-protein interaction was enhanced following genotoxic stress. Finally, the induction of pro-apoptotic genes such as Bax, Noxa, and Fas by p53 is enhanced by overexpression of STAT-1, and this is dependent on intact p53 DNA binding sites in their respective promoters.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Wild-type STAT-1(+/+)/ and STAT-1(−/−) mouse embryonic fibroblasts (MEF) were kindly provided by David E. Levy (21) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). The human leukemia cell line MEG-01 and U937-derived cell lines stably expressing STAT-1 were kindly provided by Ian Kerr (22) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Treatment with cisplatin (Cp, Sigma) and doxorubicin (Dox, Sigma) was performed in subconfluent cultures in 5% fetal bovine serum for the times indicated. Functional Promoter Analysis—Promoter reporter constructs were kindly provided by John Reed (pBaux-luciferase), Yoshishinobu Nakanishi (pFas-luciferase), Nobuyuki Tanaka (WT-pNoxa-Luc) and Frank McCormick (Mdm2-Luc) Expression vectors for STAT-1 and GST-STAT1 constructs were kindly provided by Kurt Horvath. The C-terminal wild-type and mutant STAT-1 constructs were constructed as previously described (5) Transient transfection was performed using the calcium phosphate method. Treatment with cisplatin (10 μM) or doxorubicin (1 μM) for 24 h was performed post-transfection, after which transfected cells were lysed in 100 μl/well of 1× passive lysis buffer (Promega), and 50 μl from each lysate was used to measure firefly and Renilla luciferase activities. Both luciferase assays were quantified using an Orion Renilla Luminometer. Values for firefly luciferase were corrected by their corresponding Renilla luciferase values to obtain relative luciferase units (RLU).

Band Shift Assays—This was performed as described previously (7). Briefly nuclear extracts were incubated with either a wild type Noxa DNA probe containing the p53 binding site −174 AGGGTTGGCCCGG-GCAAAGTT or a mutant (indicated by lowercase) −174 AGGAGTCGGCCGGGaAATTTG (25). Samples were also incubated with either p53 (Oncogene), or STAT-1 or STAT-3 (Santa Cruz Biotechnology) antibody for 30 min prior to incubation with the DNA probe. Functional Promoter Analysis—Promoter reporter constructs were kindly provided by John Reed (pBaux-luciferase), Yoshishinobu Nakanishi (pFas-luciferase), Nobuyuki Tanaka (WT-pNoxa-Luc) and Frank McCormick (Mdm2-Luc) Expression vectors for STAT-1 and GST-STAT1 constructs were kindly provided by Kurt Horvath. The C-terminal wild-type and mutant STAT-1 constructs were constructed as previously described (5) Transient transfection was performed using the calcium phosphate method. Treatment with cisplatin (10 μM) or doxorubicin (1 μM) for 24 h was performed post-transfection, after which transfected cells were lysed in 100 μl/well of 1× passive lysis buffer (Promega), and 50 μl from each lysate was used to measure firefly and Renilla luciferase activities. Both luciferase assays were quantified using an Orion Renilla Luminometer. Values for firefly luciferase were corrected by their corresponding Renilla luciferase values to obtain relative luciferase units (RLU).

Assessment of Apoptosis—After treatment with cisplatin or doxorubicin for 48 h cells were washed twice with ice-cold phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde for 15 min on ice and washed with PBS. The TUNEL assay (Roche Applied Science) was performed as described by the manufacturer. TUNEL-positive cells were quantified following immunofluorescence microscopy using immunofluorescence microscopy.

Western Blot Analysis—Cells were treated with cisplatin or doxorubicin for 24 h and cell extracts were prepared in lysis buffer (150 mM NaCl, 50 mM Tris base, 0.5% SDS, 1% Nonidet P-40). Samples were then boiled in SDS sample buffer for 5 min and then run on a 10% SDS-PAGE. Samples were transferred to nitrocellulose filters and subjected to Western blotting using specific antibodies to p53 (Oncogene), p14ARF, and p19ARF (Oncogene), Bax (CN Bioscience), Fas, and STAT-1 (Santa Cruz Biotechnology), phospho-STAT-1 Ser^70 (Upstate Technologies) or Tyr^701 (Zymed Laboratories Inc.).

Immunoprecipitation and GST Pull-down Assays—For detection of STAT-1 and p53 interaction, MEF STAT1(+/+) cells were either untreated or treated with cisplatin or doxorubicin for 4 h. Cell extracts were prepared in radioimmune precipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, aprotinin 1 μg/ml), and lysates were incubated with anti-p53 antibody (Oncogene) or control mouse serum and antibody complexes were isolated using protein G-agarose beads (Amersham Biosciences) and washed three times with radioimmune precipitation assay buffer. The beads were then boiled in SDS sample buffer and run on a 10% SDS-PAGE. Samples were transferred to nitrocellulose filters and subjected to Western blotting using anti-STAT-1 antibody (Santa Cruz Biotechnology).

GST pull-down assays were performed with the following bacterially expressed GST fusion proteins: GST-STAT1, encoding a full-length STAT-1 fusion protein; GST-STAT1β, encoding STAT-1 lacking the C-terminal domain; GST-STAT1C, an isolated C-terminal STAT-1 GST fusion protein. Each bacterially expressed GST/STAT1 fusion protein was incubated with Sepharose beads together with p53 expressed by in vitro translation using the TntT-coupled transcription-translation system as described by the manufacturer (Promega). The Sepharose beads were then washed and subjected to Western blot analysis using antibodies to p53.

Immunofluorescence Confocal Microscopy—STAT-1(+/+) or STAT-1(−/−) MEF cells were grown on gelatin-covered coverslips and either left untreated or treated with 50 ng/ml IFN-γ or 10 μM cisplatin for 4 h. After fixation in 20% ethanol, coverslips were incubated 60 min in 3% bovine serum albumin in PBS at room temperature, followed by incubation in 1% bovine serum albumin in PBS containing 1:200 mouse anti-p53 (CN Bioscience) and 1:200 rabbit anti-STAT-1 (Santa Cruz Biotechnology) for 60 min. After three washes in PBS, 1,200 Alexa 488 goat anti-mouse (Molecular Probes) and 1:1000 Alexa 568 goat anti-rabbit (Molecular Probes) were added together in 1% bovine serum albumin with Hoechst 33258 (Sigma) for 30 min. After three washes in PBS, coverslips were mounted with DAKO fluorescent mounting medium. Images were collected using a Leica TCS SP2 confocal microscope, and absence of antibody cross-reaction and bleedthrough of fluorophore was verified on control slides. The maximum projection of six cross-sections was taken of each fluorophore individually (as indicated in the figure) and combined (giving yellow where both proteins were present).

Statistical Analysis—All results are expressed as the mean ± S.E. of at least three independent experiments. Paired data were evaluated by Student’s t test. A one-way analysis of variance was used for multiple comparisons.

RESULTS

STAT-1-deficient Cells Are Less Susceptible to DNA Damage-induced Apoptosis—STAT-1-deficient human fibrosarcoma U3A cells were compared with the parental cell line 2TGH (which contain STAT-1) in their response to DNA damage induced by either cisplatin or doxorubicin. As shown in Fig. 1, although both cisplatin and doxorubicin induced significant apoptosis in the 2TGH cell line, these responses were reduced significantly in the STAT-1-deficient U3A cells. However, the level of apoptosis following DNA damage was restored to the levels seen in the 2TGH cells, in the U3A-STAT1 cells (U3A cells into which wild-type STAT-1 has been reintroduced by stable transfection). Similar results were also obtained using other methods to assess apoptosis such as propidium iodate staining and Annexin-V-Fluos flow cytometry analysis (data not shown).

To exclude the possibility that this was an artifactual result arising from the use of an immortalized cell line, we repeated these studies using MEF STAT1(+/+) and STAT1(−/−) cell lines. Similar results to those obtained in the 2TGH/U3A cells were seen in both the MEF STAT1(+/+) and STAT1(−/−) cells in response to either cisplatin or doxorubicin, again showing that STAT-1 is required for maximal apoptosis following expo-
STAT-1 Modulates p53 Activity

Fig. 1. STAT-1 sensitizes cells to DNA damage-induced apoptosis. Levels of apoptosis following 48 h treatment with 10 μM Cp or 1 μM Dx in the parental 2TGH, U3A (STAT-1-deficient) and U3A-ST1 (STAT-1 re-introduced into U3A cells) as assessed by the TUNEL assay. The data represent the mean ± S.E. of three independent experiments. ***, p < 0.001.

Fig. 2. STAT-1 is required for enhanced apoptosis following DNA damage. MEF STAT1(+/-) and STAT1(-/-) cells were treated for 48 h with 10 μM Cp or 1 μM Dx, and the levels of apoptosis were assessed by the TUNEL assay. The data represent the mean ± S.E. of three independent experiments. ***, p < 0.01.

Fig. 3. Reduced p53 levels in STAT1(-/-) MEF cells. A, expression of p53, Mdm2, and p19ARF levels determined by Western blot analysis of cell extracts isolated from STAT1(+/-) (STAT1(+/-)) and STAT1(-/-) (STAT1(-/-)) MEF cells treated for 48 h with 10 μM Cp or 1 μM Dx. B, RT-PCR analysis of p53 and glyceraldehyde-3-phosphate dehydrogenase mRNA in wild-type (STAT1(+/-)) and STAT1(-/-) MEF cells following doxorubicin exposure for 24 h. C, Western blot analysis of p53 and Mdm2 levels shown at 4, 8, 24, and 48 h following doxorubicin. In all cases, similar results were observed in three independent experiments.

Mdm2 observed in cells lacking STAT-1 following DNA damage. Thus, STAT-1 may be involved in the negative regulation of the Mdm2 gene resulting in enhanced p53 levels.

Therefore, we next examined the mechanism of enhanced expression of Mdm2 in STAT1(-/-) MEF cells by assessing the
transcriptional activity of the Mdm2 promoter. Transfection of the Mdm2 reporter construct demonstrated significantly higher basal activity in MEF STAT1-/-/cells (Fig. 4A), which also paralleled the protein levels of Mdm2 observed in the above studies. To determine whether STAT-1 regulates the Mdm2 promoter, we next tested whether IFN-γ, which is known to enhance the expression and activity of STAT-1 (1) is able to reduce the activity of the Mdm2 reporter in STAT1-/-/ cells but not in STAT1+/+ MEF cells.

p53 is known to activate the Mdm2 promoter and therefore modulates its own stability (22–23). Therefore we also examined whether IFN-γ/STAT-1 activation is also able to inhibit doxorubicin-induced p53 enhancement of Mdm2 promoter activity. As shown in Fig. 4A, doxorubicin enhanced Mdm2 reporter activity in both STAT1+/+ and in STAT1-/-/ MEF cells. However, IFN-γ reduced doxorubicin-induced Mdm2 promoter activity in STAT1+/+ and to a less extent in STAT1-/-/ MEF cells.

Fig. 4B illustrates that the endogenous levels of Mdm2 correlated with the activity of the Mdm2 promoter following treatment with either IFN-γ or doxorubicin alone or in combination. Thus, IFN-γ enhanced STAT-1 levels and reduced Mdm2 levels in STAT1+/+ MEF cells but not in STAT1-/-/ MEF cells. Furthermore, doxorubicin-mediated enhancement of Mdm2 levels was also inhibited by IFN-γ in STAT1+/+ MEF cells.

Fig. 4. STAT-1 modulates the expression of the Mdm2 gene. A, STAT-1 is required for IFN-γ inhibition of the Mdm2 promoter. The Mdm2 reporter construct was transfected into the STAT-1+/+ and STAT-1-/-/ MEF cells followed by treatment with IFN-γ (50 ng/ml) or doxorubicin (1 μM) or both together for 24 h. The data represent the mean ± S.E. of three independent experiments. B, Western blots; C, RT PCR showing the levels of Mdm2 and STAT-1 from STAT-1+/+ (ST1+/+) and STAT-1-/-- (ST1-/--) MEF cells treated for 24 h with 1 μM Dx or 25 ng/ml of interferon-γ (IFN) alone or together. Similar results were observed in three independent experiments.

Fig. 5. STAT-1 is required for maximal expression of pro-apoptotic proteins. A, expression levels of Bax and Fas following treatment with 1 μM Dx for 24 h as determined by Western blot analysis of cell extracts isolated from STAT-1+/+ (ST1+/+) and STAT-1-/-- (ST1-/--) MEF cells. Similar results were observed in three independent experiments. B, enhancement of the Bax, Fas, and Noxa promoter activity in response to DNA damage requires STAT-1. Transfection of the Bax, Fas, and Noxa reporter constructs into STAT-1+/+ (ST1+/+) and STAT-1-/-- (ST1-/--) MEF cells was followed by treatment with 1 μM Dx for 24 h. The data represent the mean ± S.E. of three independent experiments. **, p < 0.01.
and also to an extent in STAT1(-/-) MEF cells. We also have observed in our transient transfection experiment that overexpression of STAT-1 can also inhibit p53-up-regulation of Mdm2 promoter activity (data not shown). Thus, these data strongly show that IFN-γ, by activating STAT-1, is able to reduce the constitutive activity of the Mdm2 promoter and also reduce the stimulatory effects of p53 on Mdm2 promoter activity. However, IFN-γ signaling pathway is also able to inhibit DNA-damage p53-mediated induction of the Mdm2 promoter and protein levels in the STAT-1(+/+) and STAT-1(-/-) MEFs (Fig. 4, A and B).

To confirm whether the observed changes in the Mdm2 pro-
moter activity were also occurring at the mRNA levels following the treatments as shown in Fig. 4A, we measured Mdm2 mRNA levels by RT-PCR. As shown in Fig. 4C, IFN-γ reduced Mdm2 mRNA levels in wild-type but not in STAT1(−/−) MEF cells. Moreover, IFN-γ also reduced doxorubicin-induced Mdm2 mRNA expression in wild-type but not in STAT1(−/−) MEF cells. Thus, STAT1 activation via IFN-γ exposure also inhibits Mdm2 expression at the mRNA level.

Optimal Expression of Pro-apoptotic Genes Requires STAT-1 in Response to DNA Damage—We next examined the levels of p53 target genes that are known to stimulate apoptotic cell death. Western blot analysis demonstrated enhanced Bax, and Fas expression in STAT1(+/-) cells in response to DNA damage compared with STAT1(−/−) cells (Fig. 5A). Thus, in the absence of STAT-1, the induction of these pro-apoptotic factors is reduced. Bax, Noxa, and Fas promoters constructs were used to determine whether the alteration observed in protein levels was due to a direct effect at the transcriptional level. Transfection of these reporter constructs into STAT1(+/−) cells in response to DNA damage resulted in higher levels of apoptotic activity compared with STAT1(−/−) MEF cells showed that promoter activity was enhanced more efficiently in response to doxorubicin-induced DNA damage in wild-type cells compared with STAT1(−/−) cells (Fig. 5B).

To determine whether p53 is required for STAT-1-mediated enhancement of p53 target genes, we examined the activity of the Noxa reporter in the p53-deficient Saos-2 cell line. Interestingly, we observed that overexpression of STAT-1 had no significant effect on Noxa promoter activity in the absence of p53 (Fig. 6A). However, re-introduction of p53 by transfection using a p53 expression vector in Saos-2 cells increased Noxa promoter activity. Overexpression of p53 together with STAT-1 enhanced Noxa promoter activity even further than transfection of p53 alone (Fig. 6A). Similarly, in the STAT1(−/−) MEF cells, where overexpression of p53 resulted in a small increase in Noxa promoter activity, overexpression of STAT-1 together with p53, resulted in a further enhanced Noxa promoter activity (Fig. 6B). Similar results were also obtained using the Bax and Fas reporter constructs (data not shown). To demonstrate that these effects are mediated via the p53 DNA binding site, we next examined a Noxa reporter construct, in which the p53 DNA binding site was mutated to remove p53 responsiveness, as previously reported (25). Mutation of the p53 binding site in this promoter resulted in a significant reduction in reporter activity compared with wild-type Noxa promoter activity in STAT1(+/-) cells overexpressing STAT-1 or p53 (Fig. 6C). Once again, overexpression of STAT-1 together with p53 was able to enhance the activity of the Noxa wild-type promoter, but had no effect on a mutant Noxa promoter. These results demonstrate that STAT-1 is able to modulate p53 transcriptional activity, possibly by enhancing p53 binding to DNA.

We next examined whether the observed effects of STAT-1 on p53 functional activity are also able to modulate apoptosis. As shown in Fig. 6D, overexpression of STAT-1 plus p53 in STAT1(−/−) MEF cells resulted in higher levels of apoptotic cell death than transfection of either STAT-1 or p53 alone. Furthermore, levels of the active form of caspase-3, a downstream effector of the apoptotic pathway, were enhanced in cells transfected with STAT-1 plus p53 (Fig. 6D). Similar results were observed in Saos-2 cells (data not shown). Thus, STAT-1 plus p53 not only enhance p53 target genes, but correspondingly increase the apoptotic program by enhancing caspase-3 processing.

We next wanted to determine the molecular mechanism involved in STAT-1 modulating the functional activity of p53. Therefore, we compared wild type versus STAT-1 727 serine mutant in terms of their ability to enhance p53-dependent responsiveness using the Noxa reporter assay. As shown in Fig. 7A, wild-type but not STAT-1 serine 727 mutant transfected in...
STAT-1(−/−) MEF cells was able to enhance Noxa reporter activity. Furthermore, transfection of p53 plus a C-terminal STAT-1 expression vector, lacking the N terminus and the DNA binding domain also enhance Noxa reporter activity. However, transfection of p53 together with a mutant C-terminal STAT-1 expression vector (lacking the N terminus and the DNA binding domain), in which the serine 727 was again mutated was less efficient in enhancing Noxa reporter activity (Fig. 7A). Similar results were also observed using either a Fas or a Bax reporter construct (data not shown). These results demonstrate that the isolated C-terminal domain of STAT-1 (lacking the DNA binding domain) can enhance p53-mediated gene activation in the absence of a STAT-1 DNA binding domain. This indicates that STAT-1 is able to act as a co-activator for p53 in activating p53 target genes.

To investigate whether STAT-1 and or p53 are bound to DNA, we first tested the ability of p53 to bind to a wild type or mutant DNA using the same p53 binding sequence from the reporter Noxa promoter assessed by band shift assays. As shown in Fig. 7B, a single retarded complex was observed from nuclear extracts isolated from STAT-1(−/−) MEF cells incubated with the p53 wild-type DNA-labeled probe. Additionally, an extra retarded band appeared in nuclear extracts from STAT-1(−/−) MEF cells when we re-introduced STAT-1 back into these cells by transfection using an expression vector for STAT-1. A p53-unlabeled DNA probe was able compete away both retarded complexes. Moreover, a specific anti-p53 antibody was also able to abolish both retarded complexes, while an anti-STAT-1 but not an anti-STAT-3 antibody, only abolished the larger retarded complex. (Fig. 7B). In contrast, no retarded complexes were observed from the same nuclear extracts using the p53 mutant DNA-labeled probe (Fig. 7B). These experiments indicate that the smaller retarded complex consists of p53 bound to DNA, while the larger retarded complexes consist of p53 plus STAT-1 bound to DNA. Thus, STAT-1 is able to modulate the activity of p53 by acting as a co-activator for p53, and requires an intact p53 DNA binding site to do so.

**STAT-1 Associates with p53 in Response to DNA Damage**—Our molecular studies so far indicate that STAT-1 may interact with p53 to induce maximal induction of p53-dependent target genes. To confirm this, we next assessed whether STAT-1 is able to associate with p53 in cells exposed to DNA damage. Co-immunoprecipitation studies were performed in STAT1(+/+) MEF cells following exposure to DNA damage. As shown in Fig. 8A, STAT-1 co-precipitated with p53 in STAT1(+/-) cells but not after immunoprecipitation with control serum. Furthermore, the level of STAT-1 associated with p53 was enhanced in response to DNA damage. However, p53/STAT-1(−/−) MEF cells—associated enhanced may be due to increased immunoprecipitated p53 form cell lysates following cisplatin treatment.

We next determined which region of STAT-1 is necessary for association with p53 by performing in vitro GST-pull-down assays using different fragments of STAT-1 tagged with GST. As shown in Fig. 8B, full-length STAT-1 but not STAT-1 lacking the C-terminal 38 amino acids (STAT1β), is able to associate with p53. However a GST C-terminal STAT-1 construct (STAT1C) containing the last 38 amino acids) was able to associate with p53 (Fig. 8B). Thus, the C-terminal STAT-1 domain is required for interaction with p53, paralleling the ability of this region to enhance activation of p53-dependent promoters.

To test whether STAT-1 and p53 co-localize after DNA damage in vivo, we analyzed cells treated with the DNA-damaging agent cisplatin by immunofluorescence microscopy. As shown in Fig. 9, cisplatin treatment resulted in enhanced nuclear staining for STAT-1 and p53. As has been previously demonstrated, IFN-γ also caused enhanced STAT-1 nuclear staining, demonstrating that both cisplatin and IFN-γ resulted in STAT-1 activation, which leads to translocation of STAT-1 from the cytosol to the nucleus. Although we observed a small amount of STAT-1 and p53 nuclear staining under control conditions, the intensities of both p53 and STAT-1 in the nucleus following cisplatin treatment was enhanced, these data together with the demonstration by co-immunoprecipitation experiments that the two factors interact, suggest that STAT-1 and p53 may potentially associate in the nucleus in vitro following DNA damage.

Recently we reported that STAT-1 phosphorylation on serine 727 but not tyrosine 701 is required for STAT-1-induced apoptosis (4, 7). Therefore, we examined whether STAT-1 is phosphorylated following DNA damage. As shown in Fig. 10A, STAT-1 induction was enhanced together with the induction of phosphorylation on serine 727 and not tyrosine 701, following exposure of wild-type MEF cells to cisplatin. To test whether cisplatin promotes STAT-1 functional effects, we also showed that a reporter construct containing a STAT-1 binding site is also activated following doxorubicin treatment in STAT1(+/-) but not in STAT1(−/−) MEF cells (Fig. 10B). Hence, phosphorylation of STAT-1 on serine 727, as well as p53 is activated following DNA damage, and these proteins translocate and associate in the nucleus.
DISCUSSION

The role of STAT-1 in the regulation of apoptosis following various stress-induced stimuli has been well documented. However, the molecular mechanism of how STAT-1 mediates apoptosis is unclear. In the present study, we show that cells deficient in STAT-1 have reduced levels of p53, and are correspondingly resistant to death induced by the DNA-damaging agents, doxorubicin, and cisplatin. Our finding that the levels of p53 is reduced in STAT-1-deficient cells were secondary to increased transcription and expression of Mdm2, a protein that targets p53 for proteasomal degradation. Expression of p19ARF, a protein, which sequesters Mdm2, was unaffected. Transcription of the p53-regulated genes, Fas, Bax, and Noxa, was reduced in STAT-1(-/-) cells treated with DNA-damaging agents. This data, together with the p53/STAT-1-dependent transcriptional enhancement of Fas, Bax, and Noxa expression, suggest that STAT-1, as well as modulating p53 levels by regulating the expression of Mdm2, also functions as a p53 coactivator to modulate the functional activity of p53-responsive genes.

The importance of STAT-1 as a pro-apoptotic factor has been reported previously using a variety of cell systems. For example, TNF-α-induced apoptosis was defective in STAT-1-deficient U3A cells (3). In addition lymphocytes derived from mice deficient in STAT-1 showed reduced apoptosis and enhanced proliferation (9). We have previously demonstrated that STAT-1 plays a role in the induction of apoptosis in a human fibrosarcoma cell line and in primary cardiac myocytes exposed to hypoxia, heat shock, and ischemia/reperfusion injury (4–7). STAT-1 has already been shown to influence the expression of multiple genes involved in apoptosis such as caspase-3, Fas, and FasL (3, 7–8). STAT-1 levels have also been shown to regulate the cell cycle by modulating the expression of the cyclin-dependent kinase inhibitors p27 and p21 (9–10, 16).

The p53 tumor suppressor gene is well known to be negatively regulated by the product of one of its downstream targets, Mdm2 (11–12, 23–24). Mdm2 induces rapid degradation of p53 by ubiquitin-mediated proteolysis (11–12, 23–24). Deletion of Mdm2 is embryonically lethal although the combined deletion of Mdm2 and p53 rescues this lethal phenotype (26). It has also been shown that loss of Mdm2 induces p53-dependent apoptosis, rather than p53-mediated G1 arrest (27). In the present study, we have shown that STAT-1-deficient cells show enhanced Mdm2 promoter activity, and express higher levels of Mdm2 protein, than wild-type cells, suggesting that STAT-1 is a negative transcriptional regulator of Mdm2. This may also explain our observation that STAT-1-deficient cells are resistant to cell death following exposure to DNA-damaging agents.

Our studies show that STAT-1 differentially modulates the function of p53 on different p53-responsive genes. Thus, we show data that STAT-1 inhibits p53 up-regulation of the Mdm2 promoter, while enhancing other p53-responsive genes such as Noxa, Bax, and Fas. Although we are unable to explain these differential effects of STAT-1 and p53 on these p53 responsive genes, we have previously observed similar effects with the Brn-3a transcription factor, which also interacts with p53 and inhibits transactivation of the pro-apoptotic Bax gene promoter, while promoting activation of the growth arrest p21 gene promoter in response to p53 (15). In this case, the distinct architecture of the two promoters and positions of the two p53 binding sites determined their distinct responses to p53 (28).

**Fig. 9.** STAT-1 and p53 associate in vivo in response to DNA damage. STAT1(+/-) MEF cells were either untreated or treated with either 50 ng/ml IFN-γ or 10 μM cisplatin for 4 h, fixed, and immunostained with antibodies to STAT-1 (red) or p53 (green) and analyzed by confocal microscopy. Co-localization (yellow) of STAT-1 and p53 is observed when the panel for p53 and STAT-1 are merged.
treatment in tumors harboring wild type p53 together with overexpression of Mdm2, may contribute to p53-induced apoptosis by inhibiting the expression of Mdm2 via a STAT-1-mediated mechanism. Thus, this may have important implications in cancer therapy.

In addition to indirectly regulating p53 levels and therefore activity, through its negative effects on Mdm2 expression, STAT-1 also binds p53 and enhances its transcriptional effects on p53-responsive genes. This interaction of STAT-1 with p53 is mediated by the C-terminal 38 amino acids of STAT-1. We have previously shown that the C-terminal 59 amino acids of STAT-1, which also lacks the DNA binding domain, enhances apoptosis induced by ischemia/reperfusion injury in rat cardiac myocytes and a human fibrosarcoma cell line (4–5). This C-terminal region of STAT-1 has also been shown to interact with transcriptional regulators such as CBP (18) and BRCA1 (10), two factors, which are also known to bind, and modulate the activity of, p53 (19–20). Therefore, it is possible that the modulating effects of STAT-1 on p53 may be mediated through the formation of a multimeric complex involving STAT-1, CBP/BRCA1, and p53. Indeed, the JMY protein is known to interact with CBP to enhance the ability of p53 to induce expression of Bax and apoptosis but not cell cycle arrest (34). Furthermore, PIAS1, which interacts with and inhibits STAT-1 functional activity, has recently been shown to interact with and sumoylate p53 (35). Thus, several factors that interact with p53 are also associated with STAT-1, further suggesting that a multimeric complex may exist involving STAT-1 and p53 and which may modulate the functional activity of p53.

Other co-activators are also known to regulate the activity of p53. For example, ASSP1 interacts with p53 and enhances p53-dependent apoptosis but not cell cycle arrest (13). Similarly, the interaction of BARD1 with p53 promotes apoptosis (14). Therefore, these results show that STAT-1 is one of many co-factors that are required for maximal p53-dependent apoptosis. The generation of the C-terminal fragment of STAT-1, the sequence necessary for p53 binding, by caspase cleavage (36), suggests that the STAT-1 C-terminal/p53 interaction may act to re-enforce the apoptotic process. Moreover we have recently demonstrated that a C-terminal STAT-1 expression vector is more potent than the full length STAT-1 in promoting apoptotic cell death (4–5). Hence STAT-1 is likely to act as a co-activator for p53 in a manner independent of DNA binding.

Our present finding that cisplatin is able to induce phosphorylation of STAT-1 on serine 727 and not tyrosine 701, is similar to our previous study which showed that serine 727 but not the tyrosine 701 phosphorylation is important in promoting apoptotic cell death (7). Similarly, it has been reported that stresses such as UV and lipopolysaccharide (37–38) result only in the induction of STAT-1 phosphorylation on serine 727. Thus, these studies suggest that different stresses may induce differential STAT-1 phosphorylation, which may also determine the factors that are recruited to the DNA promoter and therefore also the genes which become activated. Moreover, this effect further supports the potential co-activator role of STAT-1 since while phosphorylation of serine 727 promotes transcriptional activation by STAT-1, tyrosine 701 phosphorylation is required for dimerization and direct DNA binding.

In summary, our data provide a new functional mechanism whereby STAT-1 is able to act at different levels to modulate the apoptotic effect following genotoxic stress. Firstly, STAT-1 negatively regulates the Mdm2 gene, the major factor that controls the p53 proteasomal degradation pathway. Secondly, STAT-1 associates with p53 to enhance p53-mediated transcriptional gene activity and apoptosis. Thus, STAT-1 may be a factor, which is integral for DNA damage-
induced checkpoint pathways and may therefore behave as a tumor suppressor.

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