The tumor suppressor p53 can induce growth arrest and cell death via apoptosis in response to a number of cellular stresses. We have shown previously that the immunosuppressant cyclosporin A (CsA) induces programmed cell death with typical features of apoptosis in rat glioma cells. We report that CsA treatment results in increased level of the p53 tumor suppressor, its nuclear accumulation, and transcriptional activation of p53-dependent genes. The increase of p53 correlates with the elevation of p21^Waf1 and Bax protein expression. The increased level of Bax protein was accompanied with changes in its subcellular localization and association with mitochondria. Importantly, we demonstrate that glioma cells stably transfected with a mutant p53 (p53Val135) fail to increase p21 and Bax protein levels and are less sensitive to CsA-induced apoptosis. Furthermore, primary fibroblasts from p53−/− knockout mice are significantly more resistant to CsA-induced apoptosis compared with their corresponding counterparts containing functional p53. Together, our results suggest that the apoptotic program activated by CsA can be mediated by activation of p53 tumor suppressor and potentiation of its ability to initiate apoptosis.

The p53 tumor suppressor is implicated in cell cycle control, DNA repair, replicative senescence, and programmed cell death (1, 2). In normal cells p53 is expressed at a low constitutive level and is localized predominantly in cytoplasm. The latent form of p53 is stabilized and activated by post-translational modifications (3). A number of cellular kinases have been proposed to directly phosphorylate p53, including casein kinase I, casein kinase II, double-stranded RNA-dependent protein kinase, ataxia telangiectasia-mutated protein, CDK7, DNA-activated protein kinase, c-Jun N-terminal kinase, and p38 MAP1 kinase (4–11). The activation of p53 occurs in response to DNA damage or stresses such as hypoxia or nucleotide deprivation (12–15). p53-mediated cell cycle arrest is largely brought about by induction of p21^Waf1, which, in turn, inhibits the activity of cyclin-dependent kinases (16, 17). Activation of p53 may also result in apoptosis, and indeed, p53 transcriptionally activates a number of pro-apoptotic proteins including Bax, Fas, p53, insulin-like growth factor-binding protein 3, PIG3, and apoptotic protease activating factor-1 (18–22). The signaling cascade induced by p53 is complex and likely differs depending on the type of tissue examined. Despite the fact that many distinct damaging agents and cell types share similar features in regulating p53 function, no single cell type or damaging agent can generalize all known components of the p53 pathway (23). The bax gene contains p53 consensus sequences within its promoter and can be transcriptionally regulated by p53 (24). Bax promotes apoptosis by facilitating release of apoptosis-inducing factor and cytochrome c from the mitochondria, thus triggering a cascade of caspase activation (25–27). Bax seems to be essential for p53-mediated cell death in different cell types (28–30).

Transcriptional activation is thought to play a major role in p53-mediated apoptosis, because most p53 mutations are missense in human cancers and map to the DNA binding domain of p53 (31). The p53 dysfunction is the frequent event occurring in gliomas, the most common adult brain tumors of glial origin that are highly resistant to chemotherapy and radiotherapy (32). Many studies concentrate on the discovery of agents that allow rescue of wild-type p53 conformation and function (23, 33). Restoration of wild-type p53 activity in human glioma cells containing mutant p53 by several gene transfer approaches results in the induction of growth arrest or apoptosis (34–37). Expression of p53 in stably transfected glioblastoma cells induced mostly growth arrest (34), whereas adenoviral p53 induction resulted in generalized cell death (35–36).

Cyclosporin A is a widely used immunosuppressive drug that acts by binding to immunophils and inhibiting a protein phosphatase, calcineurin (37). The effects of CsA have been mostly characterized in lymphocytes, but recent data point out that CsA not only affects signaling pathways in lymphocytes but also in other cellular types such as adipocytes (38) and myocytes (39–40). We have demonstrated previously that cyclosporin A (CsA) induces apoptosis in rat C6 glioma cells that is characterized by morphological changes (such as shrinkage of the cell body and loss of extensions), chromatin condensation, caspase-3 activation, and "ladder-like" DNA fragmentation (41–43). Apoptotic cell death induced by CsA is dependent on de novo protein synthesis and occurs in association with the persistent activation of c-Jun N-terminal kinase and p38 MAP kinases, members of stress-activated protein kinase family. Prolonged activation of stress-activated protein kinases results in the stabilization c-Jun and ATF-2 proteins, activation of
AP-1 DNA binding activity, and transcriptional activation of Fas ligand expression (43). This work was thus undertaken to further elucidate the mechanism of CsA-induced cell death of glioma cells. We have hypothesized that p53 tumor suppressor and a pro-apoptotic protein could be an important mediator of CsA-induced apoptosis. We provide evidence that treatment of glioma cells and primary fibroblasts with CsA results in the up-regulation of p53 level and activation of p53-dependent apoptotic cell death. In glioma cells the induction of apoptosis by CsA correlates with the p53-dependent expression of p21Waf1, a cell cycle inhibitor and a pro-apoptotic Bax protein.

**Experimental Procedures**

**Cells**—Rat C6 glioma cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Sigma) and antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin). Cells were grown in 24-well or 10-cm diameter culture plates (Corning Glass) in a humidified atmosphere of CO₂/air (5%/95%) at 37°C or 38°C where indicated. At 18 h after plating, glioma cells were treated with 60 μM CsA (Sandimmun, Sandoz) as described (41, 42).

Primary cultures of mouse embryo fibroblasts derived from wild-type and p53−/− knockout mice were obtained as described previously (44–45). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics.

At 18 h after passage, fibroblasts were treated with 45 μM CsA.

**Immunofluorescence**—Rat C6 glioma cells (2 × 10⁴ cells/cm²) were seeded in a 8-well glass slide and grown for 24 h. Control cells and cells treated with 60 μM CsA were fixed (4% paraformaldehyde, 10 min at room temperature), permeabilized (0.1% Triton X-100 in PBS, 15 min at room temperature), and blocked (2% bovine serum albumin in PBS for 30 min at room temperature). The cells were then stained overnight at 4°C with either anti-p53 antibody (NeoVostra), anti-p21, or anti-Bax (Santa Cruz Biotechnology) followed by anti-rabbit or anti-goat IgG antibody conjugated with Alexa 488 for 1 h at room temperature and TO-PRO-3 nuclear stain (Molecular Probes). For mitochondria detection, an antibody recognizing the E2 polypeptide of the mammalian mitochondrial pyruvate dehydrogenase complex followed by anti-human IgG conjugated with Cy3 was used (46). Stained cells were visualized with either TCS-NT Leica confocal imaging system or fluorescent microscopy (Olympus).

**Immunoblotting Analysis**—Cells were collected in PBS with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and leupeptin, 0.7 μg/ml pepstatin A). Cell lysis was achieved by addition of an equal volume of 2× Laemmli sample buffer followed by boiling for 5 min. After centrifugation at 15,000 rpm for 5 min at 4°C, protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amerham Biosciences). Equal protein loading was confirmed by staining the membranes with Ponceau Red (Sigma). Membranes were incubated with the corresponding primary antibodies as follows: a mouse monoclonal anti-p53 Ab-1 (Transduction Laboratories), and polyclonal anti-p21, anti-Bax, anti-Bcl-xL (Santa Cruz Biotechnology), or anti-Actin (Sigma). Antibody recognition was confirmed by staining the membranes with Ponceau Red (Sigma). Membranes were stripped between each probing. The hybridization was performed after preincubating the membranes with a solution containing 5% dry milk, 0.1% Tween 20, and 0.1% BSA in PBS. Membranes were then washed with high stringency buffer and exposed on Hyperfilm MP (Amerham Biosciences). Densitometry of the autoradiograms was performed using the Molecular Imageer FX (Bio-Rad).

**Statistical Analysis**—Data were expressed as means ± S.D. Statistical significance was assessed by the one-way analysis of variance test. p values less than 0.05 were considered as significant.

**RESULTS**

**Cyclosporin A Activates p53 in C6 Glioma Cells**—We examined p53 protein expression during CsA-induced apoptosis in C6 glioma cells. This particular clone of glioma cells is known to express wild-type p53 (47–48). Western blot analysis revealed a significant increase of p53 protein level in cells treated with 60 μM CsA for 15 h that remained elevated up to 50 h (Fig. 1A). The observed increase of p53 level preceded an induction of apoptosis. Fig. 1, B and C, shows representative results of flow cytometric analysis of C6 glioma cells untreated or exposed to CsA for various times. Fluorescence-activated cell sorter analysis shows a significant increase in the number of annexin V-FITC-positive cells at 40–50 h after CsA addition. Annexin V binds phosphatidylserine. Apoptotic changes in cell membrane biochemistry lead to increased concentration of phosphatidylserine on the outer plasma membrane, where it becomes accessible to annexin V. The increasing number of cells co-stained with annexin-FITC and propidium iodide at 60 h indicates an occurrence of secondary necrosis (Fig. 1C).

Proliferating glioma cells in the absence of CsA showed marginal p53 nuclear level, as determined by immunofluorescence staining with specific antibody. Upon CsA treatment, the level of nuclear p53 was significantly increased after 25 h and remained elevated up to 40 h (Fig. 2A). Staining with a fluorescent dye Hoechst 33258 that visualized apoptotic changes in nuclear morphology followed immunocytochemical detection of p53 proteins in glioma cells. This double staining revealed a considerable increase of nuclear p53 levels in apoptotic cells (Fig. 2A). Nuclear accumulation of p53 protein was more prominent in cells exhibiting morphological features of apoptosis, such as loss of cytoplasmic extensions, “bean”-shaped nuclei with highly condensed chromatin. These morphological features have been shown previously to precede an increase in the number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling positive cells and ladder-like DNA fragmentation.

To determine whether the above-described up-regulation of p53 is accompanied by an increase of its transcriptional activity, we studied the expression of some genes, p21<sup>Waf1</sup>, bax, and mdm2, that are potential transcriptional targets of p53 (14, 17). The activated p53 is known to increase the mdm2 (murine double minute) gene transcription and interactions between the p53 and Mdm2 form negative autoregulatory feedback loop (49). Total RNA was harvested at 0, 15, 25, and 35 h after exposure to 60 μM CsA. Northern blots were then serially probed with labeled cDNA probes. The increase of mdm2 and
p21\textsuperscript{wat\textsubscript{i}} mRNA levels was observed 15 h after CsA treatment, and mRNA levels remained higher than in untreated cells up to 35 h. In contrast, \textit{bax} mRNA increased barely at 25 h after CsA treatment (Fig. 2B). Staining of the 28 S and 18 S ribosomal RNA levels on corresponding agarose gels with ethidium bromide ensured the equal loading of total RNA in each sample.

Furthermore, we have examined changes in protein levels of p21Waf1 and Bax in CsA-treated cells. Immunoblot analysis with antibodies specifically recognizing p21 and Bax protein demonstrated that there was a significant increase in p21 and Bax protein levels 25 h after the addition of CsA (Fig. 2C). The same extracts applied for Bax immunoblot analysis were used to analyze changes of the levels of Bcl-x\textsubscript{L}, an anti-apoptotic member of Bcl-2 family. The levels of Bcl-x\textsubscript{L} protein slightly but reproducibly decreased at 35–50 h post-treatment (Fig. 2C).

The subcellular localization of the Bax protein plays an important role in the induction of apoptosis as cytosolic Bax is unable to induce cell death, and in different cell systems the association of Bax translocation to mitochondria with release of pro-apoptotic molecules and activation of apoptosis has been demonstrated (28–30). The use of Bax-specific antibodies and confocal microscopy revealed a significant change in the subcellular localization of Bax elicited by CsA treatment (Fig. 3). In particular, untreated cells showed a diffuse cytoplasmic localization of Bax, whereas in CsA-treated cultures many cells had Bax localized in association to mitochondria at 36 h after addition of 60 μM CsA (Fig. 3, overlay). Noteworthy, in CsA-treated cultures cells with a weak expression and diffuse cytoplasmic localization of Bax had normal nuclei without signs of chromatin condensation (Fig. 3).
accompanied by changes of its subcellular localization and association with mitochondria.

Mutant p53 Protects from CsA-induced Apoptosis—To investigate the role of p53 in CsA-induced apoptosis, we have generated C6 glioma cells stably expressing p53Val135. This mutant p53, carrying a substitution from alanine to valine at position 135, exhibits a dominant negative activity against wild-type p53 at 38 °C (50). Western blot analysis of protein extracts isolated from three cell lines of p53Val135 transfected cells, cultured at 38 °C, confirmed that these cells expressed high levels of p53 protein (Fig. 4A). Furthermore, we demonstrated that CsA-induced up-regulation of p21 and Bax levels was inhibited in cells expressing mutant p53 (Fig. 4B). The highest inhibition was observed in the cell line expressing the highest level of p53Val135 mutant. Apoptotic changes were analyzed in several independently derived clones of glioma cells stably transfected with empty vector or mutant p53Val135 that were treated with CsA for 40 h. Fig. 5 shows the results of the representative experiment demonstrating differences in sensitivity to apoptosis between control and p53Val135 cell lines. Such differences were particularly evident after staining the nuclei of fixed cells with the fluorescent dye Hoechst 33258. The vast majority of cells transfected with empty vector and treated with CsA exhibited chromatin condensation and deformations in nuclear shape. In contrast, the appearance of apoptotic changes was significantly blocked in p53Val135-expressing cells, and the majority of these cells still remained attached to the dish (Fig. 5A). The same results were obtained with several others independently derived p53Val135-expressing clones (not shown).

Furthermore, we have used annexin V binding and flow cytometry to obtain a quantitative estimate of the resistance of glioma cells expressing p53Val135 to CsA-induced cell death (Fig. 5B). Glioma cells expressing mutant p53 were significantly resistant to apoptosis as inferred by the diminished appearance of annexin V binding. Approximately 20% of the p53Val135-expressing cells were annexin V-positive after 50 h of CsA treatment, compared with 55% in the case of control cells (Fig. 5B). The experiment shown in Fig. 5B is the average of assays done with three independent p53Val135-expressing clones. All together, these results demonstrate that the expression of dominant negative p53 confers resistance to the induction of apoptosis by CsA.

Fibroblasts Derived from p53-null Mice Are Partly Resistant to CsA-induced Apoptosis—To critically test the importance of p53 activation during CsA-induced apoptosis, we have performed further studies on primary cells lacking functional p53...
exposure to CsA (not shown). Immunoblot shows increase in total p53 levels in cells treated with CsA for 25–50 h (Fig. 6, lower panel). The above results demonstrate that up-regulation of p53 during CsA-induced apoptosis is a response conserved between different cell types, such as rat glioma cells and primary mouse fibroblasts. We studied the expression of p21<sup>waf1</sup> and mdm2 after CsA treatment in fibroblasts from wild-type and p53 null mice. Total RNA was harvested at 0, 15, 25, and 35 h after exposure to 45 μM CsA. Northern blots were then serially probed with labeled cDNA probes. Fig. 7A shows the increase of p21<sup>waf1</sup> and mdm2 mRNA levels in wild-type fibroblasts after CsA treatment, whereas no expression of p21<sup>waf1</sup> and barely detectable level of mdm2 mRNA was observed in p53<sup>−/−</sup> fibroblasts. Blots were rehybridized with a probe coding for constitutive glyceraldehyde-3-phosphate dehydrogenase gene.

We have subsequently compared the extent of CsA-induced cell death in fibroblasts derived from wild-type and p53-null mice (Fig. 7). Morphological features of apoptosis were partially blocked or delayed in p53<sup>−/−</sup> fibroblasts (not shown). The percentage of apoptotic cells was determined by fluorescence-activated cell sorter analysis of propidium iodide-stained cells because an annexin V staining could not be performed on p53<sup>−/−</sup> fibroblasts. Representative histogram shows analysis of the cell cycle distribution in untreated or CsA-treated fibroblasts from wild-type and p53<sup>−/−</sup> mice and the percentage of cells in each phase of the cell cycle (Fig. 7B). The population of apoptotic cells is represented as cells in sub-G<sub>0</sub>/G<sub>1</sub> phase (with DNA content lower than 2n). Fig. 7C shows that the percentage of apoptotic cells was significantly decreased in the case of p53<sup>−/−</sup> fibroblasts compared with wild-type fibroblasts (17 versus 45% of apoptotic cells, respectively) at 25 h after CsA treatment.

**DISCUSSION**

We have shown here that cyclosporin A, a widely used immunosuppressive drug, activates p53 tumor suppressor and triggers apoptotic cell death of glioma cells and fibroblasts. We demonstrated that CsA treatment activates p53 as reflected by increase of protein level, nuclear localization, and activation of the expression of the target genes bax, mdm2, and p21<sup>waf1</sup>. Moreover, the p53-dependent increase of Bax and p21<sup>waf1</sup> protein levels during CsA-induced apoptosis was proved. Importantly, the inhibition of endogenous p53 protein or the absence of functional p53 significantly reduces the extent of CsA-induced apoptosis, thus providing evidence that the accumulation of p53 tumor suppressor is a critical component of CsA-mediated cell death. This is a first demonstration that an immunosuppressive drug is capable of activating the function of the tumor suppressor p53 in glioma cells. Importantly, this response appears to be common to different cell types such as tumor glioma cells and primary fibroblasts. Although the induction of p53 mRNA level through unknown mechanism by another immunosuppressant, FK506, has been reported in keratinocytes and skin biopsies of psoriatic patients (51), no link to apoptosis has been established.

Activation of functional p53 and the expression of its transcriptional targets including p21<sup>waf1</sup> and Bax occur in many cells in the response to DNA damage or stress-inducing agents (30, 52–54). Anticancer agents including adriamycin, 5-fluorouracil, ionizing radiation, and etoposide, can induce p53 function, indicating that p53 pathway can respond to physiological and exogenous tumor-suppressing agents (23). However, studies employing microarrays to identify p53-triggered gene expression revealed the heterogeneity of induction of p53-dependent genes in studied cell lines, demonstrating the diversity of the repertoire and kinetics of p53-dependent genes (18–19).

In CsA-treated glioma cells the increase of mdm2 and p21<sup>waf1</sup> mRNA levels was observed at 15 h after CsA treatment, and a maximal increase of their mRNA levels correlated temporally with the elevation of the p53 protein level. In comparison, a moderate increase of bax mRNA occurred later after CsA treatment (Fig. 2B). Although the increase of bax mRNA level was minor, strong Bax immunofluorescent staining was observed in cells exhibiting apoptotic morphology. Perhaps Bax is increased in the population of p53-expressing cells undergoing apoptosis; alternatively, this increased intensity may reflect concentration of Bax in apoptotic cells. However, the failure to detect an increase of Bax expression in apoptotic cells lacking p53 (Fig. 4B) provides evidence that the Bax response is an element of the p53-dependent apoptotic pathway.

Furthermore, we demonstrated that the increase of Bax protein expression during CsA-induced apoptosis correlates with its association with the mitochondria. Translocation from cytoplasm to mitochondria has been observed for Bax and other members of the Bcl-2 family, BID and BAD, and may represent an important activating mechanism for the propagation of apoptotic signals to the cytoplasm (25, 55–57). It has been already shown that overexpression of Bax in human glioma cells results in increased sensitivity to apoptosis. In glioblastoma cells with a wild-type p53 genotype, overexpression of Bax produced spontaneous apoptosis (58–59).

Our findings clearly indicate that p53 plays a significant role in the induction of apoptosis after CsA treatment. Inhibition of function of the endogenous wild-type p53 blocked up-regulation of Bax and p21 proteins in glioma cells. Moreover, glioma cells expressing mutant p53 were significantly resistant to CsA-induced cell death; morphological features of apoptosis, annexin V staining, and chromatin condensation were significantly lower than in empty vector-transfected glioma cells (Fig. 5). Apoptotic changes of cell morphology and chromatin condensation were considerably blocked in p53<sup>−/−</sup> fibroblasts,
CsA Induces p53-dependent Apoptosis

Fig. 7. Fibroblasts derived from p53-null mice are less sensitive to CsA-induced apoptosis. A, lack of induction of p21<sup>WAF1</sup> and mdm2 expression in CsA-treated p53 null fibroblasts. Total RNA was isolated from fibroblasts derived from wild-type and p53<sup>-/-</sup> mice treated with 45 μM CsA for various times or left untreated. Representative Northern blot analysis using a p21<sup>WAF1</sup> and mdm2 cDNA probes is shown. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene expression was used as control. Lower panel shows the 28 S and 18 S ribosomal RNA levels visualized on corresponding agarose gels by staining with ethidium bromide.

Supporting further the importance of p53 activation in CsA-induced apoptosis.

Inactivation of the p53 tumor suppressor protein contributes to the progression of the wide range of human tumors including glial neoplasms. Previous studies (34–36) have shown that human gliomas are highly sensitive to the effects of p53 activation when p53 is introduced ectopically. Inducible expression of p53 in stably transfected glioma clones induced mostly growth arrest (34–35), whereas onadoviral p53 transduction resulted in generalized cell death (36). In studies where the wild-type p53 activity was restored close to physiological levels, a low expression of p53 caused cell cycle arrest, and a high level of expression resulted in apoptosis (60). It suggests that a high level of p53 expression (experimentally achieved with adenoviral p53 transfer) is necessary to induce apoptosis of glioma cells. Moreover, some reports demonstrated that the efficacy of p53 gene therapy depends on the p53 status of gliomas. U87 human glioma cells (wild-type p53) were highly resistant to adenoviral p53-mediated apoptosis, whereas glioma cells with mutated p53 cells underwent extensive apoptosis after adenovirus-p53 infection (61, 62). Recent studies indicate that apoptosis induced by p53 transduction in glioma cells can be repressed at several steps by tumor resistance mechanisms, and simultaneous transduction of caspase-9, Apaf-1, or FasL is necessary to overcome these mechanisms (63). We have demonstrated previously a transcriptional activation of Fas ligand expression in CsA-treated cells (43). It suggests that the treatment of glioma cells with cyclosporin A induces two independent or related pathways triggering the cell death machinery. Although apoptosis induced by CsA requires activation of wild-type p53 to turn on the cell death program, our preliminary data indicate that CsA can induce cell death in human glioblastoma cells with a mutated p53. However, in such cases cell death does not exhibit features of apoptosis such as ladder-like DNA fragmentation and caspase activation.2

Because of the potential toxicity of adenoviral vectors, the experimental strategies exploring the possibility of restoring p53 function for therapeutic benefit focused recently on the post-translational regulation of the p53 protein level and the p53-dependent transactivation pathways (23, 33). Up-regulation of the functional p53 tumor suppressor and induction of p53-triggered apoptosis through pharmacological intervention with the well known drug such as cyclosporin A can be a useful strategy to induce glioma cell death. These findings may be of clinical importance for pharmacological intervention in gliomas.

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Tumor Suppressor p53 Mediates Apoptotic Cell Death Triggered by Cyclosporin A
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