The G2/M Regulator 14-3-3σ Prevents Apoptosis through Sequestration of Bax*

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Received for publication, July 9, 2001, and in revised form, September 6, 2001

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 276, No. 48, Issue of November 30, pp. 45201–45206, 2001

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In response to DNA damage and genotoxic stress, the p53 tumor suppressor triggers either cell cycle arrest or apoptosis. The G2 arrest after damage is, in part, mediated by the p53 target, 14-3-3σ (σ). Colorectal tumor cells lacking σ are exquisitely sensitive to DNA damage. Here we analyzed the mechanism of this sensitivity in σ−/− as compared with σ+/+ human colorectal tumor cells. Exposure to adriamycin resulted in rapid apoptosis only in σ−/− cells. This was further characterized by caspase-3 activation, p21CIP1 cleavage, and CDK2 activation. Moreover, Bax was rapidly translocated out of the cytoplasm, and cytochrome c was released in σ−/− cells. Transient adenovirus-mediated reconstitution of σ in the σ−/− cells led to effective rescue of this phenotype and protected cells against apoptosis. The association of σ, Bax, and CDK1 in protein complexes may be the basis for this antiapoptotic mechanism. In conclusion, σ not only enforces the p53-dependent G2 arrest but also delays the apoptotic signal transduction.

Cellular DNA damage induces the p53 tumor suppressor, which in turn trans-activates a number of downstream genes to mediate either cell cycle arrest or apoptosis. After DNA damage cells are either arrested at cell cycle checkpoints or forced to undergo apoptosis in the face of irreparable damage (1–4). Both scenarios of either arrest or apoptosis contribute to the overall maintenance of genetic stability.

At least two distinct pathways lead to apoptosis, both of which converge on caspases as downstream effectors. The TNF-R/Fas-triggered apoptotic pathway recruits caspase-8 to activate caspase-3, one of the effector caspases (5, 6). The second pathway depends on the release of cytochrome c from mitochondria and subsequent binding to Apaf-1, which is followed by the activation of caspase-9 and caspase-3. This second pathway is blocked by the anti-apoptotic Bcl-2 family proteins (7, 8). A probable third pathway for the initiation of apoptosis involving the endoplasmic reticulum and caspase-12 has recently been described (9). Although the final executors of apoptosis are proteins of the caspase family, it is not fully understood by which mechanisms p53 mediates apoptosis; many p53-induced genes might be involved (10). Recent evidence shows that p53-dependent apoptosis may proceed through the Apaf-1/caspase-9 pathway (11). However, the complete set of pathways connecting p53 to these proteins upstream in the caspase cascade remains to be elucidated.

The p53-induced G1/S arrest is primarily mediated through the induction of p21CIP1 (12, 13). p53 regulates the G2/M checkpoint through induction of 14-3-3σ (σ), a protein that sequesters CDK1 in the cytoplasm (14). Other p53-dependent effects, e.g. repression of CDK1 and cyclin B1 promoter activities, have also been reported to contribute to the G2 arrest (15, 16). σ is a component of the G2/M checkpoint because its overexpression leads to G2 arrest (14), and cells lacking σ are defective in a stable G2 arrest (17). However, additional p53-independent mechanisms exist to initiate the G2/M checkpoint, because under certain conditions cells lacking p53 also enter G2 arrest (16, 18). The p53-independent G2 arrest is regulated through phosphorylation of CDC25C phosphatase by the CHK1 and CHK2 kinases (19, 20) and cytoplasmic sequestration. Sequestered CDC25C is unable to activate CDK1 and thereby prevents entry into mitosis.

In the face of DNA damage, σ is believed to protect damaged cells from entry into mitosis by binding and sequestering CDK1/cyclin B1 complexes in the cytoplasm (17). p21 is also required for the maintenance of G2 arrest, because damaged p21−/− cells progress into mitosis but fail to complete cytokinesis (14, 21, 22). Additionally, cleavage of p21 has been reported to be part of the events in the progression of apoptosis (23, 24). Simultaneous loss of both p21 and σ renders cells to be more sensitive to DNA damage than those lacking either of the two genes, indicating their cooperative roles in the G2/M checkpoint (25).

To understand the mechanisms of cell death that underlie the sensitivity to DNA damage and to examine the role of σ after DNA damage, we compared σ−/− cells with their parental HCT116 counterparts. The results indicate that in addition to its known role in securing the G2/M checkpoint, σ functions to protect against apoptosis by affecting Bax localization.

**EXPERIMENTAL PROCEDURES**

Cell Lines, DNA Damage, and Adenoviral Infection—HCT116 wild type cells and σ−/− clone of HCT116 derived by homologous recombination were cultured as described (17). Adriamycin was used at a final concentration of 0.7 μg/ml in culture medium. Both adherent and floating cells were collected for lysate preparations. For reconstitution, σ−/−

* This work was supported by Grant FU342/2-3 from the Deutsche Forschungsgemeinschaft (to J.O.F.), the ELAN-Fonds of the University of Erlangen-Nuremberg (to J. O. F.), the Boehringer Ingelheim Fonds (to J. T. E.), and a fellowship from Bristol-Myers Squibb (to A. M.).

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cells were infected with a σ expressing adenovirus (14) for 6 h. They were then left untreated or damaged with adriamycin (ADR).

Lysis, Immunoprecipitations, and Fractionation—Both adherent and floating cells were collected for lysis and fractionation. Cell lysates were prepared by brief sonication in cell lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM NaF, 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate, either with 0.1% Tween 20 or without detergent. Immunoprecipitations were performed essentially as described (26). The beads were washed with lysis buffer containing 0.1% Tween 20. For fractionation of lysates, the cells were lysed by Dounce homogenization in 0.2M sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.4, with added protease and phosphatase inhibitors. Unlysed cells and nuclei were pelleted by centrifugation at 800 × g for 10 min. The resultant supernatant was centrifuged at 17,000 × g for 20 min at 4°C to yield cytosolic supernatant and mitochondrial-enriched pellet. The pellet was washed once with the lysis buffer and solubilized in 10× volume lysis buffer with 1% Nonident P-40 for 15 min on ice. Solubilized proteins were examined by immunoblotting. Whole cell lysates were prepared by resuspending intact cell pellets in cell lysis buffer above containing 1% Nonident P-40 for 30 min on ice followed by centrifugation at 17,000 × g.

Antibodies—Primary antibodies for the detection of the various proteins were purchased from the following sources: p53 (Ab-6), Raf-1 (Ab-2), p21 (Ab-1), and 14-3-3ζ (Calbiochem), cyclin B1, cytochrome C, and Bax (Pharmigen), caspase-3, CDK2, CDK1, 14-3-3ζ, 14-3-3ε, 14-3-3θ, Bad (Santa Cruz Biotechnology, Inc.), and activated caspase-3 (Cell Signaling Technologies). CIP1-associated regulator of cyclin B1 (CARB) antibody was generated as described (27). The affinity-purified antibody against 14-3-3ζ used here was generated earlier (17).

Kinase Assays and Apoptosis Detection—Kinase assays were performed as described elsewhere (26). Annexin-V staining and DNA laddering tests were done using Annexin-V Fluos and apoptotic DNA ladder kits, respectively, from Roche Biochemicals. For flow cytometry, the cells were trypsinized and fixed in 70% ethanol for at least an overnight. Staining was done with a solution of phosphate-buffered saline containing 0.5% Tween 20, 2% fetal calf serum, 0.5 μg/μl RNase A, and 50 μg/μl propidium iodide. The assay was done using FACScan (Becton Dickinson), and the readouts were analyzed using CellQuest software.

Immunofluorescence Staining—The cells were fixed with methanol/acetic acid (1:1) for 5 min at −20°C and stained as described (27) with minor modifications. Blocking and antibody incubations were done in phosphate-buffered saline with 2% bovine serum albumin and 30% goat serum. Double immunofluorescence stainings were performed using CY2-conjugated anti-rabbit and CY3-conjugated anti-mouse secondary antibodies (Amersham Pharmacia Biotech). Images were taken using Leica DMRB/E fluorescence microscope with I3 and N2.1 filters. DePsipher (R & D Systems) staining was done according to the manufacturer's instructions. Culture medium was used instead of the provided reaction buffer. The cells were incubated with the DePsipher solution for 15 min in the incubator.

RESULTS

Rapid Caspase-3 Activation and Loss of p21 Function Are Characteristic of Apoptosis of σ−/− Cells—Following exposure to ADR, both σ−/− and σ+− cells showed up-regulation of p53 (Fig. 1A). Unexpectedly, while p21 was up-regulated and maintained at high protein levels in the σ−/− cells, p21 levels were reduced and two distinct bands of 21 and 15 kDa were apparent in the σ−/− cells (Fig. 1A), consistent with cleavage of the protein. Cleavage of p21 at its C terminus (between amino acids 112 and 113) by caspase-3 or caspase-3-like enzymes has been reported during apoptosis of both cancer cells (24) and human umbilical vein endothelial cells (28). In accordance with this, caspase-3 was found to be activated only in the σ−/− cells, concomitant with the cleavage of p21. This coincided with the progression of apoptosis in σ−/− cells (see below), which were detaching from the plate surfaces and massively floating in the culture medium (data not shown).

Subsequently, we assayed for CDK2 kinase activity to examine the functional integrity of p21 in DNA-damaged σ−/− and σ+− cells. There was no decrease but rather an increase in CDK2 activity in σ−/− cells coinciding with p21 cleavage. In contrast to this, a profound down-regulation of CDK2 activity was observed in σ−/− cells, in which p21 was induced and not cleaved (Fig. 1A). In addition to the assessment of p21 function, we looked at the status of the functionally related CARB, which has been suggested to contribute to the retention and protection from degradation of cyclin B1 in the cytoplasm (27). Immunoblotting for CARB and cyclin B1 proteins revealed that CARB was degraded, with the appearance of a smaller fragment in damaged σ−/− cells, coinciding with a sharp decrease in cyclin B1 level (Fig. 1A).

Because p53 was up-regulated following DNA damage to identical levels in both σ−/− and σ+− cells (Fig. 1A), we looked further into the events upstream of p21 cleavage. For this purpose, cell-permeable inhibitors of caspase-3 (Z-DEVD-FMK) and caspase-9 (Z-LEHD-FMK) as well as the pan-caspase inhibitor Z-VAD-FMK were employed. Caspase-3 activation and p21 cleavage were effectively blocked in σ−/− cells by both caspase-3 and caspase-9 inhibitors (Fig. 1B). Following caspase inhibition, damaged σ−/− cells did not undergo apoptosis and stayed arrested for over 72 h (Fig. 2D and data not shown).

Because other members of the 14-3-3 protein family have also been implicated in the protection of cells from apoptosis (29, 30), we compared the endogenous levels of expression of 14-3-3ε, 14-3-3γ, 14-3-3θ, and 14-3-3β in σ−/− and σ+− cells. Whereas σ was up-regulated in σ−/− cells after ADR-induced damage, all other 14-3-3 family members were equally expressed in both cell types, irrespective of DNA damage (Fig. 1C).

The activation of caspase-3, cleavage of p21, and detachment of cells from culture plates was suggestive of an apoptotic process in σ−/− cells. To prove this functional damage as well as damaged σ−/− and σ+− cells were examined for early or late apoptotic marker events. The apoptotic phenotype of the σ−/− cells was evident by the accumulation of a sub-G1 population, positive annexin-V staining, and the occurrence of DNA laddering (Fig. 2, A–C). These results confirmed that σ−/− cells were indeed undergoing apoptosis, and there was no indication

1 The abbreviations used are: ADR, adriamycin; HCT, human colorectal tumor; CARB, CIP1-associated regulator of cyclin B1.
of G2 arrest. The $\sigma^{+/+}$ cells, however, did not undergo significant apoptosis during this time and remained arrested (Fig. 2 and data not shown).

**Altered Subcellular Distribution of Bax Predisposes $\sigma^{-/-}$ Cells to Activation of Caspase-3**—The distribution and activities of either pro- or anti-apoptotic proteins in subcellular compartments is critical to the outcome after DNA damage. Therefore, subcellular distribution of caspase-3 and Bax was examined by analyzing cytosolic and mitochondrial-enriched fractions of $\sigma^{+/+}$ and $\sigma^{-/-}$ cells exposed to ADR. Activation of cytosolic caspase-3 was detected only in $\sigma^{-/-}$ cells about 36 h after damage (Figs. 1A and 3A). In addition to its presence in the cytosolic fraction, the cleaved active form of caspase-3 was also detected in the mitochondrial fractions of $\sigma^{-/-}$ cells. The relative abundance of the active form of the enzyme in the mitochondrial fractions was higher as compared with the procaspase form in the same fractions (Fig. 3A). The detection of equal levels of Raf-1 in the cytosolic and the nonspecific band in the mitochondrial fractions (Fig. 3A) served as fractionation controls. $\sigma$ was only present in the cytosolic fractions (Fig. 3B).

Additionally, there was a profound decline in the level of cytosolic Bax in damaged $\sigma^{-/-}$ cells, and importantly, basal and DNA damage-induced Bax protein levels in whole cell lysates were identical in $\sigma^{+/+}$ and $\sigma^{-/-}$ cells (Fig. 3C). These data and the absence of any detectable cleavage products suggested relocalization of Bax (Fig. 3A). This further coincided with cytochrome $c$ release as evidenced by its rapid increase only in the cytosolic fractions of the $\sigma^{-/-}$ cells. In contrast, damaged $\sigma$ cells retained Bax in the cytosol without reduction over the duration of the examination and with no significant cytochrome $c$ release.

To exclude subsequent genetic alterations in the $\sigma^{-/-}$ cells as a cause for the increased apoptotic responsiveness and to prove that $\sigma$ was solely responsible for this phenotype, $\sigma$ was reconstituted in the $\sigma^{-/-}$ cells by adenovirus-mediated transient gene transfer (ad-$\sigma$). Thereby it was possible to reconstitute $\sigma$ in 100% of the cells as determined by immunofluorescence staining (Ref. 14 and data not shown). The time point for the examination of $\sigma$-reconstituted cells was deliberately chosen so that a $\sigma$ overexpression phenotype, as observed in wild type cells (14), did not yet occur. Following infection with either ad-$\sigma$ or, as a control, ad-GFP, the cells were damaged with adriamycin and analyzed for the presence of cytoplasmic Bax and apoptosis at different time points. Indeed, the reconstitution of $\sigma$ effectively reduced the loss of cytoplasmic Bax and lead to partial prevention of apoptosis (Fig. 4A), whereas ad-GFP infection had no detectable influence on these parameters (Fig. 4B and data not shown).

**Association of $\sigma$ and Bax in HCT Cells**—Following the observation that Bax was retained cytosolic in $\sigma^{+/+}$ but not in $\sigma^{-/-}$ cells, we examined whether this effect is mediated by binding of $\sigma$ to Bax. We hypothesized that, following DNA damage, $\sigma$ regulates the redistribution of Bax from the cytoplasm. To test this model, anti-$\sigma$ immunoprecipitates were examined for the presence of Bax and other known 14-3-3-associating proteins. We found that anti-$\sigma$ immunoprecipitates from damaged $\sigma^{+/+}$ cells contained Bax (Fig. 5A). Bad, another proapoptotic protein of the Bcl-2 family that is known to bind other 14-3-3 proteins, was not co-immunoprecipitated. In accordance with previous data (17), CDK1 also bound $\sigma$ after DNA damage to a similar degree (Fig. 5A). The reverse immunoprecipitation with anti-Bax antibodies could not be interpreted because of nonspecific bands on the protein blot at the position of $\sigma$. Control immunoprecipitates of 14-3-3$\xi$ from both $\sigma^{+/+}$ and $\sigma^{-/-}$ cells did not contain Bax, nor did $\sigma$ antibody immunoprecipitate Bax from $\sigma^{-/-}$ cells (Fig. 5B). Furthermore, we examined whether Bax associates with CDK1 complexes, which might contribute to the retention of Bax in the cytosol. Therefore immunoprecipitates of Bax and CDK1 proteins were analyzed for possible complex formation. Interestingly, anti-Bax immunoprecipitates from damaged $\sigma^{+/+}$ cells but not from $\sigma^{-/-}$ cells (data not shown).
were examined for Bax.

...continued on the right panel, or Bad (el). Relative molecular mass positions are indicated in the left panel. Control lysates were made from \( \alpha^{+/-} \) cells and immunoprecipitated with \( \alpha \)-antibody (left panel) or 14-3-3 immunoprecipitates from lysates of \( \alpha^{+/-} \) (middle panel) and \( \alpha^{+/-} \) cells (right panel) were examined for Bax. B, \( \alpha^{+/-} \) cells were left untreated or exposed to ADR for 36 h. Bax immunoprecipitates were immunoblotted for CDK1 and vice versa.

...shown) contained CDK1 and vice versa (Fig. 5C). In undamaged \( \alpha^{+/-} \) cells there was only a very weak co-immunoprecipitation of CDK1 with Bax, hinting that \( \sigma \), CDK1, and Bax may form a trimeric protein complex.

Because we observed evidence for the translocation of Bax in \( \alpha^{-/-} \) cells, we traced the mitochondrial localization and Bax redistribution before and after treatment with ADR. By immunofluorescence analysis with antibodies against Bax and the mitochondrial cytochrome oxidase, a redistribution of Bax and aggregation of mitochondria around the centrosome was observed in over 95% of the \( \alpha^{-/-} \) cells as early as 24 h after DNA damage (Fig. 6A and data not shown). The centrosomal concentration of Bax and mitochondria was confirmed by counterstaining the damaged \( \alpha^{-/-} \) cells with \( \gamma \)-tubulin, which marked the center of the site of aggregation (Fig. 6B). At this time, both \( \sigma^{-/-} \) and \( \sigma^{+/-} \) cells were still adherent to the plates, but \( \sigma^{-/-} \) cells started to detach from the plates a few hours later, when cytosolic caspase-3 was activated. The localization of \( \sigma \) and CDK1 under these conditions has previously been demonstrated (17). To rule out a fixation artifact or the possibility that the cells showing mitochondrial aggregation were only shrinking, undamaged and damaged \( \sigma^{-/-} \) cells were stained with DePsipher reagent, which stains the mitochondria in live cells without the need for fixation. Microscopic evaluation of the stained cells clearly showed that at the time of the aggregation of mitochondria, detectable 24 h after DNA damage, the cells were still adherent to the slides without evidence of shrinkage (Fig. 6C).

DISCUSSION

We elaborated a mechanism by which \( \sigma \), a p53-induced mediator of G2 arrest, potentially counteracts apoptosis. In both \( \sigma^{+/-} \) and \( \sigma^{-/-} \) cells, p53 was up-regulated to identical levels...
after ADR-induced DNA damage. Although \( \sigma^{+/+} \) cells also upregulated \( \sigma \) and arrested in G2, \( \sigma^{-/-} \) cells proceeded without delay into apoptosis, showing all the characteristic features, including caspase-3 activation. During this time, Bax remained predominantly cytosolic in \( \sigma^{+/+} \) cells, whereas in \( \sigma^{-/-} \) cells there was a pronounced shift of Bax out of the cytosolic compartment. These results indicate that \( \sigma \), up-regulated upon DNA damage, is involved in the regulation of the progression of apoptosis by affecting the distribution of Bax. The precise nature of this regulation and the question as to whether the association between \( \sigma \) and Bax is direct or indirect deserves further analysis. However, because \( \sigma \) binds to Bax equally well and under identical conditions as it binds to CDK1 (Ref. 17 and Fig. 5), this suggests that this association likely has a regulatory effect. The identical expression of other 14-3-3 family members in both \( \sigma^{+/+} \) and \( \sigma^{-/-} \) cells, the fact that these 14-3-3 proteins are not up-regulated upon DNA damage, and the effect of 14-3-3\( \sigma \) reconstitution altogether suggest that this protection from apoptosis is specific for 14-3-3\( \sigma \).

In the absence of \( \sigma \), Bax appears to be readily translocated to mitochondria and probably other organelles, and this may sensitize the cells for rapid progression into apoptosis through caspase-3 activation. Interestingly, immunofluorescence analysis of ADR-treated \( \sigma^{-/-} \) cells showed a relocalization of Bax and aggregation of mitochondria around the centrosomes. The existence of caspases-2 and -9 zymogens (31) and of pro-caspase-3 (32) in mitochondrial fractions has previously been reported. We further present evidence that active caspase-3 might also be present in the mitochondria, possibly because of influx from the cytosolic compartment.

\( \sigma \) is exclusively expressed in cells of epithelial origin (33, 34). In natural and in vitro cultured epidermal cell layers, the expression of \( \sigma \) is limited to the suprabasal layer formed by the progeny of basal cells that exit the cell cycle to initiate keratinization (35). Interestingly, the exact same layer of cells in the epidermis express the proapoptotic protein Bax, whereas the more superficial layers express other proapoptotic proteins like Bak (36). The simultaneous expression of two functionally antagonistic proteins in suprabasal cells, which are at a stage prior to the dominance of pro-apoptotic proteins, might indicate a balance between pro- and anti-apoptotic proteins before these cells undergo terminal differentiation. This also supports our conclusion that \( \sigma \) counteracts premature apoptosis. On the other hand, selective down-regulation of \( \sigma \) by promoter methylation has been shown to frequently occur in breast and hepatocellular carcinomas (37, 38). Down-regulation of \( \sigma \) in tumor cells may contribute to malignant transformation, whereas at the same time it may sensitize the cells to the effects of chemotherapeutic DNA damage. The degree to which \( \sigma \) is involved in the sensitization of cells may possibly be different for various forms of DNA damage.

It is not clear whether p21 cleavage is essential for the progression into apoptosis or whether it is just an intermediate. Previous studies showed that p21 cleavage is an early event in apoptosis (23). The requirement for the CDK inhibitory activity of p21 to prevent apoptosis has also been demonstrated; a p21 mutant incapable of binding CDK2 failed to protect cells from irradiation- or chemical-induced apoptosis (39). Furthermore, p21 cleavage might mediate apoptosis through CDK2 activation (28); alternatively, p21 cleavage might be involved during the progression of apoptosis (24). Because \( \sigma^{-/-} \) cells undergo apoptosis with p21 cleavage, our results conform with the notion that cleavage of p21 at its C terminus, with subsequent loss of its CDK-inhibitory function, is an important determinant for the progression of apoptosis. Simultaneous with the cleavage and loss of function of p21, there was also loss of CARB, a protein suggested to contribute to the cytoplasmic maintenance of cyclin B1 proximal to the centrosome (27). The observed decrease of cyclin B1 levels in \( \sigma^{-/-} \) cells may be partially attributable to the loss of CARB, because such a loss would leave cyclin B1 free to be degraded. This mechanism of loss of cyclin B1 may add to the previously reported direct effect of p53 on the expression of the protein (15, 16).

Finally, our results demonstrate that lack of \( \sigma \) in colorectal tumor cells sensitizes them to chemotherapy-induced apoptosis. Therefore, strategies to target \( \sigma \) during chemotherapy might contribute to the therapeutic efficiency. Besides its G2/M checkpoint functions, \( \sigma \) also plays a role in the prevention of the progression of apoptosis by influencing the subcellular distribution of the pro-apoptotic protein Bax. This implies that the p53-induced G2 arrest may be accompanied by a separate mechanism to delay apoptosis. The dual activities of p53 to induce either cell cycle arrest or apoptosis therefore seem to function in a manner such that one pathway is delayed or prevented if the other is induced.

Acknowledgments—We thank B. Vogelstein for cell lines, D. C. Altieri and S. Dimmeler for reagents, M. B. Lutz, A. S. Baur, A. Steinkasserer, and fellow lab members for comments, and G. Schuler for support and encouragement.

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J. Biol. Chem. 2001, 276:45201-45206.
doi: 10.1074/jbc.M106427200 originally published online September 26, 2001

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