The Molecular Mechanism of Noxa-induced Mitochondrial Dysfunction in p53-Mediated Cell Death*

Received for publication, August 8, 2003
Published, JBC Papers in Press, September 18, 2003, DOI 10.1074/jbc.M308785200

Young-Woo Seo‡§, Jin Na Shin§§, Kang Hee Ko‡§, Jong Hee Cha‡, Jae Yoon Park‡, Byoung Rai Lee‡, Cheol-Won Yun§, Young Myeong Kim**, Dai-wu Seol††, Dong-wook Kim‡‡, Xiao-Ming Yin†††, and Tae-Hyoung Kim‡‡‡

From the ‡Department of Biochemistry, Chosun University School of Medicine, Dong-Gu, Gwangju 501-759, Korea, §Korea Basic Science Institute, Gwangju Branch, Chonnam National University, Bak-Gu, Gwangju 500-757, Korea, ||Department of Microbiology, Pukyong National University, Nam-Gu, Pusan 608-737, Korea, †Vascular System Research Center, Kangwon National University, Chunchon, Kangwon-Do 200-701, Korea, Departments of ¶¶Surgery and ¶¶¶Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, and §§Molecular Neurobiology Laboratory, McLean Hospital, Harvard Medical School, Belmont, Massachusetts 02478

Genotoxic stresses stabilize the p53 tumor suppressor protein which, in turn, transactivates target genes to cause apoptosis. Although Noxa, a “BH3-only” member of the Bcl-2 family, was shown to be a target of p53-mediated transactivation and to function as a mediator of p53-dependent apoptosis through mitochondrial dysfunction, the molecular mechanism by which Noxa causes mitochondrial dysfunction is largely unknown. Here we show that two domains (BH3 domain and mitochondrial targeting domain) in Noxa are essential for the release of cytochrome c from mitochondria. Noxa-induced cytochrome c release is inhibited by permeability transition pore inhibitors such as CsA or MgCl2, and Noxa induces an ultra-structural change of mitochondria yielding “swollen” mitochondria that are unlike changes induced by tBid. This indicates that Noxa may activate the permeability transition-related pore to release cytochrome c from mitochondria into cytosol. Moreover, Bak-oligomerization, which is an essential event for tBid-induced cytochrome c release in the extrinsic death signaling pathway, is not associated with Noxa-induced cytochrome c release. This finding suggests that the pathway of Noxa-induced mitochondrial dysfunction is distinct from the one of tBid-induced mitochondrial dysfunction. Thus, we propose that there are at least two different pathways of mitochondrial dysfunction; one mediated through Noxa in response to genotoxic stresses and the other through tBid in response to death ligands.

The p53 mutation is found in more than half of all human cancer patients. Cancers with loss of p53 function are often resistant to chemotherapeutic agents mainly because of the absence of p53-dependent apoptosis (1–3). The p53-dependent apoptosis largely depends on the capability of p53 to function as a transcription factor, although recent reports show that the transcription-independent function of p53 plays a role in this process. There are many target genes (e.g. Apaf-1, DR5, p53AIP, PUMA, and Noxa) transactivated by p53 that are proposed to mediate p53-dependent apoptosis in various cancer cells (4). A recent report shows a close correlation between p53 level and Noxa induction that could be a key determinant of chemically induced hepatocellular carcinoma in mice, suggesting that Noxa is a crucial mediator of p53-dependent apoptosis in the liver (5). Noxa induced by p53 is up-regulated by x-ray irradiation and is heavily dependent on p53 and its homologue p73 (6). Noxa belongs to the “BH3-only” branch of the Bcl-2 protein family, although mouse Noxa has two BH3 domains, whereas other BH3-only proteins have a single BH3 domain. Like many Bcl-2 family proteins that translocate to mitochondria and modulate mitochondrial function, Noxa translocates to mitochondria and then leads to cytochrome c release and caspase-9 activation (7). Most of the BH3-only proteins such as Bid and Bim appear to induce efflux of mitochondrial death-stimulating proteins through Bax or Bak homo-oligomerization leading to the permeabilization of the mitochondrial outer membrane (8, 9). However, the detailed molecular mechanism by which Noxa induces mitochondrial dysfunction remains to be solved. In these studies, we characterize Noxa-induced mitochondrial dysfunction in order to provide insight into the molecular mechanism by which p53 induces apoptosis. We show that Noxa causes mitochondrial dysfunction by a distinct pathway in mediating p53-dependent apoptosis, unlike mitochondrial dysfunction induced by tBid in response to FasL or tumor necrosis factor-α.

MATERIALS AND METHODS

Cell Culture and Mitochondrial Localization—HeLa cells were seeded onto an 8-well Lab-Tek chamber slide glass (Nunc) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). The indicated GFP–Noxa plasmids were transfected into HeLa cells using Effectene (Qiagen) according to the manufacturer’s instructions and cultured for 2 additional days. In some experiments, Mitotracker Red CMXROS (Molecular Probes Inc.) was added to the medium at a final concentration of 200 nM, and cells were incubated for a further 10 min. In these experiments, the dye was visualized without a fixation step. After washing with PBS, fresh me-
Mitochondrial localization was analyzed with a laser scanning confocal microscope (Leica Microsystems TCS NT). Immunostaining of Cytochrome c—HeLa cells were rinsed with PBS three times and fixed with ice-cold 2% buffered paraformaldehyde (pH 7.4) for 10 min. After washing with PBS and blocking with a buffer containing 0.1% saponin and 0.05% BSA in PBS, pH 7.4, for 30 min, the cells were incubated with anti-cytochrome c antibody (dilution 1:250, BD Biosciences) for 1 h at 30 °C and then washed with the blocking buffer. Tetramethylrhodamine-labeled anti-mouse IgG antibody (dilution 1:250, BD Biosciences) was added to the cells, and they were incubated for 1 h more. After washing with PBS, cells were examined with the laser scanning confocal microscope.

Apoptosis Assay—HeLa cells were cultured in 24-well plates and transfected with the indicated plasmid constructs. Each well was har-
incubation for 30 °C for 1 h. After centrifugation, supernatants were subjected to Western blot analysis with anti-cytochrome c antibody. B. isolated mitochondria were treated with tBid (0.1 µg ml⁻¹) or Noxa (50 µg ml⁻¹) in the presence of Bcl-xLΔ7 (30 µg ml⁻¹) and incubated at 30 °C for 1 h. Cytochrome c was visualized in the supernatants by Western blot analysis.

FIG. 2. Noxa is able to release cytochrome c from isolated mitochondria. A, recombinant tBid (0.1 µg ml⁻¹) or recombinant human Noxa (1, 2.5, 5, 10, 25, and 50 µg ml⁻¹) was incubated with isolated mitochondria at 30 °C for 1 h. After centrifugation, supernatants were subjected to Western blot analysis with anti-cytochrome c antibody. B, isolated mitochondria were treated with tBid (0.1 µg ml⁻¹) or Noxa (50 µg ml⁻¹) in the presence of Bcl-xLΔ7 (30 µg ml⁻¹) and incubated at 30 °C for 1 h. Cytochrome c was visualized in the supernatants by Western blot analysis.

RESULTS

It has been reported that mouse Noxa has two BH3 domains and induces apoptosis, whereas human Noxa has only one BH3 domain. First, to determine whether human Noxa is able to induce cell death in HeLa cells, GFP-Noxa (1–54) was transfected into HeLa cells. As shown in Fig. 1A, GFP-Noxa (1–54) induced cell death, and Bcl-xL blocked Noxa-induced cell death. In addition, the pan-caspase inhibitor z-VAD-fmk inhibited Noxa-induced cell death, indicating that caspases play a crucial role in Noxa-induced cell death. These results indicate that one BH3 domain in human Noxa is sufficient to induce apoptosis. To determine whether cytochrome c release accompanies human Noxa-induced cell death, GFP-Noxa (1–54) was transfected into HeLa cells, and the cells were immunostained using anti-cytochrome c antibody. HeLa cells transfected with human Noxa showed diffuse cytochrome c distribution throughout the cytosol, but control cells (transfected with GFP alone) maintained the typical mitochondrial distribution of cytochrome c (Fig. 1B), indicating that cytochrome c release from mitochondria is an event induced during human Noxa-mediated cell death. To further confirm that human Noxa targets to mitochondria and causes mitochondrial dysfunction, we checked whether GFP-Noxa (1–54) was present in mitochondria after transfection. GFP-Noxa (1–54) showed a punctate distribution typical of mitochondria (Fig. 1C, left panel, green color), and mitochondrial localization of Noxa was confirmed by overlapping staining (Fig. 1C, right panel, yellow color) with Mitotracker (Fig. 1C, middle panel, red color). This finding is in agreement with a previous report showing the mitochondrial localization of Noxa.
localization of mouse Noxa when using an adenovirus expressing mouse Noxa and suggests that human Noxa is functionally identical to mouse Noxa despite having a single BH3 domain (7).

Although the data indicated that human Noxa causes the release of cytochrome c from mitochondria in HeLa cells, it was not clear whether Noxa is able to directly trigger efflux of death-stimulating proteins from the inter-membrane space of mitochondria or whether another, non-mitochondrial, factor is also necessary. To address this question, we adopted the \textit{in vitro} system of Noxa-induced mitochondrial dysfunction. In this system, recombinant human Noxa protein was able to induce cytochrome c release directly from isolated mitochondria. Noxa-induced cytochrome c release was inhibited by \textit{Bcl-xL/}{H}9004\textit{TM} (Fig. 2, \textit{A} and \textit{B}). These results strongly suggest that the presence of human Noxa in mitochondria is enough to induce mitochondrial dysfunction.

It has been shown that a death ligand-activated BH3-only protein, tBid, targets to the mitochondria and that the DEF helices of tBid play a crucial role in this localization. We hypothesized that human Noxa may have a mitochondrial targeting domain (MTD), similar to tBid. To determine the MTD of Noxa, genetic experiments, as shown in Fig. 3, were performed.

**FIG. 3.** \textbf{C-terminal region of Noxa contains the mitochondrial targeting domain.} \textit{A} and \textit{B}, schematic diagrams of plasmid constructs expressing various regions of Noxa fused to GFP are presented. Plasmid constructs containing the indicated regions of Noxa fused to GFP were transfected into HeLa cells and cultured for 2 days. Cells were visualized for co-localization of Mitotracker CMXROS (200 nm) and GFP using a confocal microscope. \textit{C}, sequence alignment of Noxa from human, mouse, cow, chicken, and pig. The BH3 domain and the MTD are indicated. \textit{D}, plasmids expressing the indicated regions of Noxa fused to GFP (200 ng/well) were transfected into HeLa cells with the reporter plasmid, luciferase (100 ng/well), and further cultured for 2 days before measuring the luciferase activity.
Noxa, various expression plasmids of Noxa fused to GFP were transfected into HeLa cells (Fig. 3A). In Fig. 1 we showed that Noxa (1–54) displays the punctate distribution that is typical of a mitochondrial staining pattern, and mitochondrial distribution of Noxa (1–54) was confirmed by overlapped staining with Mitotracker. This same punctate distribution disappeared in Noxa (1–30) and Noxa (1–40); however, Noxa (21–54) and Noxa (31–54) maintained a similar pattern, indicating that Noxa (31–54) contains the MTD. To define the MTD of Noxa, Noxa (31–54) was dissected as shown in Fig. 3B. Noxa (31–40) and Noxa (31–45) showed a dispersed cytoplasmic distribution, whereas Noxa (41–54) and Noxa (41–50) displayed the punctate pattern. These results demonstrate that human Noxa (41–50) contains the entire MTD. This MTD seems to be conserved between human, mouse, cow, pig, and chicken (Fig. 3C), suggesting that the MTD of Noxa is functionally significant for induction of cell death in p53-dependent apoptosis.

To verify whether human Noxa needs the MTD to kill cells, plasmids containing various GFP-Noxa constructs and a reporter gene were co-transfected into HeLa cells. Noxa (1–54)
and Noxa (21–54), which have intact BH3 and MTD domains, were able to induce cell death (Fig. 3D). We further confirmed that the cell killing induced by Noxa (1–54) and Noxa (21–54) was associated with an efflux of cytochrome c from mitochondria (Fig. 4). Interestingly, Noxa (11–40) and Noxa (21–40), which have the BH3 domain but not the MTD domain, failed to induce cell death (Fig. 3D). In addition, Noxa (31–54) and Noxa (41–54), which have the MTD domain but harbor a disrupted BH3 domain or no BH3 domain, respectively, failed to induce apoptosis, indicating that the MTD domain is necessary but not enough to induce cell death (Fig. 3D). These results demonstrate that the killing activity of the Noxa BH3 domain is functional only when it targets to mitochondria, and this mitochondria targeting is mediated by the MTD.

It is known that after engagement of death ligands to death receptors, caspase-8-activated tBid binds to cardiolipin, which exists exclusively in the mitochondrial membrane and is enriched at the inner membrane and the mitochondrial contact sites. The interaction of tBid with cardiolipin is a critical step for tBid targeting to mitochondria (10, 11). To understand the molecular mechanism by which Noxa targets to mitochondria in p53-dependent apoptosis, we examined whether Noxa also targets to cardiolipin like tBid. It is unlikely that Noxa binds to cardiolipin (data not shown), and it implies that p53-mediated mitochondrial dysfunction through Noxa might be distinct from mitochondria dysfunction triggered by death ligands that is mediated through tBid. To examine whether Noxa triggers an efflux of mitochondrial proteins in a manner distinct from tBid, we examined the inhibitory activity of CsA in a cytochrome c release assay. CsA showed no significant change in the release of cytochrome c induced by tBid, consistent with a previous report (12) (Fig. 5A). On the other hand, calcium, which is a strong activator of the PT pore, was able to release cytochrome c from isolated mitochondria and, as expected, was inhibited by CsA. Surprisingly, CsA also blocked Noxa-induced cytochrome c release. In addition, Mg²⁺, another inhibitor of the PT pore, was also able to inhibit Noxa-induced cytochrome c release, indicating that Noxa in p53-dependent apoptosis is likely to cause efflux of mitochondrial proteins through a PT-related pore in a manner that is distinct from the tBid-induced pathway activated by death ligands.

Bak-oligomerization is a key event of tBid-induced cyto-

Fig. 5. Differential mitochondrial dysfunction between tBid and Noxa. A, supernatants were analyzed by Western blot analysis using anti-cytochrome c antibody. Isolated mitochondria were treated with tBid (0.1 μg ml⁻¹), Noxa (50 μg ml⁻¹), CaCl₂ (50 μM), or MgCl₂ (4 mM) in the presence or absence of CsA (50 μM). Cytochrome c in the supernatant was analyzed by Western blot analysis. B, isolated mitochondria from either mouse or rat livers were treated with either tBid (0.1 μg ml⁻¹) or Noxa (50 μg ml⁻¹) and then incubated for 1 h at 30 °C. After centrifugation, mitochondrial pellets were resuspended, and either the cross-linking agent bismaleimide hexane (10 μM) or Me₂SO as a control buffer was added. The supernatants were analyzed by Western blot analysis using anti-cytochrome c antibody. After further incubation with shaking for 30 min at room temperature, mitochondrial pellets were harvested by centrifugation and then analyzed by Western blotting using anti-Bak antibody. Equivalent protein loading was verified by Western blotting using anti-cytochrome c oxidase IV antibodies.
Me2SO has been used for Bak-oligomerization analyses. As rat liver mitochondria to determine whether Bak-oligomerization is not required for cytochrome c release in response to tBid because of either insufficient or absent Bak in their mitochondria (13). Therefore, we analyzed Bak-oligomerization using mouse and rat liver mitochondria to determine whether Bak-oligomerization is an important component of Noxa-induced cytochrome c release. The cross-linker bismaleimide hexane or (as a control) Me2SO has been used for Bak-oligomerization analyses. As expected, tBid induced significant Bak-oligomerization from mouse but not rat mitochondria. Noxa did not cause significant Bak-oligomerization in either case, although it was able to effect cytochrome c release (Fig. 5B). Furthermore, our results confirmed that rat liver mitochondria only release a small amount of cytochrome c in response to tBid (Fig. 5B). On the other hand, Noxa was able to release a comparable amount of cytochrome c from both rat and mouse mitochondria without Bak-oligomerization. These results reveal that Bak-oligomerization is not required for cytochrome c release in response to Noxa. However, we do not rule out the possibility that Bax may be involved in the Noxa-induced cytochrome c release because Bax is able to compensate for Bak-deficiency.

Because CsA and Mg2+ are blocking agents of the PT pore and inhibit Noxa-induced cytochrome c release (Fig. 5A), we assumed that Noxa is likely to activate the PT pore, resulting in mitochondrial swelling. We next measured mitochondrial swelling by light scatter after Noxa treatment; however, we were unable to detect any significant high amplitude swelling (data not shown). Recently, Scorrano et al. reported that tBid causes cristae-remodeling of the mitochondrial structure, resulting in the movement of cytochrome c stores (14). We hypothesized that Noxa might also cause ultra-structural changes in the mitochondria without high amplitude swelling, and this structural change might be distinct from that induced by tBid. To examine the hypothesis, morphological changes of mitochondria treated with tBid, Noxa, or calcium were observed using electron microscopy. As shown in Fig. 6A, freshly isolated mitochondria showed an electron-dense matrix of the isolated mitochondria, the typical “condensed state” described by Hackenbrock (15). When tBid was used to treat the mitochondria, the electron-dense matrix was reorganized to have mitochondrial features showing a “sausage-shaped” electron-dense matrix (Fig. 6B), as described by Scorrano et al. (14). Noxa-treated mitochondria, however, showed an expanded and dilute matrix, and small matrical granules with tubular-shaped cristae were widely dispersed (Fig. 6C). These morphological changes are characteristics of “swollen” mitochondria as designated by Hackenbrock. It is likely that these morphological changes take place without large amplitude mitochondrial swelling because no significant changes in mitochondrial size were observed. Thus, Noxa seems to cause changes in the mitochondrial ultra-structure characteristic of swollen mitochondria without inducing large-amplitude swelling.

**DISCUSSION**

Mitochondrial dysfunction in response to FasL or tumor necrosis factor-α seems to be mediated through tBid activated by caspase-8. This process requires either Bak- or Bax-oligomerization to release cytochrome c and Smac without the opening of PT pores (8, 9, 16). However, Bid may not be essential for mitochondrial dysfunction in stress-activated death stimuli such as γ-irradiation because Bid-deficient hepatocytes are able to lose membrane potential of mitochondria in response to these death stimuli (17). This indicates that there is an alternative pathway leading to mitochondrial dysfunction in stress-activated death stimuli. Noxa may be the candidate protein for induction of this alternative pathway because Noxa is transactivated by p53 in response to genotoxic agents, and Noxa causes mitochondrial dysfunction (7). Indeed, our present results show the differences of cytochrome c releases from mitochondria induced by tBid or Noxa. First, Noxa-induced cytochrome c release is not dependent upon Bak-oligomerization, whereas Bax- or Bak-oligomerization is requisite for tBid-induced cytochrome c release. Although our data indicate that Noxa-induced mitochondrial dysfunction is not associated with Bak-oligomerization, we do not rule out the possibility of Bax participation in this process. The fact that thymocytes isolated from Bax−/− and Bak−/− mice are resistant to etoposide-induced apoptosis suggests that Bax might be associated with Noxa-induced mitochondrial dysfunction. However, this remains to be answered. Second, PT-related components might, at least in part, participate in the Noxa-induced permeabilization of the mitochondrial outer membrane because the PT pore inhibitors CsA and Mg2+ are able to inhibit Noxa-induced cytochrome c release. This is further supported by a Noxa-induced swollen electron-dilate matrix, an indicator of the opening of the PT pore. This supports the notion that stress-activated p53 utilizes Noxa which leads to mitochondrial dysfunction in a distinct pathway through PT-related pore. Together, it suggests that there are two different pathways in the permeabilization of the mitochondrial outer membrane which are activated by different death stimuli. This notion might provide an explanation for enhanced apoptotic response of some cancer cells to combinatorial treatment of genotoxic agents and death ligand. Bax-deficient colorectal cancer cells, for instance, are resistant to TRAIL alone, mainly because of the lack of cytochrome c release; however, these cells are sensitive to TRAIL and the topoisomerase I inhibitor camptothecin. It is possible that Bax-deficient cells have the defective pathway of mitochondrial death signaling induced by tBid; however, the camptothecin might restore the mitochondrial death signaling through Noxa-mediated pathway.

Acknowledgments—We thank Dr. Wendy Mars at the University of Pittsburgh for critical reading of the manuscript and Korea Basic Science Institute Gwangju branch for technical assistance with the confocal microscope.

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