Bax-inhibiting peptide derived from mouse and rat Ku70

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Abstract

Bax is a proapoptotic protein that plays a key role in the induction of apoptosis. Ku70 has activities to repair DNA damage in the nucleus and to suppress apoptosis by inhibiting Bax in the cytosol. We previously designed peptides based on the amino acid sequence of Bax-binding domain of human Ku70, and showed that these peptides bind Bax and inhibit cell death in human cell lines. In the present report, we examined the biological activities of other pentapeptides, VPTLK and VPALR, derived from mouse and rat Ku70. Cells in culture accumulated FITC-labeled VPTLK and VPALR, indicating that these peptides are cell permeable (human, mouse, rat, and porcine cells were examined). These peptides bound to Bax and suppressed cell death in various cell types including primary cultured cells. These data suggest that such Bax inhibiting peptides from three mammalian species may be used to protect healthy cells from apoptotic injury under pathological conditions.

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Bax is a member of the Bcl-2 family of proteins that plays a key role in the induction of apoptosis [1,2]. In response to apoptotic stimuli, Bax translocates from the cytosol to mitochondria and causes release of apoptogenic factors [3–5]. Bax-mediated cell death is implicated as one of the major causes of pathology in damaged tissue, such as the neurodegenerative diseases including Alzheimer's disease [6], Parkinson's disease [7], and ischemia/reperfusion-induced organ damage [8]. Ku comprises two subunits of 76kDa (Ku70) and 86kDa (Ku86) (reviewed in [9]). Ku has DNA-end joining activity required for double-strand break repair; Ku also plays a key role as a DNA-binding unit of the DNA-dependent protein kinase holoenzyme (DNA-PK), a DNA damage sensor [9]. The DNA repair function of Ku70 explains its role in the nucleus, however, Ku70 has been localized also to the cytosol [10].

In previous studies, we found that Ku70 binds Bax in the cytosol and inhibits its translocation into mitochondria [11,12]. The Bax-binding domain of human Ku70 consists of residues 578–583 [12]. Pentapeptides based on this sequence are cell permeable and suppress
Bax-mediated cell death in human cancer cell lines (these peptides were termed Bax inhibiting peptides or BIPs) [12]. Here, we report that peptide design based on Ku70 encoded by both mouse and rat genomes bind Bax and are effective in protecting cells from apoptosis. BIPs have the potential to protect normal cells from Bax cytotoxicity, and therefore may constitute an effective strategy for the reduction of organ damage during degenerative diseases. Hence, the development of improved versions of BIPs may provide useful information for the design of new cytoprotective therapeutics and thus may be of potential clinical interest.

We also previously demonstrated that BIPs effectively suppress apoptosis induced by cytotoxic drugs such as staurosporin (STS) and anti-cancer drugs [12]. However, cell death induced by toxic reagents is considered non-physiological or “artificial.” The deprivation of trophic factors triggers apoptosis as naturally occurring programmed cell death during the development and pathogenesis of certain degenerative diseases [13–15]. Bax reportedly plays a role in trophic factor (growth factors or hormones) deprivation-induced cell death [13,16], suggesting that the BIPs can control this type of cell death. In the present study, we show that BIPs effectively suppress physiologically relevant cell death induced by trophic factor deprivation.

Materials and methods

Peptide synthesis. Peptides were synthesized using standard 9-fluorenylethoxycarbonyl (FMOOC) protocols on an ABI 433 instrument (Applied Biosystems). Amino acids were activated using 2-(1H-benzotriazol-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in dimethylformamide (DMF) and deblocked using 25% piperidine in N-methylpyrrolidone (NMP). The resin was rinsed with dichloromethane (DCM) and lyophilized overnight. Peptides were cleaved from the resin using 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), and 2.5% H2O. The precipitate was washed three times in ethyl ether, dissolved in 5% acetic acid, and lyophilized. Peptides were purified by reverse-phase HPLC using a Vydac 218TP1022 column (1% TFA in acetonitrile) on a Beckman HPLC system. Peptide mass was verified using matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF), performed at the Protein/Nucleic Acid Core Facility at the Medical College of Wisconsin (Milwaukee, WI).

Peptide preparation. Dried peptide powders were stored at –30°C to –35°C. The peptides were dissolved in fresh dimethyl sulfoxide (DMSO; Sigma, D2650) at 200 mM in plastic tubes (Fisher; 05-406-16), and 5 μl of each solution was dispensed to individual 0.5-ml plastic tubes (Coaster; 3209). These 5-μl aliquots were used as stocks. All tubes were stored at –30°C, and each tube was used only one time to minimize freeze–thaw degradation.

Analysis of peptide binding by Bax. Co-precipitation was performed as previously described [12] with a slight modification. In brief, HEK293T cells (approximately 4 × 106 cells) were lysed in 2 ml CHAPS buffer (150 mM NaCl, 10 mM Hepes, pH 7.4, and 1.0% CHAPS) containing protease inhibitors (Protease Inhibitor Cocktail, Sigma P8340, diluted 1:100) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were prepared by collecting the supernatant after centrifugation (14,000 rpm) at 4°C for 30 min. The protein concentration of each lysate was adjusted to 7.5 mg/ml by dilution with CHAPS buffer. After preclearing 200 μl of the samples with 20 μl of streptavidin beads (Amersham-Pharmaica Biotech) at 4°C for 1 h, the samples were incubated at 4°C for 2 h with 200 μM of various biotin-labeled peptides (biotin-KLPVM, -IPMIK, -VPMLK, -VPTLK, or -VPALR). Streptavidin beads (20 μl) were then added to the samples and the mixtures were incubated at 4°C for 2 h, after which the beads were washed three times with 100 μl CHAPS buffer (beads were recovered and rinsed with 100 μl of 4°C for 2 h). The beads were boiled in 40 μl Laemmli buffer and 20 μl of the eluted proteins was added to 7% SDS-PAGE gels. After SDS-PAGE separation, the bands were visualized by Coomassie Blue staining. The peptide concentration in each solution was adjusted to 7.5 mg/ml by dilution with CHAPS buffer.
LaserSharp Processing software. Each point in the figures represents the mean ± SEM of two (VPTLK and VPALR) or three (VPMLK) independent experiments performed on different days. To determine the membrane permeability of the peptides, cumulus cells were incubated with FITC-labeled peptides (100 µM for mouse and rat cells; 200 µM for porcine cells). The photograph shown in Fig. 4 was taken after the cells were incubated for 24 h in medium containing the peptides.

Results

Pentapeptides derived from mouse and rat Ku70 bind Bax and suppress etoposide-induced cell death in human Hep3B cancer cells

We previously localized the Bax-binding domain of human Ku70 to the second α-helix from the C terminus [12]. The synthetic pentapeptide (VPMLK) based on the human Ku70 Bax-binding domain is cell permeable and has anti-apoptotic activity in cultured cells [12]. Since Ku70 suppresses Bax-mediated apoptosis in mouse cells [11], we were interested in knowing whether synthetic peptides based on rodent Ku70 would show similar activities. Hence, we synthesized mouse and rat Ku70 peptides (VPTLK and VPALR, respectively) based on an alignment with the sequence of the human Ku70 Bax-binding domain (Fig. 1). To test the Bax-binding activity of these peptides, biotin-labeled peptides were added to cell lysates prepared from the human kidney epithelial cell line HEK293T, and the peptides were precipitated by streptavidin beads as previously reported [12]. As shown in Fig. 2, Bax was pulled down by Ku70 peptides but not by negative control peptides, suggesting that Bax binds to the peptides derived from human, mouse, and rat Ku70.

We previously reported that the human Ku70-derived VPMLK (at 200 µM) effectively suppresses apoptosis in human cancer cell lines [12]. Based on this information, we tested new versions of Ku70 peptides at 200 µM in Hep3B cells (as human hepatoma cell line) (Fig. 3).

The human, rat, and mouse Ku70 peptides were almost equally effective in suppressing etoposide-induced cell death in Hep3B cells.

Ku70 peptides protect primary cultured cumulus cells from cell death induced by hormone deprivation

Cumulus cells serve as nurse cells for oocytes and undergo apoptosis in response to the deprivation of a trophic hormone (e.g., follicular stimulating hormone, FSH) [22–24]. Hence, cumulus cells undergo typical apoptosis when cultured in medium lacking FSH [23,25,26]. We tested whether the human, mouse, and rat Ku70 peptides prevent apoptosis in mouse, rat, and porcine cumulus cells cultured in the absence of FSH. Ku70 peptides were N-terminally labeled with FITC and then used to test cell permeability. FITC fluorescence was observed inside cumulus cells after culture in the presence of FITC-labeled peptides. Fig. 4 shows the confocal microscopic images of cumulus cells cultured for 24 h in the presence of FITC-labeled peptides. The
incorporation of FITC-labeled peptides was detected after incubation for 1.5h (data not shown). The mechanism by which these peptides enter cells is not known. The Ku70 peptides may enter the cells by endocytosis rather than by simple penetration of the plasma membrane, and therefore several hours may be required for peptides to accumulate inside cells. The human, mouse, and rat Ku70 peptides were almost equally effective in suppressing cell death induced by FSH deprivation in mouse and rat cumulus cell cultures (Figs. 5A and B). Interestingly, the human peptide, VPMLK, showed very strong protection of porcine cumulus cells compared with the mouse (VPTLK) and rat (VPALR) peptides (Fig. 5C).

Ku70 peptides suppress cell death induced by growth factor deprivation in a mouse myeloid cell line

We also tested the effects of Ku70 peptides in the IL-3-dependent myeloid cell line, 32D (EpoR wt) (Figs. 6 and 7). These cells undergo apoptosis within 24h in response to IL-3 deprivation [27]. The Ku70 peptides suppressed cell death induced by IL-3 deprivation in a dose-dependent manner (Fig. 7). For example, at 400 µM, the Ku70 peptides attenuated cell death by ~50% relative to the control. The human, mouse, and rat Ku70 peptides showed similar activity in suppressing cell death. FITC-labeled Ku70 peptides were detected in cells after 3h of incubation (data not shown). Fig. 6 shows the microscopic images of 32D (EpoR wt) cells incubated for 15h in the presence of FITC-labeled peptides.

Discussion

Three versions of Ku70 peptides derived from human (VPMLK), mouse (VPTLK), and rat (VPALR) were almost equally effective in binding Bax and suppressing cell death in human, mouse, and rat cells. Although the exact Ku70-binding domain in Bax has not been identified, these results suggest that the biochemical characteristic of the interaction is evolutionarily conserved in these species. Among these three peptides there is variability in the amino acid at the third (M, T, or A) and the fifth positions (K or R). On the other hand, the first (V), second (P), and fourth (L) positions are conserved, suggesting that these three residues are essential for Bax inhibition. This information on the residues that are critical for Bax binding will contribute to our understanding of the molecular mechanism by which Ku70 peptides inhibit Bax activation, and may prove useful for the development of potential cytoprotective therapeutics.

The Ku70 peptides suppressed cell death induced by trophic factor deprivation (e.g., myeloid cell (32D (EpoR wt)) death in the absence of IL-3 and primary cultured cumulus cell death in the absence of FSH).
It was recently reported that the human Ku70 peptide VPMLK as well as Ku70 rescues NGF-deprived primary cultured neurons from cell death [28]. It is well established that Bax plays a key role in trophic factor deprivation-induced cell death [13,14,16], and therefore the cytoprotective activities of the Ku70 peptides in this type of apoptosis are consistent with their ability to bind and inhibit Bax in vitro and in vivo. In some types of degenerative diseases, the trophic factor deprivation-induced cell death causes the dysfunctions of the tissues [13,15]. Since the Ku70 peptides are able to attenuate apoptosis induced by trophic factor deprivation, it is possible that these peptides and their mimetic may be utilized to protect cells from degenerative diseases.

Interestingly, VPMLK showed very strong activity in protecting porcine cumulus cells, whereas VPTLK and VPALR showed weak activity (Fig. 5C). On the other hand, all three peptides exhibited nearly equivalent activity with respect to mouse, rat, and human cells. The basis for the differential cytoprotective activity with porcine cells is not clear. However, part of the difference may be differential membrane permeability and/or stability inside these cells. It is also possible that VPMLK binds porcine Bax with higher affinity than do VPTLK and VPALR. We used only porcine cumulus cells in this study, and thus it is not clear whether VPMLK has more activity than VPTLK and VPALR in other porcine cell types. The studies with other cell types from porcine tissue will be necessary to address this issue.

All three BIPs that we tested are cell permeable. At present, the mechanism by which these peptides penetrate the plasma membrane is not clear. The cell permeability of the Ku70 peptides may not be dependent on the simple diffusion of a small molecule from the medium to the intracellular space. The fact that the accumulation of the FITC-labeled peptides takes a relatively long time (>1h) suggests that the process may involve a specific membrane trafficking pathway. FITC-labeled peptides were taken up by adherent cell types more efficiently than by non-adherent cell types [29]. Given that the rate of endocytosis is higher in adherent cell types than in non-adherent cell types [29], it is possible that the Ku70 peptides may enter cells via the endocytic machinery or an analog system. In all three Ku70 pentapeptides, the first four residues have non-polar or uncharged polar side chains whereas the fifth residue has a charged polar side chain. This type of peptide sequence may play an important role in interacting with certain components of the membrane trafficking system. In addition to their ability to protect cells, the Ku70 peptides may be used as tools to deliver other molecules into cells, as described for the HIV-tat signal and the antennapedia protein (reviewed in [30]). We are currently investigating the possible use of BIPs for this purpose.

In summary, the present study demonstrates that VPTLK and VPALR, derived from mouse and rat
Ku70, respectively, bind Bax and are able to suppress apoptosis. Importantly, these two peptides are also cell permeable. For these peptides to find clinical application in protecting against cell damage during degenerative diseases, further improvements in the peptide sequence or chemistry will be needed (e.g., to avoid immune reaction and to control the half-life of the peptides in vivo). The ability of various peptide sequences to bind Bax and cross the membrane will provide useful information for the development of more effective peptides or mimetics for practical uses.

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