Nix and Nip3 Form a Subfamily of Pro-apoptotic Mitochondrial Proteins*

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We have identified Nix, a homolog of the E1B 19K/Bcl-2 binding and pro-apoptotic protein Nip3. Human and murine Nix have a 56 and 53% amino acid identity to human and murine Nip3, respectively. The carboxyl terminus of Nix, including a transmembrane domain, is highly homologous to Nip3 but it bears a longer and distinct asparagine/proline-rich N terminus. Human Nix maps to chromosome 14q11.2–q12, whereas Nix/BNip3L was found on 8q21. Nix encodes a 23.8-kDa protein but it is expressed as a 48-kDa protein, suggesting that it homodimerizes similarly to Nip3. Following transfection, Nix protein undergoes progressive proteolysis to an 11-kDa C-terminal fragment, which is blocked by the proteasome inhibitor lactacystin. Nix colocalizes with the mitochondrial matrix protein HSP60, and removal of the putative transmembrane domain (TM) results in general cytoplasmic and nuclear expression. When transiently expressed, Nix and Nip3 but not TM deletion mutants rapidly activate apoptosis. Nix can overcome the suppressors Bcl-2 and Bcl-XL, although high levels of Bcl-XL expression will inhibit apoptosis. We propose that Nix and Nip3 form a new subfamily of pro-apoptotic mitochondrial proteins.

Apoptosis is regulated by molecules that both activate and suppress cell death. The molecular regulators of developmental apoptosis in Caenorhabditis elegans include cell death inducers Ced-3 and -4 (1) which are conserved in mammals (2, 3). Ced-9 is the prototypical cell death suppressor protein, and Bcl-2 and Bcl-XL are the mammalian homologs (4). However, the Bcl-2 family of proteins also contains members that function to activate apoptosis such as Bax, Bad, Bak, Bik/Nbk, and Hrk (5–8). The molecular regulators of developmental apoptosis include cell death inducers such as Fas, TNF, and death receptors for TNF-related apoptosis-inducing ligand (9). In contrast to these proteins, Bcl-2 and Bcl-XL are the mammalian homologs (4). However, the Bcl-2 family of proteins also contains members that function to activate apoptosis such as Bax, Bad, Bak, Bik/Nbk, and Hrk (5–8).

We recently identified a new cell death-inducing protein named Nip3 (9) (also called BNip3 (10)), which was initially reported to physically interact with both adenovirus E1B 19K and Bcl-2 (11). Nip3 is a mitochondrial protein that induces apoptosis and can enhance the apoptosis induced by other cell death signals when transiently expressed (9, 10). Bcl-2 transiently suppresses Nip3-induced apoptosis although this resistance is rapidly overcome (9, 10). We report the cloning of Nix (Nip3-like protein X), which closely resembles Nip3 in both structure and function. We propose that Nix and Nip3 form a new structurally related pro-apoptotic family of mitochondrial proteins.

MATERIALS AND METHODS

Cloning of Murine Nix (mNix), Human Nix (hNix), and Murine Nip3 (mNip3)—Searches of the GenBank EST§ and TIGR (Institute for Genomic Research) data bank using the human Nip3 cDNA revealed related but novel human and mouse EST clones. Using primers within these EST sequences, we generated PCR fragments, which were cloned into pCRIT vector (Invitrogen, San Diego, CA) and sequenced. The entire mNix and hNix cDNA were then polymerase chain reaction amplified from mouse 17-day embryo Matchmaker cDNA library and human fetal liver 5’-stretch plus cDNA library (CLONTECH, Palo Alto, CA). Murine Nip3 cDNA was cloned from a λGT11 3T3 fibroblast cDNA library using human Nip3 as a probe. Kpn1 and EcoRI sites were introduced into the full-length cDNA by PCR and then subcloned into expression vector pcDNA3-T7, a modified pcDNA3 vector containing a C-terminal T7-tag (9). Transmembrane domain-deleted expression vector mNix (ΔTM) was generated by PCR using splice overlap extension (12). Human Nip3 and the Nip3Δ53 transmembrane deletion mutant have been described previously (9).

Reagents and Cell Lines—Rat-1, Rat-1/Bcl-2, TC (10T1/2 parental cell line), TS X and TSX2 (Bcl-XL transfected clones), and MCF-7 cells were cultured in α-minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Murine monoclonal anti-T7 antibody was purchased from Novagen (Madison, WI). Rabbit anti-HSP60 antibody was donated by Dr. Radhey Gupta (McMaster University). FITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) and Cy5-conjugated goat anti-mouse IgG (BioCan Ltd., Mississauga) were used for fluorescence studies. Lactacystin was purchased from Calbiochem-Novabiochem (Cambridge, MA).

Northern Analysis—Total RNA (30 µg) was fractionated on a formaldehyde denaturing gel and then transferred and hybridized with nick-translated human or mouse Nix cDNA corresponding to the coding region as described (9).

In Vitro Transcription—[35S]-labeled Nix proteins were prepared by in vitro transcription and translation of cDNA cloned in pcDNA3 vector using TnT coupled reticulocyte lysate system (Promega, Madison, WI).

Western Blotting—Aliquots of 5 × 10⁶ cells transfected with Nix-T7 were separated by an SDS-PAGE method using Tris-Tricine buffer and polyvinylidene difluoride membrane that is suitable for detecting small molecular weight peptides (13). Western blots were probed with mouse monoclonal anti-T7 antibody using an ECL detection kit (Amersham Pharmacia Biotech, UK).

Transient Transfection and Apoptosis Assays—Cells were transfected with Nix using LipofectAMINE Reagent (Life Technologies, Inc.). Aliquots of 5 × 10⁶ cells were Western blotted as described above. For apoptosis experiments, Nix-expressing cells were detected with mouse anti-T7 antibody and FITC-conjugated rabbit anti-mouse IgG antibody. Apoptotic cells, exhibiting altered DNA morphology following Hoechst dye staining, were enumerated manually counting as described previously (9). Fluorescence was visualized using a Zeiss Axiopt microscope equipped with a cooled CCD camera.

For β-galactosidase assay assays evaluating the effect of removing the transmembrane domain on Nix and Nip3 apoptosis, 293T cells were cotransfected with 0.2 µg of pcDNA3-β-galactosidase plus 0.75 µg of the expression plasmid in triplicate using LipofectAMINE. Cells were fixed, stained, and evaluated 21 h later as described previously (14).

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§ The abbreviations used are: EST, expressed sequence tag; PCR, polymerase chain reaction; TM, transmembrane; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bp, base pair(s).

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RESULTS AND DISCUSSION

The cloning of hNix, mNix, and mNip3 was as follows. Using the human Nip3 cDNA as a probe, we screened a GT11 mouse fibroblast cDNA library for clones related to Nip3. The clone containing the longest insert of 1311 bp was found to contain the entire mouse Nip3 coding region of 187 amino acids (GenBank accession number AF041054). The cDNA had overall identity with human Nip3 of 90.2% (Fig. 1).

Human and murine ESTs related to but different from Nip3 were used for PCR to clone, as described under “Materials and Methods,” a human 1337-bp and murine 1351-bp cDNA containing a large open reading frame of 219 and 218 amino acids, respectively. The protein was named Nix. The predicted human Nix (hNix) (GenBank accession number AF067396) and mouse Nix (mNix) (AF067395) proteins share 56 and 53% identity, respectively, with Nip3 of the same species, and are 97% identical to each other (Fig. 1). The highest regions of homology between the four proteins resides in the C terminus. Nix proteins bear a single hydrophobic α-helical sequence at the extreme carboxyl terminus, corresponding to the location of the TM domain of Nip3 (2) (Fig. 1). Nix has a significantly longer N terminus that contains an unusual five asparagines in sequence and is proline-rich. N-terminal amino acids 1–59 of hNix and 1–32 of hNip3 are the most divergent. Another region from amino acids 97–120 of hNix is also distinct from hNip3, whereas the remainder is over 85% homologous with large blocks of identical sequences in both species.

Similar to Nip3 (9, 11), Nix contains PEST sequences comprised of high frequency stretches of Pro, Glu, Ser, Thr, and Asp, flanked by charged amino acids His, Arg, or Lys. PEST sequences are associated with proteins having high turnover rates (15) whose degradation is controlled by the proteasome. hNix and mNix are expressed as 4.5- and 1.4-kilobase mRNA transcripts, respectively (not shown). The smaller transcript corresponds to the size of the cDNAs reported above and is likely the complete sequence. The larger transcript has not been identified; however, some cDNA clones were found that bear a distinct 5'-UTR suggesting alternate splicing of the Nix mRNA, and may represent a larger transcript.

Following in vitro transcription and translation and Western blotting (Fig. 2A), mNix and hNix run as proteins of 48,000 relative molecular weight under reducing and non-reducing conditions using the Tris-Tricine SDS-PAGE method (13). hNip3 has a 40,000 relative molecular weight in Tris-Tricine-buffered SDS-PAGE (Fig. 2A). In contrast, using the Laemmli
method, Nix runs as an 80-kDa protein (not shown) and Nip3 at 60 kDa (9). The reason for the difference in mobility in the two buffers is not clear; however Nip3, which forms a homodimer that does not dissociate under reducing conditions (9), runs at its predicted molecular weight of 40,000 in the Tris-Tricine buffer. Nix runs as an 80-kDa protein (not shown) and Nip3 at 60 kDa (9). The reason for the difference in mobility in the two buffers is not clear; however Nip3, which forms a homodimer that does not dissociate under reducing conditions (9), runs at its predicted molecular weight of 40,000 in the Tris-Tricine buffer. Nix runs as an 80-kDa protein (not shown) and Nip3 at 60 kDa (9). The reason for the difference in mobility in the two buffers is not clear; however Nip3, which forms a homodimer that does not dissociate under reducing conditions (9), runs at its predicted molecular weight of 40,000 in the Tris-Tricine buffer. Nix runs as an 80-kDa protein (not shown) and Nip3 at 60 kDa (9). The reason for the difference in mobility in the two buffers is not clear; however Nip3, which forms a homodimer that does not dissociate under reducing conditions (9), runs at its predicted molecular weight of 40,000 in the Tris-Tricine buffer. Nix runs as an 80-kDa protein (not shown) and Nip3 at 60 kDa (9). The reason for the difference in mobility in the two buffers is not clear; however Nip3, which forms a homodimer that does not dissociate under reducing conditions (9), runs at its predicted molecular weight of 40,000 in the Tris-Tricine buffer. Nix runs as an 80-kDa protein (not shown) and Nip3 at 60 kDa (9). The reason for the difference in mobility in the two buffers is not clear; however Nip3, which forms a homodimer that does not dissociate under reducing conditions (9), runs at its predicted molecular weight of 40,000 in the Tris-Tricine buffer. Nix runs as an 80-kDa protein (not shown) and Nip3 at 60 kDa (9). The reason for the difference in mobility in the two buffers is not clear; however Nip3, which forms a homodimer that does not dissociate under reducing conditions (9), runs at its predicted molecular weight of 40,000 in the Tris-Tricine buffer. Nix runs as an 80-kDa protein (not shown) and Nip3 at 60 kDa (9). The reason for the difference in mobility in the two buffers is not clear; however Nip3, which forms a homodimer that does not dissociate under reducing conditions (9), runs at its predicted molecular weight of 40,000 in the Tris-Tricine buffer. Nix runs as an 80-kDa protein (not shown) and Nip3 at 60 kDa (9). The reason for the difference in mobility in the two buffers is not clear; however Nip3, which forms a homodimer that does not dissociate under reducing conditions (9), runs at its predicted molecular weight of 40,000 in the Tris-Tricine buffer.
At decreasing doses of Nix plasmid from 1.0 to 0.1 μg, the transfection efficiency decreased progressively but the apoptosis rate in transfected cells remained constant between the two cell lines (not shown). Examining Nix apoptosis in Bcl-XL expressing cell lines TX22 and TX5, we find that only the higher Bcl-XL expressing cell line TX5 was effective at blocking Nix although both lines were resistant to staurosporine-induced apoptosis when compared with the parental line TC (Fig. 5, B and C).

The suppressive action of Bcl-2 can be reversed by heterodimerization with pro-apoptotic members of the Bcl-2 family through the BH3 (Bcl-2 homology-3) domain (8, 17, 18). Nip3 protein appears to have a region that bears some structural similarity to the BH3 domain of the Bcl-2 family (9, 10), which may partially function to promote Nip3 cell death (10). However, it is also clear that Nip3 and Nix are more structurally related to each other than to pro-apoptotic members of the Bcl-2 family.

A recent report identified a human cDNA called BNip3L that is homologous to hNip3 and maps to chromosome 8p21 (19). The protein encoded by the BNip3L cDNA is identical to the human Nix. For comparison, we have now mapped the chromosomal location of human Nip3 and, using a radiation hybrid panel, we find it is on chromosome 14; 4.08 centi-Ray from the marker WI-6506. Using the Whitehead Institute/MIT YAC map of chromosome 14, the CEPH YAC 951d6 was positive, placing the gene slightly centromeric to WI-6506 at the band 14q11.2-q12. The distinct mapping location of the two genes warrants a unique name for Nix rather than BNip3L because Nix is not a long form of Nip3.

Expression of BNip3L in cervical cancer cell lines reduced their colony-forming ability and suggested to the authors that the protein may have growth-suppressive function. Because we find that both hNix and mNix are pro-apoptotic when overexpressed in tumor cell lines, induction of apoptosis can account for the observed growth suppression and poor colony formation in the BNip3L-transfected carcinoma cells.

In conclusion, we have described Nix, a homolog of the Nip3 protein, which shares the ability to induce apoptosis, localize to mitochondria, and undergo proteasome-dependent degradation when overexpressed. Nip3 and Nix form a new subfamily of mitochondrial cell death proteins.

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