**Requiem: A Novel Zinc Finger Gene Essential for Apoptosis in Myeloid Cells**

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To identify genes mediating programmed cell death triggered by interleukin 3 (IL-3)-deprivation of myeloid cells, the IL-3-dependent murine myeloid cell line FDCP-1 was cloned that prevented the programmed cell death response following IL-3 deprivation by causing antisense suppression of an endogenous 2.4-kilobase (kb) mRNA. A 2.3-kb cDNA containing the identical 892-base pair overlapping sequence was cloned that encoded a deduced 371-amino acid protein containing a single Kruppel-type zinc finger and a cluster of 4 cysteine/histidine-rich repeats resembling atypical zinc fingers. The 2.4-kb mRNA was found to be ubiquitously expressed in murine tissues and its abundance in FDCP-1 cells was not altered in response to IL-3 deprivation. Since expression of this 2.4-kb mRNA was a prerequisite for the apoptosis response following IL-3 deprivation, the gene encoding it was named requiem. Requiem is likely to encode a transcription factor required for the apoptosis response following survival factor withdrawal from myeloid cells.

Hematopoietic stem and progenitor cell proliferation, lineage commitment, differentiation, and programmed cell death are regulated by hematopoietic growth factors and cytokines (1, 2). Hematopoietic progenitor cells committed to the myeloid lineage express high affinity receptors for GM-CSF and IL-3, proliferate in response to receptor occupancy (3), and acquire a distinct survival-dependence for GM-CSF or IL-3 which is manifest by a programmed cell death response triggered within hours of GM-CSF or IL-3 deprivation (4). The gain in regulatory sensitivity conferred by this dual response system would be expected to result in a tighter linkage between cytokine availability and progenitor cell abundance in vivo.

Certain genes involved in the effector arm of the apoptosis response pathway are conserved in multicellular organisms as exemplified by the ced-3 gene of Caenorhabditis elegans and its mammalian homologue cysteine protease, interleukin-1β-converting enzyme (5, 6). Likewise conserved are antagonists of the apoptosis response pathway: ced-9 of C. elegans and its mammalian functional homologue bcl-2 (7, 8). Less is known about the molecular control of initiation in comparison to downstream effectors or inhibitors of apoptosis (reviewed in Ref. 9). In some cellular systems, including IL-3-dependent myeloid cells, the apoptosis response requires new mRNA and protein synthesis (4, 9). The latter finding implies the presence of preformed transcriptional regulators that trigger the apoptosis response in these cell types as a result of post-translational modification.

Murine myeloid cell lines that proliferate in response to IL-3 and undergo rapid programmed cell death within 1–2 days of IL-3 deprivation are suitable for adaptation to an expression cloning system for candidate genes that antagonize apoptosis. For this purpose, the murine IL-3-dependent cell line FDCP-1 was chosen because its apoptosis response has been well characterized (4), and it has been used as a mammalian host cell line to demonstrate the utility of stable retroviral cDNA expression libraries for cloning of the cDNAs for IL-3 and GM-CSF (10).

**EXPERIMENTAL PROCEDURES**

**General Molecular Techniques**—Unless otherwise specified, standard molecular biology protocols were performed according to Sambrook et al. (11).

**Selection of an Enriched Plasmid Pool from a Murine Spleen Cell cDNA Library**—The murine myeloid IL-3-dependent cell line FDCP-1 was used to generate a polyoma virus T antigen-expressing G418-resistant stable subline, FDCP-1T (kindly provided by Y. C. Yang, Indiana University, Indianapolis, IN), that remained absolutely dependent on...
IL-3 for proliferation and survival.\(^2\) FDCP-1T cells were passaged twice weekly at 10\(^5\) cells/ml in RPMI containing 1.5 mg/ml G418, 10% fetal bovine serum (FBS) immediately prior to electroporation. Following electroporation the cells were allowed to recover for 2 h then diluted to 2 \( \times 10^5\) cells/ml in the same medium and subsequently maintained in IL-3-free medium for 10 days. The episomal plasmid DNA from FDCP-1T cells surviving at day 10 after transfection (approximately 5 \( \times 10^5\)) was recovered from the cells surviving at day 10 (12). This plasmid was excised with EcoRI then cloned into the EcoRI site of the selectable mammalian expression vector pSRneo (14). DNA sequence analysis (15) was used to confirm the selection of a pSRneo-clone 5 plasmid containing the insert in the same direction as in the original pcDNAI-clone 5. The empty pSRneo vector and pSRneo-clone 7 (a clone from the original pool that was shown to have no effect on IL-3-dependent growth) were used as negative controls. FDCP-1T cells were transfected by electroporation then selected by growth in G418-containing media for 2 weeks in the presence of optimal concentrations of IL-3. The G418-selected cells were washed and placed in fresh media at an initial cell density of 2 \( \times 10^5\)/ml and viable cell number, as reflected by trypan blue exclusion, was determined daily. Parent FDCP-1T cells grown in optimal concentration of IL-3 (filled circles), pSRneo-clone 5 transfected cells grown in the absence of IL-3 (filled triangles), pSRneo control FDCP-1T cells grown in the absence of IL-3 (open triangles), and pSRneo-clone 7 control FDCP-1T cells grown in the absence of IL-3 (open squares) are shown.

**RESULTS AND DISCUSSION**

For the present studies a random- and oligo(dT)-primed non-directional cDNA library from mouse spleen in the mammalian expression vector pcDNAI was used as a source of cDNAs that might confer an apoptosis-resistant phenotype to IL-3-deprived murine myeloid cells. In mammalian host cells that express polyoma virus T antigen, the pcDNAI plasmid is carried episomally in moderate copy number for 7–10 days following transfection. Therefore, the parent FDCP-1T cell line was used to generate a stable T antigen-expressing host cell line, FDCP-1T, that remained absolutely dependent on the presence of IL-3 for proliferation and survival.

The pcDNAI library was transfected into FDCP-1T by electroporation in IL-3-deficient medium and a plasmid pool was recovered from the cells surviving at day 10 (12). This plasmid pool was transformed into *E. coli* and 12 cDNA clones were

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\(^2\) Y. C. Yang, personal communication.
purified and individually retransfected into FDCP-1T cells. One of these 12 cDNA clones, clone 5, was capable of promoting the survival and proliferation of FDCP-1T on repeated retransfection whereas the other 11 cDNA clones or the empty pcDNAI-clone vector had no effect. The sequence of the 892-bp clone 5 cDNA insert was determined (13) and was found to have no significant homology to entries in the GenBank database (4–23–94). The longest open reading frame in the sense direction of clone 5 plasmid was only 162 base pairs and the peptide encoded by this open reading frame had no sequence homology within GenBank.

Because of the consistent apoptosis-resistant phenotype conferred by clone 5 in FDCP-1T cells, Northern blots from FDCP-1 cells were probed with riboprobes that would hybridize to the sense or antisense strand of the clone 5 plasmid in order to determine the size and orientation of the putative endogenous mRNA. These revealed the presence of a 2.4-kb endogenous mRNA in reverse orientation to clone 5 plasmid. This mRNA species was present in approximately equal abundance before or after IL-3 deprivation (Fig. 1). Expression of the clone 5 plasmid in FDCP-1T cells resulted in suppression of the endogenous 2.4-kb mRNA (Fig. 2), suggesting that antisense suppression was responsible for the apoptosis-resistant phenotype. Suppression of the endogenous 2.4-kb mRNA in FDCP-1T cells transfected with pCDNAI-clone 5 was also documented by probing the Northern blot with a cDNA probe that was distinct from and nonoverlapping with clone 5 (prepared from the 5' end of the full-length cDNA, see below) (Fig. 2).

![Fig. 4. Nucleotide sequence of requiem cDNA.](image)

The complete nucleotide sequence of the 2320-bp requiem cDNA was determined for both strands (13) (GenBank accession number U10435) and is shown with its encoded protein below in single letter code. The consensus nuclear localization signal (16) is in bold letters, the Kruppel-type zinc finger (17) is continuously underlined, and the cysteines and histidines that may be involved in metal binding as putative atypical zinc fingers are in bold and underlined.

**Characteristics of parent FDCP-1 cells expressing clone 5 cDNA** but never placed under the selection pressure of IL-3 deprivation, the insert was cloned into the selectable mammalian expression vector pSRneo (14) in the same orientation to pCDNAI-clone 5. Parent FDCP-1 cells were transfected with pSRneo-clone 5, then selected in G418 for 14 days in the continuous presence of IL-3. The resultant G418-resistant FDCP-1 cell population that had never been under the selection pressure of IL-3 was immediately able to proliferate in the absence of IL-3 with a doubling time that was approximately half the rate of the parent FDCP-1 cells in optimal IL-3 (Fig. 3). Similar results were obtained on three separate pSRneo-clone 5 transfections and the resultant stable neo-resistant clone 5-expressing FDCP-1 cells have been continuously passaged in the absence of IL-3 for up to 6 weeks. Control stable transfectants with empty pSRneo or pSRneo-clone 7, one of the cDNAs from the original pool that had no effect when retransfected into FDCP-1T as pcDNAI-clone 7, remained completely dependent on IL-3 for survival (Fig. 3A).

When stable clone 5-expressing FDCP-1 cells were compared with parent FDCP-1 cells for cell death following removal of exogenous IL-3, marked differences were observed (Fig. 3B). The ability of clone 5 DNA to confer IL-3-independent survival was not due to secretion of IL-3 or other growth factors into the medium. Conditioned medium from FDCP-1 cells surviving in the absence of exogenous IL-3 at day 9 after transfection with pCDNAI-clone 5 plasmid was prepared and concentrated 10-fold as in the preparation of WEHI-conditioned medium used as a source of IL-3. The IL-3-containing WEHI-conditioned medium contained a readily detectable band of IL-3 on Western blot analysis, whereas a 16-fold higher amount
sequences from the Kruppel-type and four atypical zinc fingers (F1, F2, F3, F4) of Neuro-d4 (15) and Requiem are aligned. Cysteines and histidines that serve as putative metal binding residues are shown in bold. Identical amino acids are underlined.

Fig. 5. A, physical map of requiem cDNA and encoded protein. The 2320-bp requiem cDNA is shown in relationship to the original 892-bp clone 5 cDNA. The 1116-bp open reading frame (open bar) encodes a 371-amino acid protein (Req) with open boxes indicating a consensus nuclear localization signal (NLS) (16), a glutamic and aspartic acid-rich domain (acidic), and four atypical zinc fingers labeled F1-F4. B, amino acid homology of Neuro-d4 and Requiem zinc fingers. The amino acid sequences from the Kruppel-type and four atypical zinc fingers (F1, F2, F3, F4) of Neuro-d4 (15) and Requiem are aligned. Cysteines and histidines that serve as putative metal binding residues are shown in bold. Identical amino acids are underlined.

Fig. 6. Mouse multiple tissue Northern blot hybridized with requiem. Oligo(dT)-purified RNA from the indicated mouse tissue was loaded at 2 μg/tube, electrophoretically separated, then transferred to Nytran (Clontech Laboratories Inc., Palo Alto CA). The Northern blot was hybridized with the 1428–2320-bp oligonucleotide fragment of requiem according to the manufacturer’s protocols using Express Hyb hybridization solution (Clontech Laboratories).

The cDNA insert from pcDNAI-clone 5 was used to clone a 2.3-kb cDNA from a mouse spleen phage λ cDNA library that had an identical overlapping 892-bp sequence. The nucleotide sequence of this 2320-bp cDNA (Fig. 4) was 63% identical to neuro-d4 (EMBL accession no. X66022), a neurospecific developmentally regulated rat gene encoding a protein containing a single Kruppel-type zinc finger and a cluster of new zinc finger motifs (15). Because of its putative role in mediating cell death, it would suggest that the function of the related neuro-d4 gene in developmental neural apoptosis should be tested.

Reg mRNA abundance following IL-3 deprivation. FDCP-1 cells growing in an optimal concentration of IL-3 were harvested 48 h after a fresh media change. After an initial aliquot was removed (time 0), the remaining cells were washed in RPMI supplemented with 10% fetal bovine serum in the absence of IL-3 then incubated in the same IL-3-deficient media for the indicated times. Total cellular RNA was prepared from each aliquot of cells then 20 μg/lane was electrophoretically separated on a 1% agarose gel and transferred to nitrocellulose as described in the legend to Fig. 1. The Northern blot was hybridized with a probe prepared as described in the legend to Fig. 2 from the 1428–2320-bp fragment of requiem (req) cDNA. To control for RNA loading the blot was also hybridized with an oligonucleotide probe from the constitutively expressed mouse gene cho-B.
Genetic Regulation of Apoptosis in IL-3-dependent Cells

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REFERENCES