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Functional Implications in Apoptosis by Interferon Inducible Gene Product 1-8D, the Binding Protein to Adenovirus Preterminal Protein

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Adenovirus (Ad) precursor to the terminal protein (pTP) plays an essential roles in the viral DNA replication. Ad pTP serves as a primer for the synthesis of a new DNA strand during the initiation step of replication. In addition, Ad pTP forms organized spherical replication foci on the nuclear matrix (NM) and anchors the viral genome to the NM. Here we identified the interferon inducible gene product 1-8D (Inid) as a pTP binding protein by using a two-hybrid screen of a HeLa cDNA library. Of the clones obtained in this assay, nine were identical to the Inid, a 13-kDa polypeptide that shares homology with genes 1-8U and Leu-13/9-27, most of which have little known functions. The entire open reading frame (ORF) of Inid was cloned into the tetracycline inducible expression vector in order to determine the biological functions related with adenoviral infection. When Inid was introduced to the cells along with adenoviruses, fifty to sixty percent of Ad-infected cells expressing Inid had rounded morphology, which was suggestive of apoptosis. Results from the terminal deoxynucleotidyl transferase (TdT) and DNA fragmentation assays confirmed that Inid induces apoptosis in Ad-infected or in uninfected cells. The Inid binding to pTP may target the cell for apoptotic destruction as a host defense mechanism against the viral infection.

Key words: adenovirus preterminal protein, interferon-inducible gene, apoptosis

Adenoviruses (Ads) cause acute, persistent infections. Like other complex viruses including the herpesvirus and immunodeficiency virus, Ads encode numerous proteins that subdue the host defense mechanism. Among them E1A, E1B/19K, and E1B/55K are well known to either induce or inhibit cellular apoptotic pathways (Burgert *et al.*, 2002). The other early proteins, however, are not well characterized in relation to the host defense machinery.

Adenovirus preterminal protein (Ad pTP) is an immediate early protein encoded by the viral E2B region along with adenovirus DNA polymerase (Ad Pol). pTP is a key participant in viral replication, in which it forms a multiprotein complex with Ad Pol and other cellular factors and binds to the Ad DNA origin. The initiation of replication involves the covalent addition of a dCMP residue to the β-hydroxyl of serine 580 of pTP (for reviews, see Hay *et al.*, 1995; Van der Vliet, 1995). It has become abundantly clear that the Ad replication complex is tightly bound to the nuclear matrix (NM) through interaction with the pTP (Schaack *et al*, 1990; Angeletti and Engler, 1996). The NM is a proteinacious network to which replication, transcription, and mRNA splicing machinery are bound in organized domains (Xing *et al.*, 1993, Pombo *et al*, 1994). It has been suggested that pTP may have a function separate from DNA replication dependent on its binding to the NM.

The pTP binding proteins, however, involved in functions other than DNA replication, have remained unclear. Therefore, we searched cellular proteins that are involved in binding or searched ones that affect the pTP function using the yeast two hybrid system. We presented data that interferon inducible gene product 1-8D binds to Ad pTP and induces apoptosis.

Materials and Methods

Cell lines, viruses, and antibodies

HeLa Tet-on cells were obtained from Clontech (USA) and maintained in Dulbecco's modified Eagle's medium, which was supplemented with 10% fetal bovine serum and 500 μg/ml G418 at 37°C. HeLa CCL2 cells obtained from American Type Tissue Culture Collection (USA) were grown in the same conditions, with the exception of G418. Adenovirus serotype 2 (Ad2) were amplified in HeLa CCL2 cells and purified as described elsewhere (Tollefson *et al.*, 1999). Adenovirus infections were carried out for 20

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h at a multiplicity of infection (m.o.i.) of 10.

The 3-1A polyclonal antibody recognizing pTP was raised to a carboxy-terminal peptide (PEPPLPPGARPRR-RC) (Fredman and Engler, 1993). The monoclonal antibody directed to the FLAG epitope (DYKDDDDE) was purchased from Sigma (USA).

Plasmid constructs

The complete Ad2 pTP ORF was cloned directly into the pGBT-9 plasmid (Clontech, USA) between an *Eco*RI and a *Bam*HI sites creating a fusion with the Gal4 DNA binding domain. The resultant plasmid was referred to as pGBT-pTP.

An epitope-tagged version of Inid was cloned in the pTRE vector (Clontech, USA) for eukaryotic expression. A FLAG/Hisx7 epitope linker was designed with a Kozak consensus (ACC-ATGG) translational start sequence (Kozak, 1986). The Hisx7/FLAG epitope tag had the following predicted amino acid sequence, MGHHHHHHHDYKDDDD. The Hisx7/FLAG linker was constructed with *Sac*II and *Eco*RI sites on the ends and was ligated to the Inid ORF, which was excised from pGAD-GH vector by digestion with *Eco*RI and *Xho*I, and then blunt ended. The product from this ligation was cloned into the pTRE vector using *Sac*II and the blunt-ended *Xba*I sites. The subsequent clone contained cDNA encoding Flag-Inid that was downstream of the tetracycline responsive promoter $P_{hCMV*-1}$ and was referred to as pTRE-Inid.

Two-hybrid screening of a HeLa cDNA library using pTP as bait

The Matchmaker two-hybrid system was purchased from Clontech (USA). Using standard lithium acetate transfection, the pGBT-pTP plasmid was transformed into the HF7c yeast strain (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL-HIS3, URA3::(GAL4 17-mers)$_3$-CYC1-lacZ*), which was grown on tryptophan minus dropout plates, and colonies were analyzed for expression of the Gal4B-pTP fusion using Western blot. The pGBT-pTP fusion was then co-transformed into the HF7c strain with a HeLa cDNA library in the pGAD-GH plasmid. Approximately 1×10^6 transformants were screened for growth in the absence of histidine, tryptophan, and leucine. The colonies were then further tested for two-hybrid interactions using β-galactosidase activity as a secondary reporter in a filter-lift assay. Plasmids were isolated from His^+ *lacZ*$^+$ colonies and were transformed into an *E.coli leu*$^-$ strain of HB101 in order to rescue the plasmids of library origin. Specific interaction of pTP with the isolated plasmids was confirmed in a different yeast strain, SFY526, in which *lacZ* is under the control of the *GAL1* promoter, a different promoter than the one used in the HF7c strain. The β-galactosidase assay was performed both in the SFY526 and HF7c strains after the co-transformation of the isolated plasmid with pGBT-pTP. cDNA inserts of the positive plasmids were determined by sequencing analysis.

TdT assay for apoptosis

HeLa Tet-on cells grown on coverslips in 6-well plates were transfected with 2 μg of pTRE-Inid using FuGene-6 (Roche, USA). In order to induce Inid expression, 2 μg/ml of doxycyclin was added to the media. Twenty-four h later, cells were infected with Ad2 and further incubated for 20 more h. Then, they were fixed with 5% paraformaldehyde. The coverslips were rinsed with phosphate buffered saline (PBS) and then incubated with a 50 μl of TdT label (FITC-dUTP) and a terminal deoxynucleotidyl transferase enzyme (Roche, USA) for 30 min at 37°C. In conjunction with the TdT assay, cells were stained for the presence of either pTP (3-1A) or Flag-Inid with Texas Red-conjugated goat anti-rabbit or goat anti-mouse antibodies, respectively. Images were captured on a Leitz Orthoplan microscope and were analyzed with IPlab Spectrum software (Signal Systems, USA).

DNA Fragmentation Analysis

HeLa Tet-on cells were transfected with pTRE or pTRE-Inid and were incubated in the presence of 2 μg/ml of doxycyclin. Transfected cells were either uninfected or infected with Ad for 20 h. After the incubation, the low molecular weight chromosomal DNA was purified. Briefly, cells were washed with PBS and were lysed in 500 μl of lysis buffer (5 mM Tris-HCl, pH 7.4, 20 mM EDTA, and 0.5% Triton X-100). Samples were incubated on ice for 5 min, and insoluble materials were removed by centrifugation. The supernatant was transferred to a new tube, and the nucleic acid fraction was purified twice by extraction with phenol/chloroform followed by an ethanol precipitation. The precipitates were dissolved in 15 μl of TE (10 mM Tris-HCl, pH 8.0 and 1mM EDTA) containing 2 μg/ml of RNase A and were incubated for 30 min at 37°C. Each sample was separated through 1.8% agarose gel electrophoresis, and the DNA was visualized by ethidium bromide staining

Western Blot analysis

HeLa Tet-on cells prepared as above were lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, and protease inhibitors for 10 min at 4°C, and then, they were cleared by centrifugation. Ten μg of lysates were resolved on 12% SDS polyacrylamide gel electrophoresis. The fractionated proteins were transferred to a nitrocellulose membrane and were probed with anti-pTP or anti-Flag antibodies. Bound antibodies were detected using chemiluminescence (ECL detection kit, Amersham, USA) according to the manufacturer's instructions.

Results

Isolation of Inid using a two-hybrid screen for pTP interacting proteins

In order to isolate the associating protein of Ad pTP, the

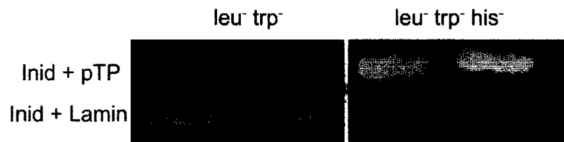


Fig. 1. Specific interaction of pTP with Inid in the yeast two hybrid system. Yeast strain HF7c was transformed with plasmids carrying pGBT-pTP with a positive isolate fused to Gal4A. Colonies containing different combinations of plasmids were streaked on leu^-trp^- or $leu^-trp^-his^-$ plates and incubated for 2 days at 30°C. For the control Gal4B-Lamin construct was co-transformed with Gal4A-Inid.

entire pTP ORF fused to the Gal4 DNA binding domain (pGBT9-pTP) was used for screening a HeLa cDNA library. The cDNA librabry was fused to the Gal4 activation domain (Gal4A). In a yeast strain HF7c, interaction of Gal4B-pTP with any protein fused to the Gal4 activation domain will activate the transcription of reporter genes (*His3*, *lacZ*). 1×10^6 transformants were screened for the ability to grow under growth selection (see Materials and Methods) and for β-galactosidase activity. Positive colonies were tested in a second yeast strain (SFY526) in the presence of 3-aminotriazol. Nine colonies were isolated in which β-galactosidase positivity was dependent on the presence of pTP; the clones did not show signals when combined with pGBT-9 or combined with each other. The deduced sequences from nine isolates were identical to Interferon inducible gene 1-8D (Inid) when queried against the GenBank database (Genebank accession number; X57351, Lewin *et al.*, 1991). The complete 396 bp ORF of Inid was contained in each of these clones. Gal4A-Inid cDNA, when co-expressed with Gal4B-pTP in the HF7c yeast strain conferred the His^+ phenotype. A control bait, Gal4B-Lamin, however, when co-expressed with Inid fusion genes, failed to induce the His^+ phenotype (Fig. 1). This result showed the specific interaction between Inid and pTP *in vivo*.

Inid induces apoptosis in Ad-infected or uninfected cells

To investigate the biological functions the Flag-epitope tagged Inid cDNA(pTRE-Inid) was expressed in HeLa Tet-on cells that stably expressed the reverse tetracycline-responsive transcriptional activator (rtTA). In this cell line Inid was expressed in response to doxycyclin. Among the Ad-infected cells expressing Inid and pTP, 50 to 60% displayed rounded morphology. Examples of these cells are shown in Fig. 2, panels A-C. Under phase contrast these cells were reduced in size and had collapsed nuclear morphology that was suggestive of apoptosis (Fig. 2C). In cells that had not been treated with doxycyclin, little to no altered cell morphology was observed (data not shown). In order to substantiate the possibility that Inid induced apoptosis in these cells, we assayed for the presence of degraded DNA using the TdT assays. Cells transfected

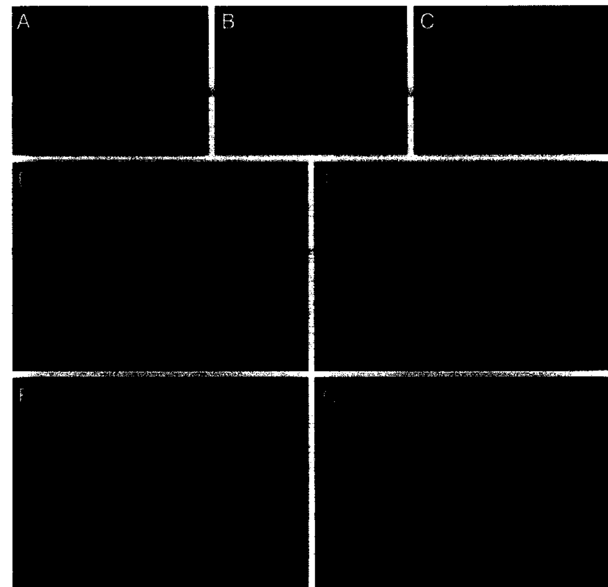


Fig. 2. Inid induces apoptosis in Ad infected cells. HeLa Tet-on cells transfected with pTRE-Inid were infected with Ad2 for 20 h and the expression of Inid was induced with doxycyclin. The cells were fixed and were either probed with anti-Flag monoclonal antibody followed by FITC-conjugated goat anti-mouse antibody to detect the presence of Inid or anti-pTP polyclonal antibody followed by Texas-Red conjugated goat anti-rabbit antibody. Ad infected cells expressing pTP (A) and Inid (B) had a "rounded" morphology which was evident by phase contrast microscopy (C). Alternatively, the cells were tested for apoptosis using the TdT assay which employed the addition of FITC-dUTP, and in which case, Inid was detected with a Texas-Red conjugated goat anti-mouse secondary antibody. Infected cells transfected with pTRE-Inid in the absence of doxycyclin were assayed for the presence of (pTP; D) and for apoptosis by the TdT assay (F). Doxycyclin untreated cells showed little evidence of apoptosis (F). Cells treated with doxycyclin showed that the "rounded" cells stained strongly for Inid (E). These cells were also strongly positive for apoptosis (TdT assay; G). Magnification was 40X for A-C and 10X for D-G.

with pTRE-Inid were either incubated with doxycyclin or were untreated. Then these cells were infected with Ad2 for 20 h and were assayed for the addition of terminal dUTP-FITC. In cells expressing pTP without the induction of Inid expression, little dUTP-FITC incorporation was detected (Fig. 2D, F). In contrast, cells showed massive apoptosis when Inid, along with Ad infection, was expressed (Fig. 5E, G). Analysis of these fields showed that 80 to 90% of the cells positive for apoptosis were also expressing Inid.

However, it was not clear whether Inid was capable of inducing apoptosis alone, or if this activity represented an anti-viral response dependent on the presence of Ad replication proteins, such as pTP. In order to address this question, HeLa Tet-on cells were transfected with pTRE-Inid along with or without Ad infection, and the apoptosis was checked by the DNA fragmentation gel assay. Little to no DNA fragmentation in cells was observed without Inid expression. Whereas, cells treated with doxycyclin

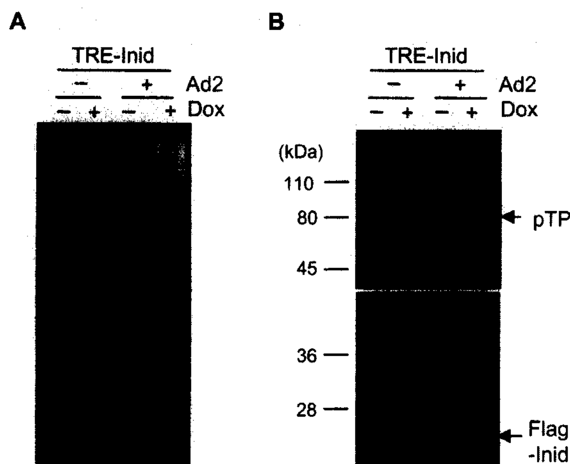


Fig. 3. Inid induces apoptosis in uninfected cells. HeLa Tet-on cells transfected with pTRE-Inid were either infected with Ad2 (Ad2+) or remained uninfected (Ad2-). In Doxycyclin untreated cells (Dox-) minimal DNA fragmentation was detected, whereas, cells treated with Doxycyclin (Dox+) clearly showed apoptosis as determined by the smearing or fast moving fragmented DNA (A). The expressions of Inid and pTP were confirmed in the Western Blot analysis. Each protein was indicated by arrows (B).

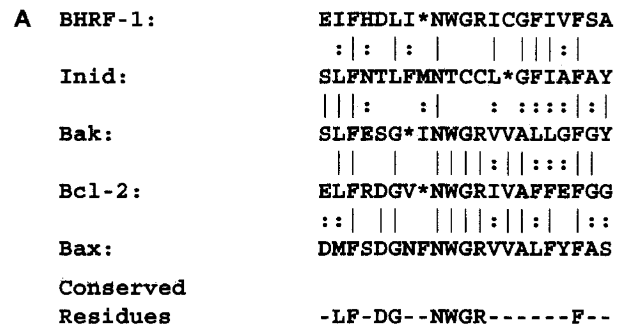
underwent rapid apoptosis characterized by the smeared DNA on the gel, confirming that Inid did induce apoptosis (Fig. 3). In addition, DNA fragmentation was observed in Inid expressed cells without Ad infection. These results suggest that Inid is capable of inducing apoptosis independent of an Ad infection.

Inid has similar features to Bcl-2 family apoptotic genes

Upon making the observation that Inid induced apoptosis, we questioned whether Inid might be related to other well-known apoptosis genes. Low stringency blast searches of the GenBank database with the Inid ORF revealed no immediate similarities with known apoptosis genes. However, when we aligned the BH1 (Bcl-2 homology domain 1) with Inid we discovered that there was significant identity with Inid, up to 40% identity when compared to the EBV BHRF1 BH1 region (Fig. 4A, Kieffer *et al.,* 1995). Furthermore, when hydropathy plots of the C-terminal amino acids of Bcl-2 (residues: 136-240) and Inid (residues: 60-132) were generated, a distinct similarity in profile was apparent (Fig. 4B). Bcl-2 contains a C-terminal hydrophobic transmembrane tail in addition to dimerization signals (White *et al,* 1996). The Kyte-Doolittle hydropathy plot of Inid also shows a hydrophobic C-terminus, which was previously predicted to be a transmembrane domain in early molecular analysis of the protein (Kyte and Doolittle, 1982, Lewin *et al.,*1991). These data support the possibility that Inid is related to Bcl-2 apoptosis genes.

Discussion

In this study, we showed that pTP binds to Inid. Heretofore,



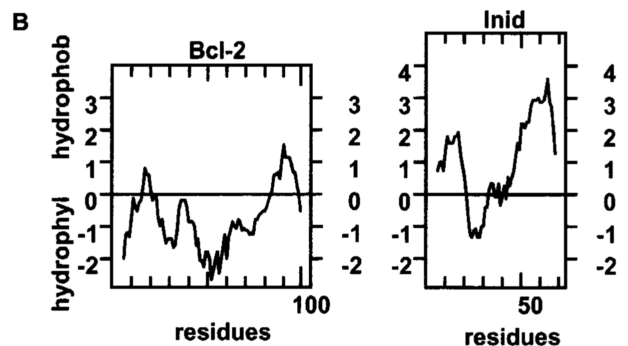


Fig. 4. Inid shares similar features to Bcl-2 family apoptotic gene products. Inid amino acid residues 60-80 were directly compared in a sequence alignment to BH1 regions (Bcl-2 homology domain 1) of various Bcl-2 related gene products: BHRF-1, Bak, Bcl-2, and Bax (A; Kieffer *et al,* 1995). The alignments reveal a significant homology of Inid with the conserved residues of the BH1 domain. Kyte-Doolittle hydropathy plots of Bcl-2 and Inid were generated with the use of DNA Strider 1.2 (CEA, France). The y-axis is in arbitrary units, where +4 is very hydrophobic and -2 is very hydrophilic. The x-axis represents amino acid units, where each increment equals 10 residues. The hydropathy plots of Bcl-2 and Inid show distinct similarities, particularly in the hydrophobic C-terminal domains.

the function of the Inid protein is unknown. Although, it is known that Inid contains two protein kinase C (PKC) sites, a casein kinase II site, and a hydrophobic C-terminus that is predicted to be a transmembrane domain. It has been shown that casein kinase II sites can function as nuclear localization signals in their phosphorylated form (Mcvey *et al.,* 1989). Inid is also known to be upregulated by interferon-α and γ (Kelly *et al.,* 1985). This fact raised the question of whether Inid might have anti-viral functions.

In eukaryotic expression studies, we observed Inid-induced apoptosis in Adenovirus infected cells. Inid expressing cells had features that are characteristic of apoptosis, such as collapsed nuclei and cytoplasms and a granular appearance (Collins *et al.,* 1992). Additionally, we found evidence of DNA fragmentation exclusively in Inid expressing cells, which is a well accepted hallmark of apoptosis. Inid has been implicated in ionizing radiation induction of apoptosis (Clave *et al.,* 1987). Further evidence that interferon-γ induces apoptosis in HeLa cells may correlate with our observed Inid-dependent cell death (Deiss *et al.,* 1995). However, it is not clear whether asso-

ciation between Inid and pTP is critical for causing apoptosis since Inid itself is capable of inducing apoptosis. Interestingly, we also observed that Inid reorganized the pTP foci on the NM (unpublished result). The disruption of pTP domains on the NM could represent an early stage of Inid-induced apoptosis. Indeed, an early stage to apoptosis involves the interleukin-1β-converting enzyme (ICE)-dependent proteolysis of lamin, the major structural component of the NM (Rao *et al.*,1996). However, we cannot rule out the possibility that the pTP-rearrangement is an Inid function which is separable from its obvious apoptosis effects. The fact that Inid both binds pTP and induces apoptosis supports the concept that it could provide targeted anti-viral killing of infected cells.

Acknowledgments

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