Apo2L/TRAIL induction and nuclear translocation of inositol hexakisphosphate kinase 2 during IFN- β -induced apoptosis in ovarian carcinoma

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Previously, we have reported that overexpression of IHPK2 (inositol hexakisphosphate kinase 2) sensitized NIH-OVCAR-3 ovarian carcinoma cell lines to the growth-suppressive and apoptotic effects of IFN- β (interferon- β) treatment and γ -irradiation. In the present study, we demonstrate that Apo2L/TRAIL (Apo2L/tumour-necrosis-factor-related apoptosis-inducing ligand) is a critical mediator of IFN-induced apoptosis in these cells. Compared with IFN- α 2, IFN- β is a more potent inducer of Apo2L/TRAIL and IHPK2 activity. Overexpression of IHPK2 converts IFN- α 2-resistant cells into cells that readily undergo apoptosis in response to IFN- α 2. In untreated cells transfected with IHPK2-eGFP (where eGFP stands for enhanced green fluorescent protein), the fusion protein is localized to the cytoplasm and perinuclear region. After treatment with IFN- β , IHPK2-eGFP translocated to the nucleus. In cells transfected with mutant IHPK2-NLS-eGFP (where NLS stands for nuclear localization sequence), containing point mutations in the NLS, the fusion protein remained trapped

in the cytoplasm, even after IFN- β treatment. Cells expressing mutant NLS mutation were more resistant to IFN- β . The IC₅₀ value of IHPK2-expressing cells was 2–3-fold lower than vector control. The IC₅₀ value of NLS-mutant-expressing cells was 3-fold higher than vector control. Blocking antibodies to Apo2L/TRAIL or transfection with a dominant negative Apo2L/TRAIL receptor (DR5 Δ) inhibited the antiproliferative effects of IFN- β . Thus overexpression of IHPK2 enhanced apoptotic effects of IFN- β , and expression of the NLS mutant conferred resistance to IFN- β . Apo2L/TRAIL expression and nuclear localization of IHPK2 are both required for the induction of apoptosis by IFN- β in ovarian carcinoma.

Key words: Apo2L/tumour-necrosis-factor-related apoptosis-inducing ligand (Apo2L/TRAIL), apoptosis, inositol hexakisphosphate kinase 2, interferon, ovarian carcinoma, translocation.

INTRODUCTION

During induction of apoptosis in NIH-OVCAR-3 ovarian carcinoma cells, IFN- β (interferon- β) induces both Apo2L/TRAIL (Apo2L/tumour-necrosis-factor-related apoptosis-inducing ligand, TNFSF10) and IHPK2 (inositol hexakisphosphate kinase 2, also known as IP6K2) [1]. When administered exogenously, Apo2L/TRAIL induces apoptosis in several different malignant cell types either as a single agent or in combination with other agents. Most normal cells are relatively resistant to Apo2L/TRAIL. Apo2L/TRAIL induces apoptosis through its two DRs (death receptors), DR4 (TRAIL-R1, TNFSF10A) and DR5 (TRAIL-R2, TNFSF10B). In addition, the ligand may also bind the DcRs (decoy receptors), DcR1 (TRAIL-R3, TNFSF10C) and DcR2 (TRAIL-R4, TNFSF10D), which lack an intracellular signalling domain, thus negatively regulating Apo2L/TRAIL-induced apoptosis [2,3]. It has been postulated that normal cells are Apo2L/TRAIL-resistant due to DcR expression. The ovarian carcinoma cells used in the present study express mutant p53 [4]. Type I IFNs (IFN- α and - β) are known to induce apoptosis in a p53-independent manner [5].

IHPK2 is a kinase that catalyses the synthesis of diphosphoinositol pentakisphosphate (InsP₅PP, contains 7 phosphates) using IP₆ (inositol hexaphosphate, contains 6 phosphates) as a substrate

in the presence of ATP [6]. IHPK2 affects several different cellular processes. The substrate for this enzyme, IP₆, inhibits nucleosomal mobility and chromatin remodelling [7]. The clathrin assembly proteins (AP-2, AP-3 and AP-180) that are required for membrane fusion [8–10], and synaptotagmins, proteins required for exocytosis [11], all bind IP₆. Overexpression of IHPK2 causes prolongation of the cell cycle, without arrest in a specific phase [12]. We have also observed that the inhibition of IHPK2 expression and enzymic activity by a dominant negative mutant resulted in cellular resistance to the apoptotic effects of IFN- β and cytotoxic agents, such as cisplatin and etoposide. Expression of IHPK2 appeared to be critical for death induction, since IFN- α 2, which also induced Apo2L/TRAIL, induced neither IHPK2 nor apoptosis.

Other investigators have demonstrated the importance of Apo2L/TRAIL as a mediator of IFN- β -induced apoptosis in malignant melanoma cells [13]. Even though they bind the same receptor (IFNAR-1) [14], IFN- α 2 and - β have differential effects in terms of antiproliferative activity. In most malignant cell lines, IFN- β had greater growth inhibitory and apoptotic effects than IFN- α 2. Importantly, IFN- β strongly induced Apo2L/TRAIL in sensitive cell lines, but failed to induce Apo2L/TRAIL in resistant cell lines. The specific aims of the present study were: (i) to determine whether there was a functional relationship between IHPK2 and

Abbreviations used: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; TNF α , tumour necrosis factor α ; Apo2L/TRAIL, Apo2L/TNF-related apoptosis-inducing ligand; DcR, decoy receptor; DR, death receptor; eGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; HEK-293T cells, human embryonic kidney 293T cells; GAP3DH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IFN, interferon; IHPK2, inositol hexakisphosphate kinase 2; IP₆, inositol hexaphosphate; mAb, monoclonal antibody; NLS, nuclear localization sequence; PI, propidium iodide; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; Z-VAD, benzyloxycarbonylvalyl-alanyl-aspartic acid (O-methyl)-fluoromethylketone.

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Apo2/TRAIL, and (ii) to identify factors responsible for the lack of apoptosis in IFN- α 2-treated ovarian carcinoma cells.

EXPERIMENTAL

Reagents

Human IFN- β (Serono, Copenhagen, Denmark), specific activity 2.7×10^8 units/mg, and IFN- α 2b (Schering-Plough, Kenilworth, NJ, U.S.A.), specific activity 3×10^8 units/mg, were used in these studies. Affinity purified anti-Apo2/TRAIL antibody [clone M180; Immunex (Amgen), Thousand Oaks, CA, U.S.A.] neutralizes the biologic activity of recombinant human Apo2/TRAIL. In ELISAs, the antibody does not cross-react with rhAPRIL, rhBAFF, rhFas ligand, rhGITR ligand, rhLIGHT, rhTNF- α , rhTRANCE or rhVEGI.

Cell growth assays

Cells were treated with IFNs during growth in RPMI 1640 medium (Mediatech, Herndon, VA, U.S.A.) and 5% (v/v) FBS (fetal bovine serum; Hyclone, Logan, UT, U.S.A.). Cells were confirmed to be mycoplasma-free by PCR. Growth was monitored using a colorimetric assay [15]. Each treatment group contained eight replicates. Cells were fixed and stained with sulphorhodamine B after 7 days. Bound dye was eluted from cells, and absorbance (A_{exp}) was measured at 570 nm. One plate was fixed 8 h after plating to determine the absorbance representing starting cell number (A_{ini}). Absorbance with this plate and that obtained with untreated cells at the end of the growth period (A_{fin}) were taken as 0 and 100 growth respectively. Thus:

$$\text{percentage of control growth} = 100\% (A_{\text{exp}} - A_{\text{ini}}) / (A_{\text{fin}} - A_{\text{ini}})$$

Expressed as a percentage of untreated controls, a decrease in cell number (relative to starting cell number) is a negative number on the y-axis.

Construction of IHPK2 mutants

IHPK2 cDNA was cloned into the pCXN2myc mammalian expression vector [16] in which the chicken actin promoter regulates expression of the myc-His-tagged insert. An NLS (nuclear localization sequence) mutant of IHPK2 was created using PCR-based site-directed mutagenesis using full-length IHPK2 as a template in the pGEM7zf vector. The putative bipartite NLS (in bold) (YLRRRELLGPVLK~~KL~~TEL) was replaced with point mutations to yield amino acid residues (YLAAELLGPVLA~~AA~~TEL), using primers 5'-pGCCAGGAGTTCAGCGCCAGG-TACCGCCCATTTGTG-3' and 5'-pCCTGTGCTCGCAGCACTG-ACTGAGCTCAAGGCA-3' (p indicates phosphorylated 5'-nucleotide). The PCR product was self-ligated and then transformed into JM109 *Escherichia coli*. IHPK2-NLS DNA was digested from pGEM7zf with *Eco*RI and *Xho*I, and cloned into the pCXN2myc mammalian expression vector. Subcloning into peGFP-C2 (BD Biosciences, ClonTech) to create a fluorescent fusion protein was performed in a similar manner. All mutations were confirmed by sequencing. Constructs were transfected into NIH-OVCAR-3 cells with the cationic lipid reagent LIPOFECTAMINETM 2000 (Invitrogen, Carlsbad, CA, U.S.A.), and stable transfectants were selected with G418. After 2 weeks of selection, surviving clones were pooled for further studies. Expressions of mutants were monitored by Western blotting.

Transfection of plasmids

Falcon CultureSlides (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) were coated with 0.1% (w/v) poly(L-lysine) (Sigma,

St. Louis, MO, U.S.A.) for 1 h, washed with sterile water and air-dried. In each chamber, 300 000 cells were plated the day before transfection. NIH-OVCAR-3 cells were 80% confluent on the day of transfection. Transfection was performed according to the manufacturer's instructions. For each transfection, 200 ng of IHPK2-NLS-peGFP or IHPK2-peGFP plasmid was used. DNA and LIPOFECTAMINETM 2000 were mixed and incubated at room temperature (25°C) for 20 min. The medium was replaced with 0.45 ml of serum-free RPMI 1640 medium and 50 μ l of the complex was added into each chamber. After 4 h incubation at 37°C, serum-free medium was replaced with 1 ml of fresh complete medium. After 24 h, cells were treated with IFN- β (100 units/ml for 16 h). After 24 h, the medium was removed and cells were fixed with 0.5 ml of 4% (w/v) paraformaldehyde for 15 min at room temperature. Slides were covered with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.). Cells were analysed by light and fluorescence microscopy. Transfection of HEK-293T cells (human embryonic kidney 293T cells) was performed similarly, but cells were washed in serum-free Dulbecco's modified Eagle's medium and plated in the same medium containing 3% FBS before exposure to tamoxifen (Sigma).

Northern-blot analysis

Total cellular mRNA (40 μ g) was separated on 1% formaldehyde-agarose gels and transferred on to nylon membrane. Membrane was incubated with a ³²P-labelled cDNA probe specific for Apo2L/TRAIL or GAP3DH (glyceraldehyde-3-phosphate dehydrogenase) mRNA. The membrane was washed, dried and subjected to autoradiography.

Caspase activity assay

Caspase 3 activity was measured by using a caspase 3 assay kit [Pharmingen (BD Biosciences), San Diego, CA, U.S.A.]. Cells were treated with IFN- β (0–72 h), washed twice with cold PBS and lysed on ice in lysis buffer (10 mM Tris/HCl, pH 7.5, 130 mM NaCl, 1% Triton X-100 and 10 mM Na₂HPO₄). Cell lysates were centrifuged at 10 000 g for 10 min, and total protein concentration was determined using Bio-Rad protein assay reagent. The assay was performed in 96-well plates, and triplicate measurements were taken for each sample. For each reaction, 20 μ g of protein extract, 200 μ l of Hepes buffer and 5 μ g of Ac-DEVD-AMC (acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; a fluorogenic substrate) were mixed and incubated at 37°C for 1 h. As controls, cell lysates or substrates alone were incubated in parallel. Where indicated, extracts from IFN-treated cells (72 h) were pre-incubated with the caspase 3 inhibitor Ac-DEVD-CHO (acetyl-Asp-Glu-Val-Asp-aldehyde; 0.5 μ g) for 5 min before the addition of substrate. Caspase enzymic hydrolysis was measured by AMC liberation from Ac-DEVD-AMC at 380 nm/460 nm using a spectrofluorimeter. Relative fluorescence of substrate control was subtracted as background emission.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay

DNA fragmentation was detected in IFN- α 2- and IFN- β -treated cells by TUNEL staining using the APO-BrdUrd kit (Pharmingen). IFN-treated cells were washed with cold PBS, trypsinized, and fixed in 1% paraformaldehyde for 15 min on ice. Fixed cells were washed twice with PBS, pelleted at 1000 g and suspended in 70% ethanol. The cells were kept for 12–24 h at 20°C in 70% ethanol. Cells were labelled with Br-dUTPs by washing twice with PBS and labelled with Br-dUTP by enzyme

TdT (terminal deoxynucleotidyl transferase) for 2 h at 37 °C. After labelling, cells were washed and stained with FITC-conjugated anti-BrdUrd mAb (monoclonal antibody) for 30 min in a low-light environment. RNase-PI (where PI stands for propidium iodide) was added, and samples were incubated for an additional 30 min at room temperature. The percentage of FITC-positive cells were analysed by FACS (Becton Dickinson Facsvantage, BD Biosciences, San Diego, CA, U.S.A.).

Annexin V/PI assay

Annexin V staining of exposed membrane phospholipid phosphatidylserine was done using the Annexin V assay kit (Pharmingen). Cells were harvested according to the manufacturer's instructions, washed with PBS twice and resuspended in 100 μ l of binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl and 2.5 mM CaCl_2). Annexin V-FITC and PI were added to individual samples and incubated for 15 min in a low-light environment. The reaction was stopped by adding 3 vol. of binding buffer. Cells were analysed by FACS (Becton Dickinson Facsvantage).

Immunohistochemistry

Primary antibody monoclonal anti-IHPK2 clone 4F10-3 made in our laboratory and secondary antibody goat anti-mouse IgG (H + L)-HRP (horseradish peroxidase) conjugate (Bio-Rad, Hercules, CA, U.S.A.) were used in these studies. Cells were grown as monolayers in Lab-Tek chamber slides (Nalge Nunc, Naperville, IL, U.S.A.) and treated with 200 units/ml IFN- β for 16 h and fixed with cold acetone (−20 °C), air dried, washed three times in PBS and blocked with 10 % FBS (Hyclone) in PBS. IHPK2 mAbs (undiluted) were applied at 37 °C for 1 h. After washing in PBS, samples were incubated with goat anti-mouse IgG (H + L)-HRP conjugated secondary antibody, diluted 1:25 in PBS with 10 % FBS and then incubated in peroxidase 3,3'-diaminobenzidine (Sigma). After washing in water, cells were stained in Gill's formulation #2 haematoxylin, and immediately washed with several changes of tap water, dehydrated with alcohol to xylene. Cells were examined by light microscopy.

RESULTS

Antiproliferative and apoptotic effects of IFNs

NIH-OVCAR-3 human ovarian carcinoma cells were more sensitive to the antiproliferative effects of IFN- β (black bars) compared with IFN- α 2 (white bars) (Figure 1, upper panel). The IC_{50} values for IFN- α 2 and - β were > 1000 and 12 units/ml respectively. Concentrations of IFN- β above 200 units/ml induced cell death, represented by data points below the x-axis. Cells readily became apoptotic in response to IFN- β but not to IFN- α 2 (Figure 2). TUNEL assays indicated that untreated cells exhibit a low level of apoptosis (7.5 %); IFN- β (1000 units/ml for 48 h) induced 64.4 % TUNEL-positive cells, whereas IFN- α 2 treatment (1000 units/ml for 48 h) induced 13.6 % TUNEL-positive cells. Annexin V cell-surface staining, also detected by flow cytometry, displayed a similar pattern. Increasing the concentration of IFN- β yielded an increase in Annexin V-positive cells, whereas high concentrations of IFN- α 2 (1000 units/ml) were no different from control cells after 48 h treatment (Table 1).

Effects of IFNs on Apo2L/TRAIL and IHPK2 expression

Since we [1] and others [13] had previously noted that IFN- β was a strong inducer of Apo2L/TRAIL, an inducer of apoptosis, we next determined whether both IFNs could stimulate Apo2L/TRAIL mRNA expression, and whether IHPK2 overexpression resulted

Figure 1 Antiproliferative effects of IFNs in NIH-OVCAR-3 cells expressing IHPK2

Upper panel, antiproliferative effects of IFN- β and - α in ovarian carcinoma cells. NIH-OVCAR-3 cells were stably transfected with vector alone (pCXN2) and grown in the presence of 5–1000 units/ml IFN- β (black bars) or IFN- α 2 (white bars). After 7 days, cells were fixed and stained with sulphorhodamine B. A_{570} of bound dye was measured and expressed as percentage of untreated controls. Each data point represents means $\pm$ S.E.M. for eight replicates. Negative values on y-axis indicate death of initially plated cells. Lower panel, NIH-OVCAR-3 cells were stably transfected with IHPK2 cDNA ligated into the pCXN2 mammalian expression vector. Cells were grown in the presence of IFN- β or - α as above for 7 days. Abbreviation: U, unit.

in higher levels of Apo2L/TRAIL. In untreated cells, Northern-blot analysis (Figure 3A) indicated that Apo2L/TRAIL transcripts were undetectable. IFN- β induced Apo2L/TRAIL mRNA within 2 h in pCXN2 (vector) expressing cells, and remained detectable up to 16 h (lanes 5–7). Apo2L/TRAIL mRNA was also induced after IFN- α 2 treatment, albeit at reduced levels when compared with IFN- β (lanes 2–4). Apo2L/TRAIL induction by both IFNs displayed similar intensity and kinetics in cells overexpressing IHPK2 (Figure 3A, bottom half) when compared with vector-transfected cells. Hence forced IHPK2 expression did not enhance Apo2L/TRAIL mRNA. To determine whether different type I IFNs modulated IHPK2 expression, immunoblots were performed. IFN- β induced IHPK2 protein after 4 h exposure (Figure 3B), whereas IFN- α 2 failed to do so.

Blockade of Apo2L/TRAIL signalling abrogates IFN- β -induced cell death

To determine whether Apo2L/TRAIL functioned as a mediator of IFN- β -induced death in ovarian carcinoma cells, we treated cells with neutralizing mAb to Apo2L/TRAIL (clone M180; Immunex) during IFN- β treatment and assayed TUNEL staining by flow cytometry. NIH-OVCAR-3 cells were treated with 1000 units/ml IFN- β , IFN- β + anti-Apo2L/TRAIL (1 μ g/ml) or

Figure 2 IFN-β induces apoptosis in NIH-OVCAR-3 cells

TUNEL assay: NIH-OVCAR-3 cells stably expressing pCXN2 were treated with PBS, IFN-α2 or -β (1000 units/ml) for 48 h, labelled with Br-dUTP using TdT, and stained with FITC-conjugated anti-BrdU. Percentage of FITC-positive cells was determined by flow cytometry.

Table 1 Effect of IFNs on induction of apoptosis in NIH-OVCAR-3 cells

Numbers indicate percentage of positive cells determined by flow cytometry, means for three separate experiments.

Transfected	Treatment (48 h)	Units/ml	Annexin V	TUNEL
pCXN2	None		10.3	7.5
pCXN2	IFN-α2	500	12.1	7.5
pCXN2	IFN-α2	1000	14.8	13.6
pCXN2	IFN-β	500	38.1	42.0
pCXN2	IFN-β	1000	57.6	64.4
IHPK2	None		12.6	9.1
IHPK2	IFN-α2	500	27.7	34.0
IHPK2	IFN-α2	1000	60.8	49.2
IHPK2	IFN-β	500	55.4	58.6
IHPK2	IFN-β	1000	72.3	81.7

Figure 4 Effect of anti-Apo2L/TRAIL blocking antibody

NIH-OVCAR-3 cells were stably transfected with pCXN2 vector (white bars) or IHPK2 (black bars). Cells were grown in the presence of PBS, affinity-purified anti-Apo2L/TRAIL antibody (mAb), IFN-β (1000 units/ml), mAb + IFN-β, IgG (isotype control) + IFN-β, IFN-α2 (1000 units/ml), mAb + IFN-α2 or IgG + IFN-α2. TUNEL assay was performed as in Figure 2. Each data point represents means ± S.E.M. for three replicates.

Figure 3 (A) Induction of Apo2L/TRAIL mRNA by IFNs and (B) induction of IHPK2 protein by IFNs

(A) NIH-OVCAR-3 cells were treated for 2–16 h with IFN-α2 or -β (1000 units/ml). Total RNA (40 µg) from these cells was subjected to Northern-blot analysis. Blots were hybridized with ³²P-labelled specific probes for Apo2L/TRAIL mRNA (upper) and GAP3DH mRNA (lower) as control. (B) Cells were treated with IFNs as above. Cell lysates were subjected to Western-blot analysis. The blot was probed with anti-actin mAb as control.

IFN-β + murine IgG (1 µg/ml, isotype control) (Figure 4). Similar combinations of IFN-α2 and mAb were also performed. IFN-β induced 58 % TUNEL-positive cells. Treatment with Apo2L/TRAIL neutralizing antibody in the presence of IFN-β resulted in only 12 % TUNEL-positive cells, which was not

significantly different from control. Isotype control IgG did not block the apoptotic activity of IFN-β. IHPK2-transfected cells displayed higher levels of TUNEL staining when compared with vector-transfected cells. Consistent with the annexin V studies (Table 1), overexpression of IHPK2 resulted in more apoptosis following IFN-α2. At equal concentrations, IFN-β caused more cell death than IFN-α2. Hence Apo2L/TRAIL induction is a critical event in IFN-induced cell death.

To confirm the role of the Apo2L/TRAIL receptors, we transfected NIH-OVCAR-3 cells with a truncated version of the DR5 receptor (DR5Δ) [17] and obtained stable transfectants. DR5Δ functions as a dominant negative mutant and blocks Apo2L/TRAIL signalling mediated through both Apo2L/TRAIL DRs DR4 (TRAIL-R1, TNFRSF10A) and DR5 (TRAIL-R2, TNFRSF10B). After 72 h treatment with IFN-β, caspase 3 enzymic activity in cells expressing vector alone (Figure 5, white bars) was 5-fold higher than that in cells expressing DR5Δ (Figure 5, black bars), indicating that functional Apo2L/TRAIL receptors and secretion of ligand were both required for IFN-β-mediated apoptosis. The caspase 3 inhibitor Ac-DEVD-CHO effectively blocked caspase 3 activity in IFN-β-treated cells.

Comparing IFN-α2 and -β, the disparity in levels of apoptosis induced by these cytokines (Figure 2) could not be explained solely on the basis of the Apo2L/TRAIL induction (Figure 3A). We then performed multiprobe RNase protection assays [1] to

Figure 5 Inhibition of Apo2L/TRAIL receptor blocks IFN- β -induced apoptosis

Vector alone (white bars) or a truncated version of the Apo2L/TRAIL DR5 receptor that functions in a dominant negative manner (DR5 Δ ; black bars) was stably transfected into NIH-OVCAR-3 cells. Cells were treated with IFN- β or - α 2 (1000 units/ml) for 72 h. Caspase 3 enzymic activity was determined by measuring cleavage of Ac-DEVD-AMC (a fluorogenic substrate) in cell extracts. Where indicated, extracts from IFN-treated cells (72 h) were preincubated with the caspase 3 inhibitor Ac-DEVD-CHO before the addition of substrate. Relative fluorescence of substrate control was subtracted as background emission.

identify mRNAs that coded for other regulators of apoptosis that were differentially regulated by IFN- α 2 and - β ; however, no such differential expression was observed (results not shown).

Since our previous studies suggested that IHPK2 sensitized cells to IFN- β -induced cell death [1,12], we hypothesized that forced expression of IHPK2 might also sensitize cells to IFN- α 2. Indeed, NIH-OVCAR-3 cells transfected with IHPK2 (Figure 1, lower panel) had an IC₅₀ value of approx. 60 units/ml IFN- α 2, compared with vector-transfected cells (Figure 1, upper panel), in which the IC₅₀ value was > 1000 units/ml IFN- α 2. For any given dose of IFN- α 2, the percentages of TUNEL-positive and Annexin V-positive cells were at least 2-fold higher in cells expressing IHPK2 compared with cells expressing vector alone (Table 1). Hence efficient induction of apoptosis in IFN- α 2-treated NIH-OVCAR-3 cells depends on Apo2L/TRAIL induction and the presence of IHPK2.

Nuclear translocation of IHPK2 during apoptosis

To localize endogenous IHPK2 within untreated dividing cells and during IFN- β -induced apoptosis, we performed immunohistochemistry. NIH-OVCAR-3 cells grown to 70 % confluence

were treated with PBS or IFN- β or - α 2 (200 units/ml) for 16 h. In cells treated with PBS, IHPK2 was distributed in both the nucleus and cytoplasm, with approx. 50 % of cells showing increased nuclear expression compared with the cytoplasm (Figure 6). After IFN- β treatment, IHPK2 protein concentrated in the nucleus, without detectable change in cytoplasmic staining. IFN- α 2 did not induce IHPK2 or cause nuclear translocation.

To localize episome-encoded IHPK2 protein during apoptosis, a fusion protein encoding eGFP (enhanced green fluorescent protein) fused to IHPK2 was expressed in NIH-OVCAR-3 cells. In untreated cells, IHPK2 resided mainly in the cytoplasm (Figure 7, top left). Exposure to IFN- β resulted in translocation of IHPK2 into the nucleus, especially in areas of condensed chromatin (Figure 7, middle left). Kinetically, protein translocation preceded increase in TUNEL or annexin V staining. IHPK2 translocation was evident at 16 h, whereas increases in TUNEL and annexin V did not occur until 24 h (results not shown) to 48 h (Table 1 and Figure 2). A mutant fusion protein (NLS) was created, in which four point mutations were created in the putative NLS. Distribution of the NLS mutant was similar to wild-type in untreated cells (Figure 7, top right). After IFN- β treatment, the NLS mutant remained trapped in the cytoplasm (Figure 7, middle right). Cells expressing eGFP-IHPK2 displayed nuclear translocation of the fusion protein, whether treated with IFN- β or - α 2 (Figure 7, bottom left). Similarly, cells transfected with the NLS mutant and treated with IFN- α 2 showed the fusion protein trapped in the cytoplasm and few dying cells (Figure 7, bottom right).

Blockade of IHPK2 translocation inhibits apoptosis

The biological relevance of the NLS mutation was determined in antiproliferative assays. Wild-type IHPK2 and the NLS mutant were cloned into the pCXN2 mammalian expression vector. These constructs were electroporated into NIH-OVCAR-3 cells and stable transfectants were obtained. The sensitivity of wild-type (untransfected) cells to IFN- β was indistinguishable from cells expressing vector alone (Figure 8A). Overexpression of IHPK2 sensitized cells to IFN- β , as the IC₅₀ value fell from 8 to 2 units/ml. In contrast, the NLS mutant conferred resistance to IFN- β , as the IC₅₀ value increased from 8 to 25 units/ml. Since there was a possibility that the eGFP tag might have perturbed protein structure or altered interaction with the apoptotic machinery, we examined nuclear translocation in the presence of a general caspase inhibitor. Indeed, Z-VAD [benzyl-oxycarbonylvalyl-alanyl-aspartic acid (*O*-methyl)-fluoromethylketone] treatment did not prevent translocation of the eGFP-IHPK2 fusion protein (Figure 8B). Similarly, Z-VAD treatment

Figure 6 Expression of endogenous IHPK2

NIH-OVCAR-3 cells grown on chamber slides were treated with PBS or IFN- β or - α 2 (200 units/16 h per ml). Cells were stained with monoclonal anti-IHPK2 antibody followed with HRP-goat-anti-mouse secondary antibody. The chromogen diaminobenzidine is shown in dark grey.

Figure 7 Expression of eGFP fusion proteins

Tagged fusion proteins were created as described in the Experimental section. NIH-OVCAR-3 cells were stably transfected with eGFP-IHPK2 or eGFP-NLS, and then treated with PBS or IFN- β (200 units/ml $\times$ 16 h). Nuclei were stained blue with DAPI (4,6-diamidino-2-phenylindole) and the fusion proteins fluoresce in green. Nuclear translocation of eGFP-IHPK2 occurs during apoptosis, but eGFP-NLS remains in the cytoplasm.

did not alter cellular distribution of eGFP-NLS, which remained trapped in the cytoplasm (results not shown).

Finally, we examined the cellular localization of IHPK2 in a cell line derived from non-ovarian histology, and a non-cytokine-based agent to induce apoptosis. Earlier reports from the laboratory of Snyder and co-workers [18] indicated that episomally expressed eGFP-tagged IHPK2 was located exclusively in the nucleus in HEK-293T cells. We transfected HEK-293T cells with IHPK2-eGFP and NLS-eGFP as in Figure 7. The IHPK2-eGFP fusion protein localized to both the cytoplasm and nucleus in untreated cells (Figure 9A, upper left). Tamoxifen (30 μ M, 24 h), which induces apoptosis in HEK-293T cells [19], caused translocation of IHPK2 into the condensing nuclei (Figure 9A, upper right). Transfection with NLS-eGFP resulted in restricted expression of the fusion protein in the cytoplasm in untreated and in apoptotic cells (Figure 9A, lower panels). Antiproliferative assays were performed to determine the functional consequences of NLS mutation. Expression of the NLS mutant rendered HEK-293T cells tamoxifen-resistant, whereas overexpression of IHPK2 increased cellular sensitivity to tamoxifen (Figure 9B). Data

points below the x -axis represent net cell death (fewer cells remaining at the end of the assay than were initially plated). TUNEL assays confirmed an apoptotic mode of death (results not shown).

DISCUSSION

Although IFN- α 2 has been employed more widely than IFN- β in the clinical treatment of human cancers, in many experimental systems IFN- β appears to be the more effective anti-tumour agent [13,20–23]. In the present study, IFN- β was a more potent inducer of cell death compared with IFN- α 2. The expression of IHPK2 clearly affected cellular sensitivity to the induction of apoptosis by IFNs. Not only did overexpression of IHPK2 sensitize cells to IFN- β , cells that were IFN- α 2-resistant were rendered IFN- α 2-sensitive. Hence IHPK2 modulates cellular responses to type I IFNs.

Neutralization of extracellular Apo2L/TRAIL with mAb blocked death induction by IFN- β , indicating that Apo2L/TRAIL was a crucial mediator of apoptosis after the administration of IFN- β . Moreover, Apo2L/TRAIL induction was necessary but not

Figure 8 (A) Effect of NLS mutation on IFN- β antiproliferative activity in ovarian carcinoma cells and (B) effect of caspase inhibitor on eGFP-IHPK2 translocation

(A) Untransfected NIH-OVCAR-3 cells (WT, wild-type), or cells stably transfected with vector alone (pCXN2), IHPK2, or the NLS mutant were grown in the presence of IFN- β for 7 days. Cells overexpressing IHPK2 were more sensitive, whereas cells expressing the NLS mutant were more resistant to IFN- β . (B) Cells transfected with eGFP-IHPK2 were exposed to IFN- β (200 units/ml) and either PBS or Z-VAD (300 μ M), a general caspase inhibitor for 16 h. The fusion protein fluorescence is shown in white.

sufficient for death induction in IFN- α 2-treated NIH-OVCAR-3 cells. Hence IHPK2 functions as a permissive factor for death induction in IFN- α 2-treated cells. Since Apo2L/TRAIL clearly played a role in this process, we initially hypothesized that in cells overexpressing IHPK2, IFN- β might be a more effective inducer of Apo2L/TRAIL. IFN- β was a more effective inducer of Apo2L/TRAIL when compared with IFN- α 2 (Figure 3). Augmentation of cellular IHPK2 (by transfection) was required to facilitate death induction by IFN- α 2 (Figure 1 and Table 1), yet cells that express high levels of IHPK2 (compared with vector-transfected cells) produce similar amounts of Apo2L/TRAIL. These results suggest that Apo2L/TRAIL is required for death by type I IFNs, and postulates an additional, unidentified factor specifically induced by IFN- β and absent after IFN- α 2 treatment. RNase protection assays, comparing induction of apoptotic regulatory factors by both IFNs, have not demonstrated clear differences (results not shown). Microarray experiments may identify an IFN- β -induced protein that interacts with IHPK2.

The Apo2L/TRAIL pathway may have clinical relevance in ovarian carcinoma. Patients whose ovarian tumours display high Apo2L/TRAIL expression exhibit prolonged survival when com-

pared with low expressors [24]. Extracellular proteins may inhibit apoptosis *in vivo*. Interleukin-8 (IL-8), present in ascitic fluid of ovarian carcinoma patients, appears to function as an antagonist of Apo2/TRAIL-induced apoptosis; indeed, IL-8 induces Apo2/TRAIL resistance in NIH-OVCAR-3 cells [25]. Even though many chemoresistant ovarian carcinomas are resistant to Apo2/TRAIL as a single agent, some of these cell lines undergo apoptosis when Apo2/TRAIL is combined with another agent (cisplatin, doxorubicin or paclitaxel) [26], reinforcing the concept that Apo2L/TRAIL expression is necessary but not sufficient to induce cell death.

The cellular localization of IHPK2 determines whether or not it can affect the death process. IHPK2 clearly could be found in both cytoplasm and nuclei of exponential phase dividing cells. During apoptosis the protein concentrated in the nuclei, especially in regions of condensed chromatin. The NLS mutant that did not translocate exerted a dominant negative effect on endogenous wild-type IHPK2. Cells expressing the mutant were relatively resistant to IFN- β -induced apoptosis (Figure 8A) and tamoxifen-induced apoptosis (Figure 9B). Hence trapping IHPK2 in the cytoplasm blunted its role in the death process.

Figure 9 (A) Localization of IHPK2 in HEK-293T cells and (B) effect of NLS mutation on tamoxifen antiproliferative activity in HEK-293T cells

(A) HEK-293T cells were transfected with the eGFP tagged constructs as in Figure 7. The fusion proteins fluoresce white. Tamoxifen (30 μ M, 24 h) was used to induce apoptosis. (B) Cells were stably transfected with vector alone (pCXN2), IHPK2, or the NLS mutant and grown in the presence of tamoxifen for 7 days. Cells overexpressing IHPK2 were more sensitive, whereas cells expressing the NLS mutant were more resistant to tamoxifen.

Several lines of evidence indicate that IHPK2 functions as a generalized pro-apoptotic regulator in disparate systems using different death inducers. Initially, IHPK2 was identified in a genetic screen as a protein that protected from IFN- β -induced death in ovarian carcinoma cells [12]. Subsequently, IHPK2 was found to sensitize cells to ionizing radiation [1]. Data above indicate that IHPK2 sensitizes embryonal kidney cells to apoptosis induced by tamoxifen (Figure 9B). Ongoing studies will identify IHPK2 binding partners that impinge on the apoptotic machinery.

Snyder and co-workers [18] have shown that episomally expressed eGFP-tagged IHPK2 was targeted exclusively to the nucleus in HEK-293T cells. In our investigation, transfection of these cells yielded detectable protein in both cytoplasm and nucleus. During the induction of apoptosis, IHPK2 concentrated in the nucleus (Figure 9A), as in NIH-OVCAR-3 cells. The apparent discrepancy regarding protein localization in the resting (non-apoptotic) state may be due to the very high levels of protein expression achieved with the pCXN2 vector. Saturation of the nuclear transport mechanism may result in back-up of excess IHPK2 into the cytoplasmic compartment.

Inositol phosphates affect several different cellular processes, including mRNA export from the nucleus [27,28], regulation of calcium stores and DNA repair [29]. Recently, Majerus and co-workers [30] have noted that the inositol 1,3,4-trisphosphate 5/6-kinase (5/6 kinase) inhibited TNF α (tumour necrosis factor α)-

induced apoptosis. This kinase, part of the same metabolic pathway as IHPK2, blocked TNF- α induced death through interference with the pro-apoptotic adapter protein TRADD at the DISC (death-inducing signalling complex). The receptors and signalling mechanisms of Apo2L/TRAIL and TNF- α are similar (but not identical), as they are members of the same superfamily. Further studies are required to determine whether IHPK2 and 5/6 kinase perform antagonistic roles.

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