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Inositol hexakisphosphate kinase 2 sensitizes ovarian carcinoma cells to multiple cancer therapeutics

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Abstract

We recently identified inositol hexakisphosphate kinase 2 (IP6K2) as a positive regulator of apoptosis. Overexpression of IP6K2 enhances apoptosis induced by interferon-β (IFN-β) and cytotoxic agents in NIH-OVCAR-3 ovarian carcinoma cells. In this study, we contrast and compare IFN-β and radiation-induced death, and show that IP6K2 expression sensitizes tumor cells. Unirradiated NIH-OVCAR-3 cells transfected with IP6K2 formed fewer colonies compared to unirradiated vector-expressing cells. IP6K2 overexpression caused increased radiosensitivity, evidenced by decreased colony forming units (CFU). Both IFN-β and radiation induced caspase 8. IFN-β, but not γ-irradiation, induced TRAIL in NIH-OVCAR-3 cells. Gamma irradiation, but not IFN-β, induced DR4 mRNA. Apoptotic effects of IFN-β or γ-irradiation were blocked by expression of a dominant negative mutant death receptor 5 (DR5Δ) or by Bcl-2. Caspase-8 mRNA induction was more pronounced in IP6K2-expressing cells compared to vector-expressing cells. These data suggest that overexpression of IP6K2 enhances sensitivity of some ovarian carcinomas to radiation and IFN-β. IP6K2 may function to enhance the expression and/or function of caspase 8 and DR4 following cell injury. Both IFN-β and γ-irradiation induce apoptosis through the extrinsic, receptor-mediated pathway, IFN-β through TRAIL, radiation through DR4, and both through caspase 8. The function of both death inducers is positively regulated by IP6K2.

Keywords

inositol hexakisphosphate kinase; apoptosis; radiation; interferon

Introduction

We have previously reported that inositol hexakisphosphate kinase 2 (IP6K2) mediates growth suppressive and apoptotic effects of IFN-β in ovarian carcinoma cells (Morrison *et al*., 2001). IP6K2 is a cytoplasmic kinase that catalyses the synthesis of diphosphoinositol pentakisphosphate ($InsP_5PP$, contains seven phosphates) using inositol hexaphosphate (IP_6, contains six phosphates) as a substrate in the presence of ATP (Saiardi *et al*., 1999). IP6K2

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maps to chromosome 3p21, a region highly susceptible to aberrant chromosomal rearrangements and deletions in human cancers (Bronner *et al*., 1994;Kok *et al*., 1987) including ovarian carcinomas (Decristofaro *et al*., 2001;Rimessi *et al*., 1994). Overexpression of IP6K2 causes prolongation of the cell cycle, without arrest in a specific phase (Morrison *et al*., 2001). Additionally, we found that inhibition of IP6K2 expression and enzymatic activity by use of antisense mRNA or a dominant negative mutant (SUB) resulted in cellular resistance to the antiproliferative effects of IFN-β and cytotoxic agents such as cis-platinum and etoposide. Hence, IP6K2 has many features of a tumor suppressor. Our studies utilized NIH-OVCAR-3 ovarian carcinoma cells, which lack functional p53 (Yaginuma and Westphal, 1992). This is consistent with earlier observations that normal p53 gene expression is not necessary for induction of death by type I IFNs (IFN-α and IFN-β) (Sangfelt *et al*., 1997).

Because IP6K2 overexpression sensitized several different cells to the apoptotic effects of cytotoxic agents, we hypothesized that this kinase may also play a role in γ-irradiation-induced apoptosis. Therefore, we designed the present experiments to determine if IP6K2 could radiosensitize ovarian tumor cells, and to further probe the mechanism by which IP6K2 regulates apoptosis in cells carrying mutant p53. Ionizing radiation results in activation of multiple genes, DNA strand breaks, and apoptosis in many different cell types. Radiation-induced apoptosis has been characterized as p53 dependent, as in thymocytes (Clarke *et al*., 1993) or p53-independent as in gliomas (Yount *et al*., 2001) and fibroblasts (Lackinger *et al*., 2001).

It is likely that γ-irradiation induces apoptosis by activation of both the mitochondrial (intrinsic) pathway and the TNF receptor superfamily (extrinsic) pathway. Irradiation of mitochondria has been shown to initiate apoptosis in a cell free system (Taneja *et al*., 2001). Induction of TNF-related apoptosis-inducing ligand, TRAIL/Apo2L has been observed following γ-irradiation of leukemic cells (Gong and Almasan, 2000). Upregulation of TRAIL receptors DR4 (Guan *et al*., 2001) and DR5 (Burns *et al*., 2001) and TRADD (Yount *et al*., 2001) following γ-irradiation has also been described. To assess the mode of death in ovarian carcinoma cells, we examined whether ionizing radiation would affect expression of any of these death associated gene products and whether overexpression of IP6K2 would affect their activity.

Results

Effect of γ-irradiation and IFN-β on expression of IP6K2

To study the effect of γ-irradiation and IFN-β on IP6K2 expression, we examined whether its mRNA levels were inducible. IP6K2 mRNA was not induced in NIH-OVCAR-3 cells following either treatment with IFN-β (Morrison *et al*., 2001) or γ-irradiation (data not shown). To determine whether γ-irradiation or IFN-β resulted in elevated IP6K2 protein levels, immunoblot (Western blot) analysis was performed using whole cell lysates. Densitometric quantitation of Western blots revealed that elevation of IP6K2 protein levels occurred at 8 h and 16 h after γ-irradiation (Figure 1a). Maximal increase occurred at 8 h (∼ threefold) at low, therapeutically relevant doses of γ-irradiation (1 – 2 Gy), and declined afterwards. Similarly, IP6K2 protein levels were induced at 8 through 48 h after IFN-β treatment (200 U/ml) (Figure 1b). Maximal elevation occurred at 8 h (fourfold induction) and declined thereafter. Hence, two diverse apoptotic stimuli resulted in elevation of IP6K2 protein levels in a time dependent manner. Both γ-irradiation and IFN-β induced IP6K2 protein via a post-translational mechanism.

Effect of IP6K2 on clonogenic survival following γ-irradiation

Clonogenic assays were performed using NIH-OVCAR-3 cells that expressed full length IP6K2 or different regions of the IP6K2 protein. To determine which domain of IP6K2 is responsible for cytotoxicity, the IP6K2 protein was expressed as three individual regions, A, B and C (Figure 2a). The B region contains the putative IPBD, which may be required for inositol phosphate (substrate) binding (Togashi *et al*., 1997). Expression of various myc-tagged IP6K2 constructs was verified in NIH-OVCAR-3 cells by Western blotting (Figure 2b). These included full length IP6K2 (FL), as well as the dominant negative mutant (SUB) that contains point mutations in the IPBD. Overexpression of the SUB mutant inhibits endogenous IP6K enzyme activity (Morrison *et al*., 2001).

Unirradiated cells expressing full length IP6K2 formed fewer and smaller colonies compared to unirradiated vector expressing cells (Figure 3a). Full length IP6K2 overexpression caused increased sensitivity to γ-irradiation, resulting in decreased colony forming units (CFU) (Figure 3b). Compared to unirradiated cells, in vector-expressing cells (white bars), the percentage of CFU formed following 2 and 4 Gy were reduced by 34 and 84%, respectively. In comparison, in IP6K2-expressing cells (black bars), the percentage of CFU formed following 2 and 4 Gy were reduced by 80 and 96%, respectively. Thus, IP6K2 expressed caused a 3.3-fold and 4.4-fold reduction in CFU formation at 2 and 4 Gy, respectively. A linear-quadratic model was used to fit the dose survival curves. The linear parameter α, and the quadratic parameter β for vector expressing (pCXN2) and IP6K2 expressing cells, are listed in Table 1. To assess the effect of IP6K2 expression on radiosensitivity, Dose Modification Factor (DMF) was calculated by determining D_{50}, the radiation dose associated with 50% CFU for each cell line. Hence, DMF is the ratio of pCXN2 D_{50}/IP6K2 D_{50}, and IP6K2 overexpression translated into a DMF of 1.81.

Expression of IP6K2 region B alone (vertical stripes) conferred a twofold enhancement of radiosensitivity. In contrast, expression of IP6K2 region A or C alone had no effect on sensitivity to γ-irradiation. NIH-OVCAR-3 cells expressing the dominant negative mutant SUB (gray bars) displayed radioresistance, reflected as 20 and 56% increases in CFU at both dose levels. Cells that overexpressed Bcl-2 (stippled bars) were most radioresistant of all NIH-OVCAR-3 transfectants; compared to vector-expressing cells, CFU were increased 1.4-fold and threefold after 2 and 4 Gy, respectively. DR5Δ was also effective at blocking radiation-induced death; CFU were increased 1.3-fold and 2.1-fold after 2 and 4 Gy, respectively. We also studied the effects of IP6K2 expression in Caov-3 and Hey ovarian carcinoma cells, which are both relatively resistant to many cytotoxic agents compared to NIH-OVCAR-3 cells. Caov-3 cells exhibited a phenotype very similar to NIH-OVCAR-3 cells, whereas IP6K2 overexpression did not radiosensitize Hey cells (data not shown).

IP6K2 enhances IFN-β-induced growth suppression

We have previously demonstrated that IFN-β caused growth inhibition in NIH-OVCAR-3 cells *in vitro* (Morrison *et al*., 2001). To study the effect of IP6K2 on IFN-β-induced growth suppression, we treated NIH-OVCAR-3 cells expressing vector alone, full length IP6K2, Bcl-2 or DR5D (Figure 4a). Cells were grown in the presence of different doses of IFN-β. After 1 week, cell growth was measured using a colorimetric assay based on binding of the chromophore sulforhodamine B (Skehan *et al*., 1990). The amount of dye retained directly correlates with cell number. IP6K2 expression caused enhanced growth suppression in response to IFN-β. Complete cytostasis occurred at ∼200 U/ml for IP6K2 express-ing cells compared to ∼500 U/ml for pCXN2 expressing cells. Cell death was evident at 250 – 500 U/ml in IP6K2 transfected cells. Bcl-2 effectively blocks growth suppressive effects of IFN-β, reducing the per cent inhibition from 95 (vector expressing cells) to 50% (Bcl-2 expressing cells) at the highest dose tested. DR5Δ overexpression resulted in near-complete protection

against IFN-β. These cells were inhibited only 15% in their growth at the highest dose of IFN-β. Thus, IFN-β mediates growth suppression and apoptosis through pathways that can be suppressed by Bcl-2 and blockade of TRAIL receptors.

To assess the effect of combination treatment we first irradiated NIH-OVCAR-3 cells and then grew them in the presence of IFN-β as above. IFN-β as a single agent generated a dose response curve similar to Figure 4a. As expected, γ-irradiation resulted in increasing growth inhibition as the dose was increased from 0.2 – 4 Gy (Figure 4b). The degree of growth inhibition in the combination group was only marginally greater than that seen following IFN-β as a single agent. Median effect analysis (of which, isobol analysis is a subset) was used to determine the mode of interaction between these two forms of treatment (Chou and Talalay, 1984) (Inset, Figure 4b). The x-axis represents fraction affected and the y-axis represents combination index (C.I.). C.I.>1 corresponds to antagonism, C.I.<1 corresponds to synergism, and C.I. = 1 corresponds to additivity. At all three doses tested, the C.I. indicated an additive mode of interaction between radiation and IFN-β-induced growth inhibition, rather than a synergistic mode of interaction. This analysis suggested that IFN-β and γ-irradiation may utilize a common pathway for growth suppression and induction of apoptosis.

Effect of IFN-β and IP6K2 on radiation-induced gene expression

To determine whether IFN-β was inducing death-associated gene mRNAs, ribonuclease protection assays (RPAs) were performed. In untreated cells, caspase 8 and TRAIL mRNAs were barely detectable; IFN-β induced their expression in a dose-dependent manner after 8 h (Figure 5a). Caspase 8 and TRAIL expression could be completely blocked with actino-mycin D, indicating a transcriptional control of their expression. Therefore, cell death induced by IFN-β is associated with caspase 8 and TRAIL expression and death can be abrogated by a dominant negative mutant TRAIL receptor (DR5Δ).

Since upregulation of death-associated genes by γ-irradiation has been described in several different cell lines (Burns *et al*., 2001;Gong and Almasan, 2000;Guan *et al*., 2001;Yount *et al*., 2001) we used RPAs to assess caspase and death receptor expression in ovarian carcinoma cells. In untransfected (not shown) or vector-transfected NIH-OVCAR-3 cells (Figure 5b, lanes 5 – 7) we observed induction of caspase 8 and DR4 following 2 Gy. Quantitation of these band densities indicated a 2 – 4-fold induction of caspase 8 and DR4, with the greatest increase at the 4 h time point (Figure 5c, white bars). Importantly, expression of both of these genes was markedly enhanced in IP6K2-overexpressing cells (Figure 5b, lanes 8 – 14). Induction was detectable at lower radiation doses (1 Gy) and at earlier time points (Figure 5c, gray bars). Peak induction was sevenfold and ninefold for caspase 8 and DR4, respectively. Death receptor and caspase mRNA induction was blocked by pretreatment with actinomycin D (not shown). A different pattern of gene expression was observed in the radioresistant Hey cells (Figure 5b, lanes 15 – 21). They expressed higher levels of fas, relative to NIH-OVCAR-3 cells, but there was no induction of caspase 8 or DR4 mRNA following irradiation.

Gamma-irradiation, IFN-β, and IP6K2 expression enhance caspase 8 activity

To determine whether the increases in mRNA translated into increased enzymatic activity, cell lysates were analysed for caspase 8 activity following various treatments. After 2 Gy, vector-transfected cells displayed a time-dependent increase in caspase 8 activity, peaking at 24 h and declining thereafter (Figure 6a, white bars). Cells overexpressing IP6K2 had enhanced caspase 8 activity compared to vector controls (Figure 6a, gray bars), peaking at 48 h. Maximal fold induction of caspase 8 activity was 2.6 and 25.3 in vector- and IP6K2-transfected cells, respectively. Greater enzymatic activity in IP6K2-transfected cells is consistent with the increase in caspase 8 mRNA observed in RPAs (Figure 5b). Assays performed with IFN-β-treated cells demonstrated a similar pattern of activity. IP6K2-expressing cells (Figure 6b, gray

bars) had enhanced caspase 8 activity compared to vector-transfected cells (Figure 6b, white bars). Similar assays were performed in Hey cells. There was no induction of caspase 8 activity, in either vector- or IP6K2-expressing cell lines following γ-irradiation or IFN-β(not shown). Hence, in NIH-OVCAR-3 cells, but not Hey cells, caspase 8 mRNA and enzymatic activity were elevated following death induced by two different stimuli.

Discussion

In ovarian carcinoma cell lines, IFN-β and γ-irradiation induce apoptosis using similar but not identical pathways. In sensitive cells, IFN-β upregulates TRAIL, and to a lesser extent, caspase 8 (Figure 5a). IFN-β-induced apoptosis has been associated with TRAIL induction in several different tumor cell lines (Chawla-Sarkar *et al*., 2001;Zhang *et al*., 1999). γ-irradiation clearly upregulates caspase 8 and DR4 mRNA (Figure 5b) as well as caspase 8 activity (Figure 6a). This is consistent with our previous study showing induction of apoptosis following ionizing radiation (Gong and Almasan, 2000). Transcriptional induction of DR4 following radiation has also been reported in lung carcinoma cell lines (Guan *et al*., 2001). Our studies are consistent with others, who have described caspase 8 activation following radiation of Jurkat cells (Belka *et al*., 2000). In three different ovarian carcinoma cell lines, we observed no induction of TRAIL following radiation treatment. This is in contrast to hematologic cells in which both TRAIL and Fas upregulation has been described (Gong and Almasan, 2000). Combination therapy utilizing TRAIL and cytotoxic agents (cisplatin, doxorubicin, or paclitaxel) has been proposed, in an effort to overcome the chemoresistance that usually develops following treatment of women with ovarian carcinoma (Cuello *et al*., 2001). The p53 status of ovarian carcinoma cells did not correlate with sensitivity to death inducers. In fact, cell lines with mutant p53 (NIH-OVCAR-3, Caov-3) were more sensitive to IFN-β and γ-irradiation than cells containing wild-type p53 (Hey).

We found that DR5Δ protects against apoptosis initiated by both IFN-β and γ-irradiation, confirming that TRAIL : DR-mediated signaling occurs following treatment with both death inducers. Thus, downstream effects of TRAIL and one of its receptors can be blocked by DR5Δ. Combination treatment with both modalities resulted in additive, rather than synergistic antiproliferative effects (Figure 4b). Such additivity is consistent with upregulation of the ligand TRAIL and its receptor DR4, components of the same apoptotic pathway. DR4 and DR5, both agonistic receptors for TRAIL, form homomeric and heteromeric complexes upon ligand binding (Kischkel *et al*., 2000). Recent reports indicate that ligation of DR4 (Sprick *et al*., 2000) and DR5 (Bodmer *et al*., 2000) lead to caspase 8 activation, using a signaling paradigm that is similar, but not identical, to those induced by TNF-α and FasL. Caspase 8 is activated as part of the death-inducing signaling complex (DISC). We show that caspase 8 activity was induced more strongly by radiation compared to IFN-β (Figure 6a,b). Yet caspase 8 activity following combination treatment with IFN-β and γ-irradiation was no greater than that following radiation alone (not shown). It is possible that in NIH-OVCAR-3 cells, caspase 8 activity is already maximized following radiation.

Overexpression of IP6K2 enhanced the degree of apoptosis induced by several different stimuli, including chemotherapeutic drugs, IFNs, and γ-irradiation. Expression of the dominant negative SUB mutant interferes with kinase activity (Morrison *et al*., 2001) and abrogates death induced by all these agents. IP6K2 protein levels are elevated following IFN-β and irradiation (Figure 1). These findings suggest that IP6K2 functions as a positive regulator of cell death pathways. The mechanism by which this inositol phosphate kinase promotes apoptosis is unclear. IP6K2 converts IP_6 to $InsP_5PP$. It is possible that $InsP_5PP$, which contains a pyrophosphate linkage, may function as an energy source that facilitates DISC assembly or signal transduction to the caspase cascade. Proteins that bind IP_6 include the clathrin assembly proteins AP-2, AP-3, and AP-180 (involved in membrane fusion) (Norris *et al*.,

1995;Voglmaier *et al*., 1992;Ye *et al*., 1995), the synaptotagmins (involved in exocytosis of synaptic vesicles) (Mehrotra *et al*., 2000) and arrestin (which acts as an adapter during endocytosis) (Gaidarov *et al*., 1999). All these proteins are closely associated with the plasma membrane. Studies in yeast have suggested that the higher inositol phosphates play a role in mRNA export from the nucleus (Saiardi *et al*., 2000;York *et al*., 1999). Finally, IP_6 was found to specifically stimulate repair of double-strand breaks in DNA by the process of nonhomologous end-joining *in vitro* (Hanakahi *et al*., 2000). If the functions of $InsP_5PP$ and IP_6 prove to be antagonistic, $InsP_5PP$ may inhibit DNA repair and thereby promote apoptosis.

Materials and methods

Reagents

Mouse anti-IP6K2 monoclonal antibody raised against full-length bacterially expressed rhIP6K2 (made in our laboratory); Human IFN-β specific activity 2.7×10^8 U/mg protein (Ares Serono); Rnasin (Promega); GACU, T7 RNA polymerase, T1 RNase (GIBCO BRL); DNase I (Boehringer Mannheim); Multi-Probe Template Set hAPO-3c, (Pharmingen); and monoclonal anti-actin antibodies (Sigma) were employed in these studies.

Cell lines and treatments

NIH-OVCAR-3 (mutant p53) and Caov-3 cells (mutant p53) were obtained from American Type Culture Collection (Rockville, MD, USA). Hey cells (wild-type p53) were obtained from Dr Yan Xu (Cleveland Clinic Foundation). Gamma irradiation was delivered by a ^{137}Cs source emitting at a fixed-dose rate of 5 Gy/min.

Plasmids

Three regions of the IP6K2 cDNA were cloned into the pCXN2 vector (Murakami *et al*., 1995) to create truncation mutants: (A) residues 1 – 153; (B) residues 154–338; (C) residues 339–419, utilizing a PCR technique described previously (Morrison *et al*., 2001). A C-terminal myc tag was added, which allowed detection of expressed IP6K2 proteins by means of anti-myc antibodies (Zymed). A substitution mutant (SUB) was created using PCR based site-directed mutagenesis (Stratagene) with full-length IP6K2 as template, creating point mutations at seven of nine residues within the highly conserved putative inositol phosphate binding domain (IPBD) (Morrison *et al*., 2001). PCR products were digested with *DpnI*, ligated, then transformed into JM109 *E. coli*, and plasmids isolated. Mutations were confirmed by sequencing. Similarly, Bcl-2 and DR5Δ were cloned into pCXN2. DR5Δ lacks the death domain of DR5, and functions as a dominant negative mutant, interrupting the function of endogenous DR5 (Gong and Almasan, 2000;MacFarlane *et al*., 1997). Constructs were transfected into cells using Lipofectamine (Gibco BRL) and stable transfectants were selected and maintained with G418. After 3 weeks selection, surviving clones were pooled for further studies. Expression of IP6K2 mutants was monitored by Western blotting using anti-myc antibodies.

Clonogenic assay

NIH-OVCAR-3 and Hey ovarian carcinoma cells were stably transfected with pCXN2 vector alone or with various IP6K2 constructs, or Bcl-2 or DR5Δ. Cells were irradiated with a ^{137}Cs source (Shepherd) at a constant rate of 5 Gy/min for a total dose of 0, 2, or 4 Gy and were plated in 10 cm dishes and incubated at 37°C. After 30 days growth, cells were fixed with 10% TCA (trichloroacetic acid), stained with 0.4% sulforhodamine-B (SRB) in 1% acetic acid, and washed in 1% acetic acid. Colony forming units (CFU) were counted and individual colony area was determined (Kodak 1DS system). A colony was defined as a single mass of 50 cells or greater.

Western blot analysis

Total cell protein (80 μg) was separated on 10% SDS – PAGE and transferred to PVDF membrane. To detect endogenous IP6K2, membranes were incubated with IP6K2 mouse monoclonal antibody. After washing, membranes were incubated with Goat Anti-Mouse IgG (H+L)-HRP conjugate (BIO-RAD) and developed using Western blot Chemiluminescence Reagent Plus (NEN Life Science Products). Myc-tagged IP6K2 constructs were detected with mouse anti-c myc antibodies (Zymed). Blots were stripped and re-probed with anti-actin antibodies (Sigma). IP6K2 band densities were quantified and expressed as a ratio compared to actin.

Ribonuclease protection assay (RPA)

NIH-OVCAR-3 cells were stably transfected with pCXN2 vector alone or with IP6K2. Some cells were pre-treated with actinomycin D (10 μg/ml) for 1 h. NIH-OVCAR-3 and Hey cells received 0, 1 or 2 Gy, as was done in the clonogenic assay. At 0, 1, 2 and 4 h, total RNA was isolated with RNAzol (Teltest). Radiolabeling of the multi-probe template set was performed as described (Gong and Almasan, 2000) according to the manufacturer's recommendation (hAPO-3c, Pharmingen). Total RNA (10 μg) was hybridized with multi-probe for 16 h. After RNase treatment, R Nase-protected fragments were resolved on 4.5% denaturing polyacrylamide gels and subject to autoradiography. Band intensities were digitally quantified and expressed as a ratio compared to the ribosomal gene L32.

Cell growth assay

NIH-OVCAR-3 cells transfected with pCXN2 vector alone, or with IP6K2, or Bcl-2, or DR5Δ were treated with IFN-β during growth in RPMI-1640 (Cellgro) and 5% FBS (HyClone). Growth was monitored using a colorimetric assay that measured cell number (Skehan *et al*., 1990). Each treatment group contained eight replicates. Cells were fixed with TCA 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,

$$\text{Per cent Control Growth} = 100\,\% * \left(A_{exp} - A_{ini}\right) / \left(A_{fin} - A_{ini}\right) \quad (1)$$

Expressed as a per cent of untreated controls, a decrease in cell number (death) is represented as a negative value on the y-axis.

Caspase-8 activity assay

Cells received 0 or 2 Gy, or 200 U/ml IFN-β. At various times, cells were counted and lysed for each caspase 8 assay (Clonetech). The substrate, IETD-AFC, consists of a synthetic peptide (sequence: IETD) conjugated to the chromophore, 7-amino-4-trifluoromethyl coumarin (AFC), that is cleaved by activated caspase 8 and is detected fluorometrically using a 400 nm excitation filter and a 505 nm emission filter. Reactions were performed in triplicate.

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Abbreviations

AFC, 7-amino-4-trifluoromethyl coumarin; CFU, colony forming unit; DISC, death-inducing signaling complex; DR, death receptor; DMF, dose modification factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gy, gray; IETD, Ile-Glu-Thr-Asp; IFN, interferon; IP6K2, inositol hexakisphosphate kinase 2; RPA, ribonuclease protection assay; SRB, sulforhodamine B; TRAIL, TNF-related apoptosis-inducing ligand.

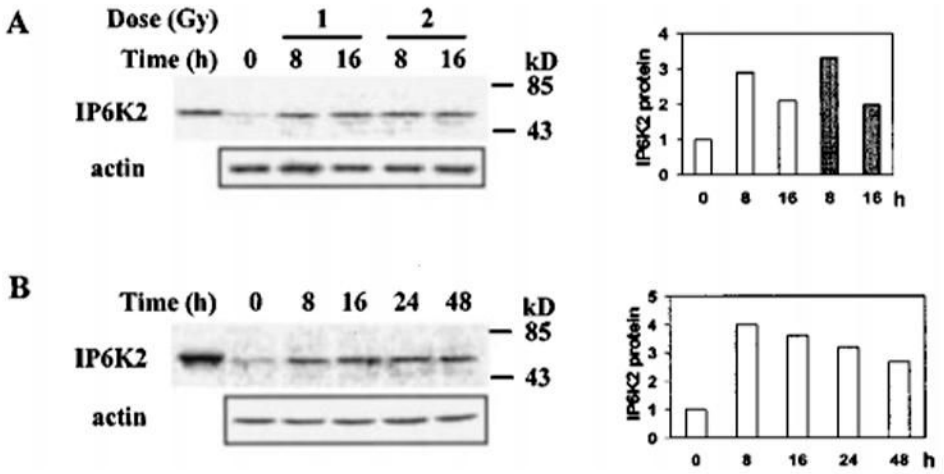

Figure 1.

Effect of γ-irradiation and IFN-β on IP6K2 protein levels. (**a**) NIH-OVCAR-3 cells growing at 75% confluence were trypsinized, suspended in 1 ml PBS, and subject to 0, 1 (white bars) or 2 Gy (gray bars) irradiation and replated in complete medium. At 8 and 16 h after plating, cells were harvested, lysed and subject to Western blot analysis using monoclonal anti-IP6K2 antibodies. To control for loading, blots were probed with anti-actin antibodies; bands were digitized and quantified. IP6K2 signal intensity was normalized to actin, then expressed as fold induction, with unirradiated cells representing a protein level of 1. Purified rIP6K2 was included as a positive control (lane 1). Numbers to right of blots indicate MW markers. Only relevant portion of the blots are shown. (**b**) NIH-OVCAR-3 cells were grown in the presence of 200 U/ml IFN-β for 0 – 48 h and then subject to Western blot analysis and quantified as above

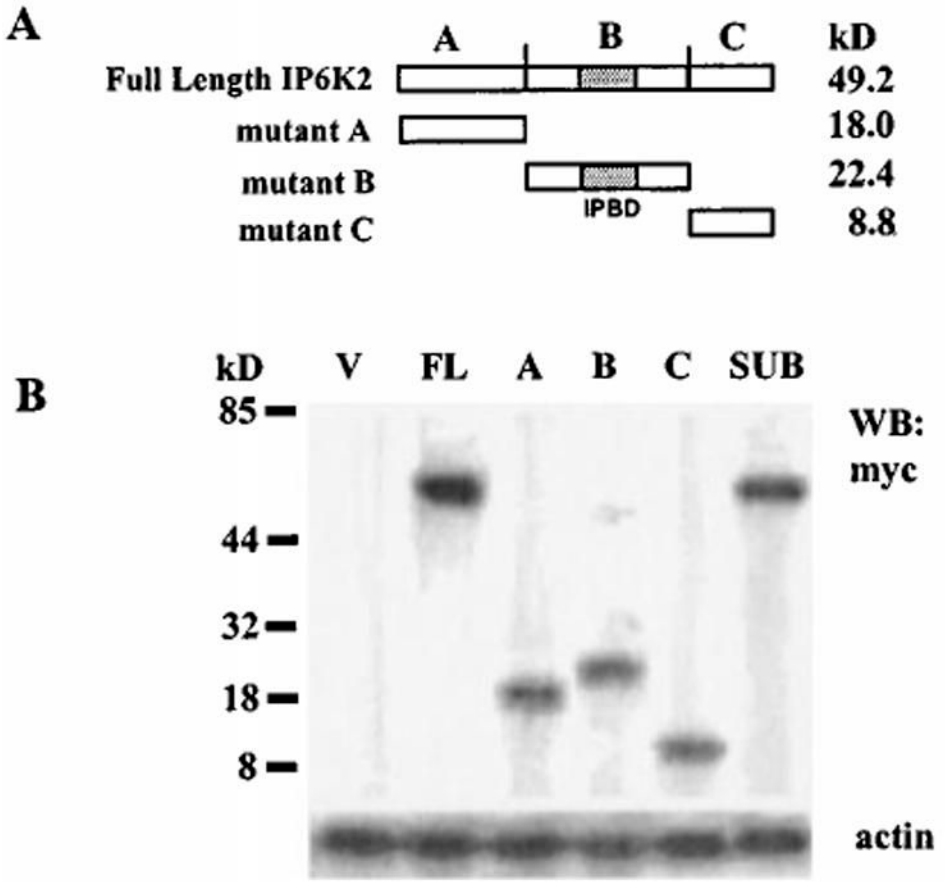

Figure 2.

IP6K2 constructs. (**a**) Diagrammatic representation of IP6K2 protein. The putative inositol phosphate binding domain (IPBD) is shown in gray. SUB, the IP6K2 dominant negative substitution mutant, contains seven point mutations within the IPBD. SUB expression inhibits cell death and IP6K enzymatic activity in NIH-OVCAR-3 cells (Morrison *et al*., 2001). (**b**) Western blot of myc-tagged IP6K2 proteins. Stably transfected NIH-OVCAR-3 cells were probed with anti-myc to verify expression of full length (FL) IP6K2 or mutants depicted above. No myc signal was detected in vector-transfected cells (V). Numbers to left of blot indicate MW markers

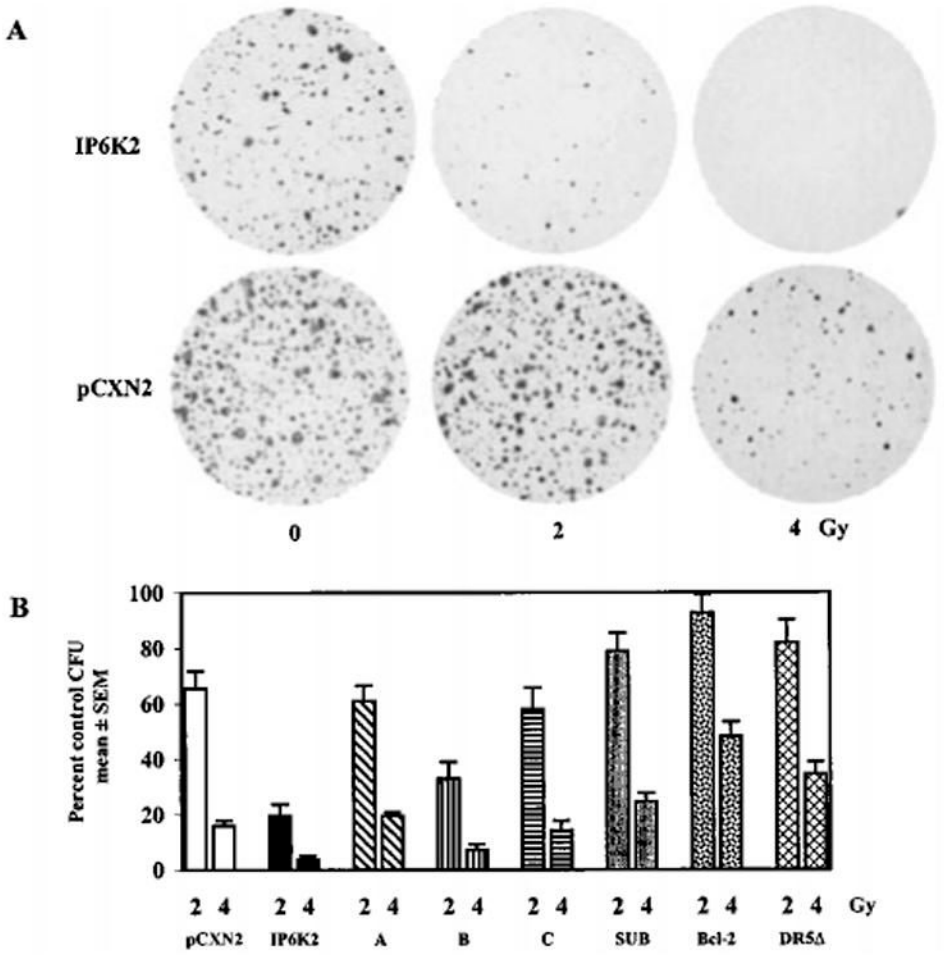

Figure 3.

Clonogenic assay: SUB mutant, Bcl-2, and DR5Δ are radioprotective. (**a**) NIH-OVCAR-3 cells stably expressing pCXN2 vector or IP6K2 received 0, 2 or 4 Gy irradiation and were plated at a density of 5000 cells per 10 cm dish and grown for 30 days. Colonies were fixed with TCA and stained with sulforhodamine B. Representative plates from each treatment group are shown. (**b**) Colonies were identified and counted using the Kodak 1DS system. Average colony size was smaller in IP6K2 expressing cells compared to vector transfected cells at equivalent dose levels (not shown). CFU were determined for the indicated NIH-OVCAR-3 cells stably expressing various IP6K2 mutants and indicated proteins. Compared to pCXN2 vector-expressing cells, IP6K2 and B-region expressing cells displayed greatly reduced CFU. In contrast, SUB, Bcl-2, and DR5Δ expression caused increased CFU, whereas A-region, C-region CFU were no different from vector control

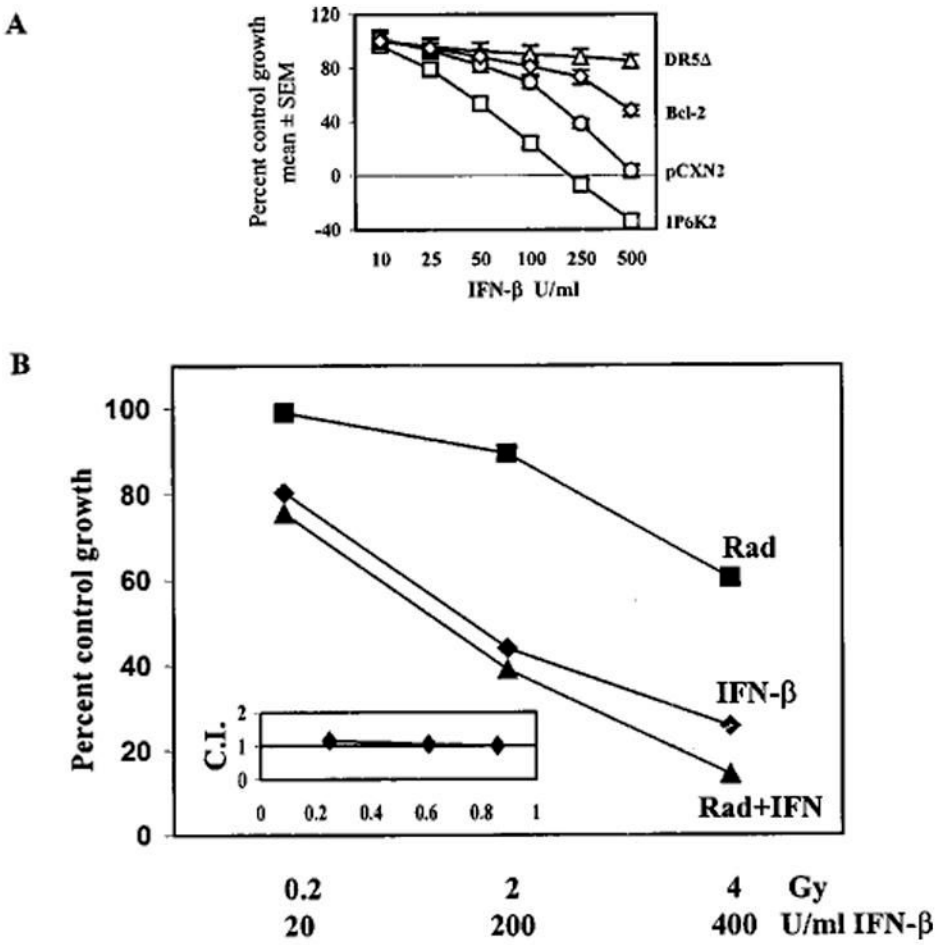

Figure 4.

Antiproliferative effects of IFN-β are TRAIL-dependent. (**a**) NIH-OVCAR-3 cells stably expressing pCXN2, IP6K2, Bcl-2, or DR5Δ were grown in 96-well plates for 7 days in the presence of 0–500 U/ml IFN-β. At the end of the growth period, cells were fixed and stained with sulforhodamine B. Absorbance (570 nm) of bound dye was measured and expressed as a per cent of untreated controls. Each data point represents mean ± s.e. of eight replicates. Negative values on the γ-axis indicate death of initially plated cells. (**b**) Combination γ-irradiation and IFN-β treatment. Cells received 0.2, 2, or 4 Gy and were grown in the presence of 20, 200, or 400 U/ml IFN-β. After 7 days cells were fixed and growth was expressed as a percentage of control (untreated) cells. Inset graph represents combination index (C.I.) plotted as a function of cell fraction affected (FA). No growth inhibition corresponds to FA = 0 and total growth inhibition corresponds to FA = 1. C.I. is calculated for each of the three dose levels. C.I. = 1 indicates that antiproliferative effects exerted by γ-irradiation and IFN-β treatment are additive

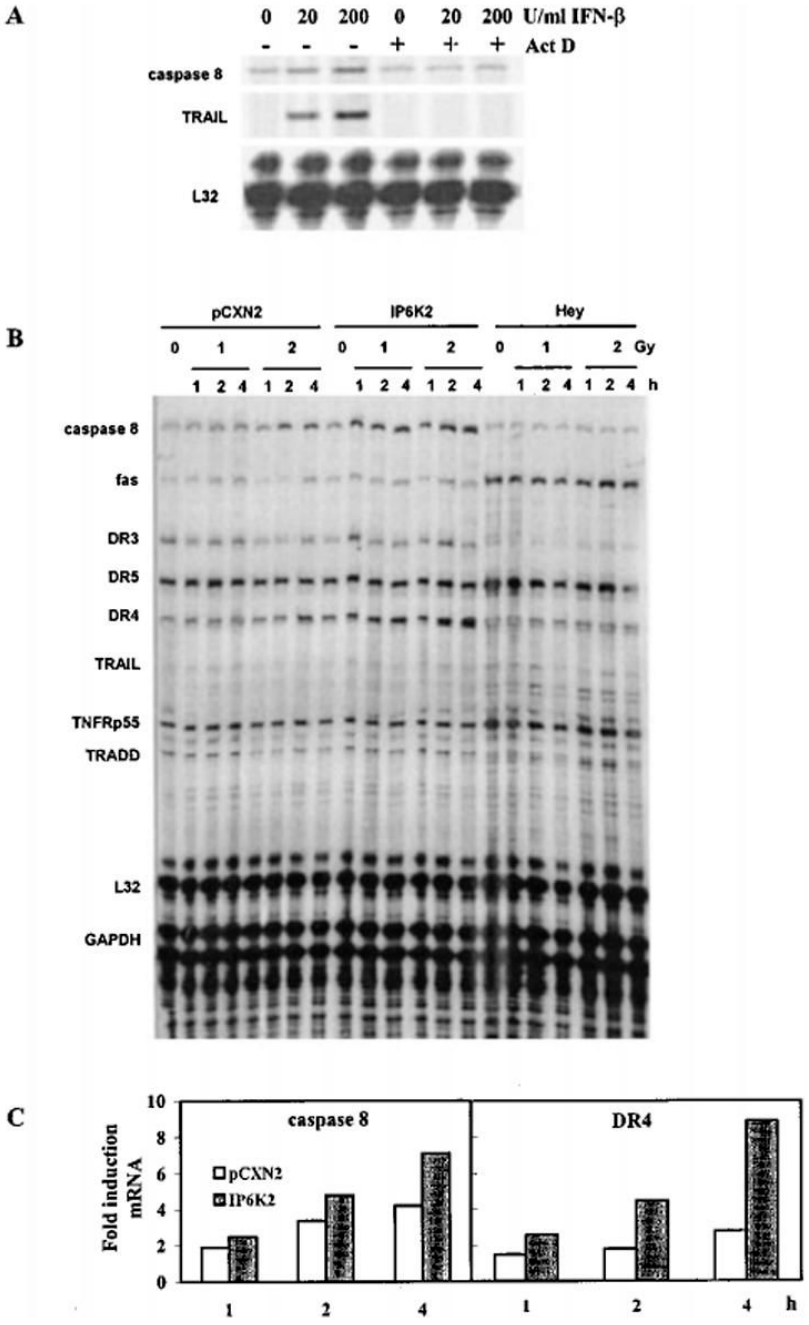

Figure 5.

Ribonuclease protection assay for death-associated genes. (**a**) Ribonuclease protection assay for caspase 8 and TRAIL mRNAs. Untransfected NIH-OVCAR-3 cells were cultured in the presence of 20 or 200 U/ml IFN-β for 8 h. Some cells received 10 μg/ml actinomycin D for 1 h prior to IFN-β treatment. Total RNA was harvested and analysed by RPA. L32 ribosomal RNA indicates equal amounts of RNA have been loaded. Only the relevant portions of the autoradiogram are shown. (**b**) NIH-OVCAR-3 cells expressing pCNX2 or IP6K2, and Hey cells, received 0, 2, or 4 Gy prior to harvesting total RNA. Transfected plasmids, dose of radiation, and times of harvest are indicated at the top of gel. Equal amounts of RNA (10 μg) were subject to ribonuclease protection assay after hybridization to a set of commercially

available death associated gene probes labeled with ^{32}P. Protected RNAs were resolved on polyacrylamide gels, subject to autoradiography and digitized by phosphorimager. The positions of death-associated genes and controls (ribosomal protein L32, and glyceraldehyde-3-phosphate dehydrogenase, GAPDH) are indicated. A negative control reaction (yeast tRNA) showed no evidence of these bands (data not shown). (**b**) Bands representing induced caspase 8 and DR4 mRNAs were quantitated by normalizing their intensities to L32 band densities. These band intensity ratios are expressed as fold induction for vector-transfected (white bars), and IP6K2-transfected (gray bars) cells

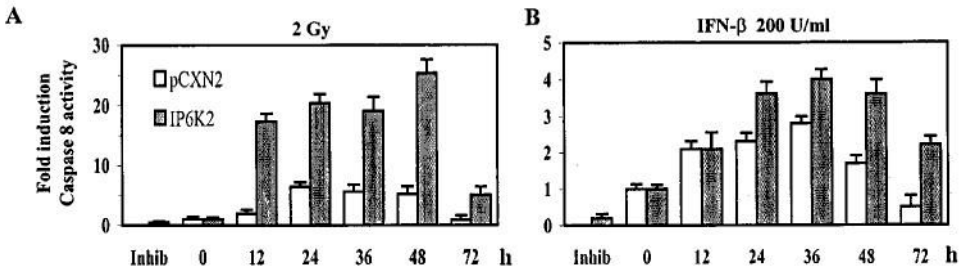

Figure 6.

Caspase 8 activity is increased by γ-irradiation and IFN-β. NIH-OVCAR-3 cells expressing pCNX2 (white bars) or IP6K2 (gray bars) were exposed to (**a**) 2 Gy or (**b**) 200 U/ml IFN-β and were assayed for caspase 8 activity at 0 – 72 h. Bars represent caspase 8 activity ± s.e.m. of three independent experiments. Enzyme activities were normalized to values obtained with untreated cells (0 h) and expressed as fold induction for each cell type. Column 1 in each graph represents caspase 8 activity in the presence of IETD-fmk (Inhibitor)

Table 1

Effect of IP6K2 expression on NIH-OVCAR-3 cell radio-sensitivity

	pCXN2	***IP6K2***
α	0.2873 ± 0.0266[a]	0.2874 ± 0.0334
β	0.0182 ± 0.0026	0.0208 ± 0.0032
D_{50} (Gy)	2.61 (2.53 − 2.69)[b]	1.44 (1.40 − 1.49)
DMF	-	1.81

[a] α and β represent linear and quadratic parameters. respectively, mean ± s.e.m.

[b] Numbers in parentheses are 95% confidence intervals