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RNAi-Mediated Silencing of Insulin Receptor Substrate-4 Enhances Actinomycin D- and Tumor Necrosis Factor-α-Induced Cell Death in Hepatocarcinoma Cancer Cell Lines

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ABSTRACT

Insulin receptor substrate-4 (IRS-4) transmits signals from the insulin-like growth factor receptor (IGF-IR) and the insulin receptor (IR) to the PI3K/AKT and the ERK1/2 pathways. IRS-4 expression increases dramatically after partial hepatectomy and plays an important role in HepG2 hepatoblastoma cell line proliferation/differentiation. In human hepatocarcinoma, IRS-4 overexpression has been associated with tumor development. Herein, we describe the mechanism whereby IRS-4 depletion induced by RNA interference (siRNA) sensitizes HepG2 cells to treatment with actinomycin D (Act D) and combined treatment with Act D plus tumor necrosis factor-α (TNF-α). Similar results have been obtained in HuH 7 and Chang cell lines. Act D therapy drove the cells to a mitochondrial-dependent apoptotic program involving cytochrome *c* release, caspase 3 activation, PARP fragmentation and DNA laddering. TNF-α amplifies the effect of Act D on HepG2 cell apoptosis increasing c-jun N-terminal kinase (JNK) activity, IκB-α proteolysis and glutathione depletion. IRS-4 depleted cells that were treated with Act D showed an increase in cytochrome *c* release and procaspase 3 and PARP proteolysis with respect to control cells. The mechanism involved in IRS-4 action is independent of Akt, IκB kinase and JNK. IRS-4 down regulation, however, decreased γ-glutamylcysteine synthetase content and cell glutathione level in the presence of Act D plus TNF-α. These results suggest that IRS-4 protects HepG2 cells from oxidative stress induced by drug treatment. J. Cell. Biochem. 108: 1292–1301, 2009.

KEY WORDS: HEPATOCARCINOMA; APOPTOSIS; CASPASE-3; CYTOCHROME *C*; GLUTATHIONE

Insulin receptor substrate (IRS) proteins are tyrosine phosphorylated and mediate multiple signals during activation of the receptors for insulin, insulin-like growth factor-I (IGF-I) and various cytokines [White, 2002]. Upon receptor activation, IRS proteins are rapidly phosphorylated at the carboxyl-terminus, resulting in activation of the phosphatidylinositol 3-kinase (PI3K) and the extracellular signal-regulated kinase (ERK) pathways [White, 2002]. The modulation of these pathways by IRS-1, IRS-2 and IRS-4 plays different roles in apoptosis [Uchida et al., 2000; Tseng et al., 2002]. Our previous results suggest that the insulin receptor [Escribano et al., 2002], insulin-stimulated IRS-4 [Escribano et al., 2003] and IGF-I-stimulated IRS-4 [Cuevas et al., 2007] play a role in

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proliferation of rat hepatocytes and human hepatoblastoma cells. Recently, some authors have ascribed a new oncogenic role to IRS proteins [Dearth et al., 2006, 2007]. In this context, IRS-4 is expressed at a low level in normal human liver and is highly expressed in the tumors of patients affected with hepatocellular carcinoma [Cantarini et al., 2006], suggesting its role in this disease.

Hepatocellular carcinoma (HCC) is the fifth most common malignancy, resulting in approximately 662,000 deaths annually [Bosch et al., 2005]. HepG2 cells are a classical model of hepatoblastoma resistant to TNF-α, but rendered susceptible to the cytokine in the presence of actinomycin D. Currently, several clinical trials are evaluating the efficacy of local TNF delivery and systemic treatment with actinomycin D or TNF-α plus actinomycin D in the therapy of advanced solid tumors [McLoughlin et al., 2005] and paediatric cancer [Meany et al., 2008]. In humans, however, TNF-α administration is hampered by systemic side-effects [Lejeune et al., 1994]. The maximum tolerated dose range is at least ten fold less than the efficient dose in animals [Lejeune et al., 1994]. It has been previously shown that an anti-IRS-1 RNAi strategy can enhance tamoxifen-induced death of cancer cells [Cesarone et al., 2006]. The present study explores the role of IRS-4 on the sensitization of HepG2 cells to actinomycin D alone or in combination with TNF-α in the induction of apoptosis. The mechanism of action of IRS-4 is also explored.

MATERIALS AND METHODS

REAGENTS

The antibody against IRS-4 was from Upstate Biotechnology (Lake Placid, NY). Antibodies against ERK1/2, pMEK1/2, phospho-54/46 JNK (c-jun N-terminal kinase) and β-tubulin were from Cell Signaling Technology Inc. (Davers, Massachusetts/MA). Antibodies against phospho-ERK, IκB-α, caspase-3, cytochrome *c* and phospho-AKT (Ser473) were from Santa Cruz Biotechnology Inc. Antibodies against PARP and FAK were from BD Biosciences Pharmingen (Franklin Lakes, NJ). The anti-GCS antibody was from NeoMarkers (Fremont, CA). All other reagents were of the highest purity grade available.

CELL CULTURES AND TRANSFECTION WITH siRNA OLIGOS

HepG2, HuH 7 and Chang cells (Sigma Chemical Co., Madrid, Spain) were grown in MEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution (both from Invitrogen, Barcelona, Spain), and incubated in a humidified 5% CO_2 atmosphere at 37°C. TNF-α plus actinomycin D treatment and their controls were conducted in the presence of 5% FBS.

Cells were transfected with IRS-4 siRNA (R) or scramble (S) oligos (Dharmacon Research Inc, Surrey Hills, Australia) as previously described [Cuevas et al., 2007]. SiRNA oligos corresponded to nt 819–839 (Oligo 1) and to nt 2300–2320 (Oligo 2) of the human IRS-4.

Hepatocarcinoma cell lines were transfected in presence of oligofectamine in a final volume of 1 ml of OPTIMEM, according to the manufacturer's instructions (Invitrogen). Cells were maintained at 37°C for 72 h with IRS-4 siRNA (R) or scrambled (S) oligos. Once the different treatments were administered, the cells were disrupted with lysis buffer for further analysis.

PROTEIN EXTRACTS AND WESTERN BLOTS

Liver biopsies were obtained in compliance with the Helsinki Declaration. All patients gave informed consent for the use of biopsies for research purposes and this was approved by the Ethical Committee of our Institution.

Tissue and cell lysates were prepared as previously described [Cuevas et al., 2007]. For the analysis of cytochrome *c* release, the intact cells collected by centrifugation were suspended in ice-cold buffer (20 mM Hepes pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose, 0.1 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml leupeptin), treated with 100 μg/ml digitonin (20 min, 4°C) and centrifuged (25,000*g*, 10 min). The supernatants, containing cytochrome *c* released from the mitochondria (cytosolic fraction) were collected, whereas the pellets (mitochondrial fraction) were resuspended in lysis buffer (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% SDS) for 10 min at 4°C and sonicated for 1 min. Nuclear and cytosolic fractions were obtained after homogenization in ice-cold buffer (10 mM Tris pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml leupeptin) by differential centrifugation.

IMMUNOCYTOCHEMISTRY AND MICROSCOPY

Immunocytochemistry was performed as previously described [Cuevas et al., 2007]. Cells were incubated with an anti-IRS-4 rabbit antibody (1/100) or antibody-free PBS for unspecific controls, and then labeled with FITC-conjugated anti-mouse or anti-rabbit Ig antibodies (1/1,000) (Molecular Probes, Eugene, OR). Nuclei were stained using propidium iodide (Molecular Probes) and HepG2 cells were examined in a laser scanning confocal microscope (Leica TCS-SL).

CELL CYCLE AND APOPTOSIS ASSESSED BY FLOW CYTOMETRY

The transfected cells were incubated during the last 18 h with actinomycin D (0.8 μM) plus TNF-α (35pM). Then the adhered cells were collected by trypsinization and washed in PBS. Apoptotic cells were determined by flow cytometry detection of their sub-G_1 DNA contents [Nicoletti et al., 1991].

Mitochondrial membrane potential loss was assessed using a JC-1 probe (5,5′,6,6′-tetrachloro-1,1′,3,3′ tetra-ethylbenzimidazolylcarbocyanine iodide/chloride; Mito-screen, BD Bioscience, San Jose, CA). In viable cells, JC-1 accumulates as aggregates in the mitochondria, resulting in red fluorescence (570 nm). In cells with damaged mitochondria, JC-1 occurs in a monomeric form, resulting in green fluorescence. Simultaneously, we identified apoptotic cells by labeling with Annexin V stain conjugated to allophycocyanine (APC); 1×10^6 cells/0.5 ml were stained with JC-1 (10 min, 37°C), washed and then resuspended in Ca^{2+} binding buffer (Hepes 10 mM, NaCl 150 nM, MgCl 1 mM, $CaCl_2$ 1.8 mM, and KCl 5 mM; pH 7.4). Cells were labeled with Annexin V-APC (Caltag, San Francisco, CA) diluted in Ca^{2+} binding buffer for 10 min. Samples were analyzed in a FacsAria cytometer (Becton Dickinson, San Jose, CA) using FacsDiva software.

DNA FRAGMENTATION GEL ASSAY

The DNA fragmentation gel assay was performed as previously described [Román et al., 1998].

GSH CONTENT AND MTT REDUCTION ASSAY

Intracellular GSH levels were determined according to the method described previously [Hissin and Hilf, 1976]. The MTT reduction assay was performed as previously described [Alley et al., 1988].

JNK ACTIVITY

Transfected cells were incubated (15, 60, and 120 min) with actinomycin D (0.8 μM), TNF-α (35 pM) or both. After treatment, JNK activity was determined as previously described [Calleros et al., 2006].

STATISTICAL ANALYSIS

Unless otherwise indicated, data were compared by ANOVA. The level of significance was set at $^{**}P < 0.01$ or $^{***}P < 0.001$.

RESULTS

CHARACTERIZATION OF IRS-4 IN HEPATOCARCINOMA CELLS (HepG2, Huh 7, AND Chang) AND OPTIMIZATION OF IRS-4 SILENCING CONDITIONS

The expression of IRS-4 and ERK 1/2 in human liver and in hepatocarcinoma cell lines (HepG2, Huh7 and Chang) is shown in Figure 1A. Increased IRS-4 expression was observed in hepatocarcinoma cell lines as compared with human liver biopsies from two patients (Fig. 1A). To localize IRS-4, HepG2 cells were subjected to immunofluorescence analysis (Fig. 1B) and subcellular fractionation (Fig. 1C). In resting conditions (in the presence of MEM for 24 h), IRS-4 was observed in both cell cytoplasm and membrane, with scarce nuclear staining. Cells treated with 5% FBS for 24 h, however, showed intense nuclear staining, suggesting translocation of IRS-4 to the nucleus in the presence of growth factors. To corroborate this hypothesis, we isolated the nucleus and the cytosol from resting (in the presence of MEM for 24 h) and activated (in the presence of 5% FBS for 24 h) cells. The presence of IRS-4 in the nucleus increased by 80% with no changes in it distribution in the cytosol after FBS treatment (Fig. 1C). To control the enrichment of the subcellular fraction, we used PARP as a marker for the nucleus and ERK1/2 and β-tubulin as markers for the cytosol (Fig. 1C). To optimize the IRS-4

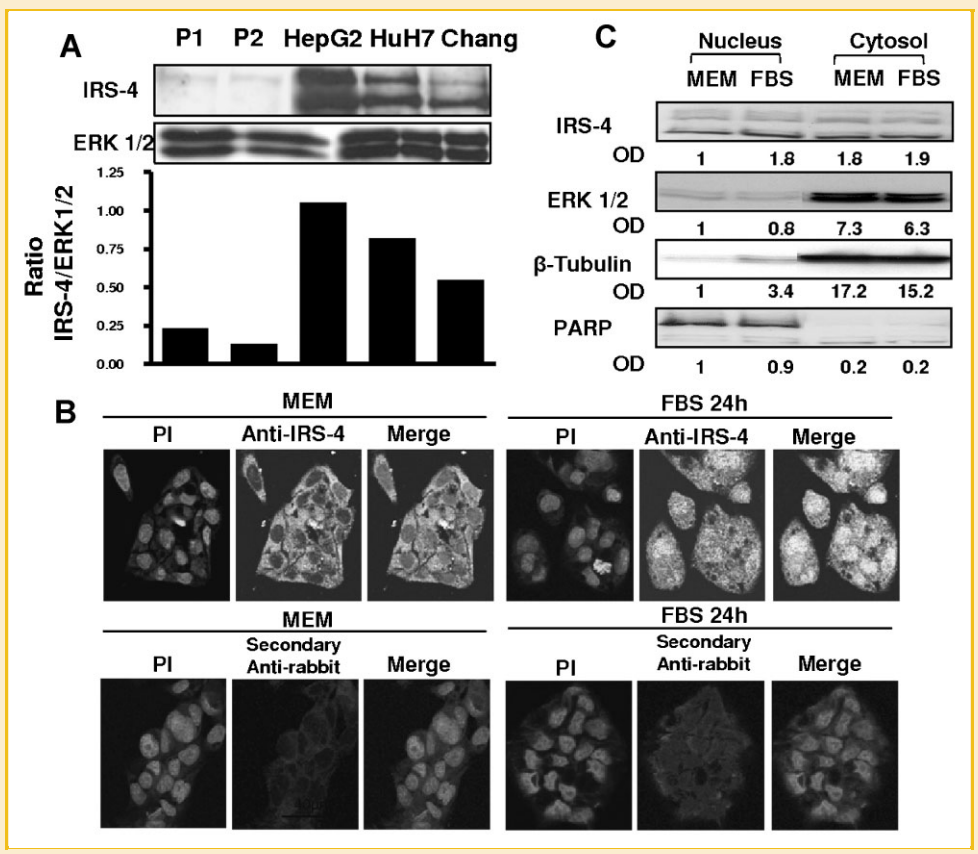

Fig. 1. A: Western blot analysis of IRS-4 and ERK in human liver normal biopsies and hepatocarcinoma cell lines. B: Immunocytochemistry and confocal microscopy results of HepG2 cells treated with or without FBS (5%) for 24 h. Cells were stained with anti-IRS-4 antibodies plus propidium iodide (PI) used to stain nuclei. For the control of unspecific staining, the corresponding secondary-FITC antibodies were used. C: Western blot analysis of IRS-4, ERK 1/2, β-tubulin and PARP in HepG2 cells treated with FBS (5%) for 24 h in cytosolic and nuclear extracts. Representative fluorescence micrographs and Western blots are shown. OD, optical density.

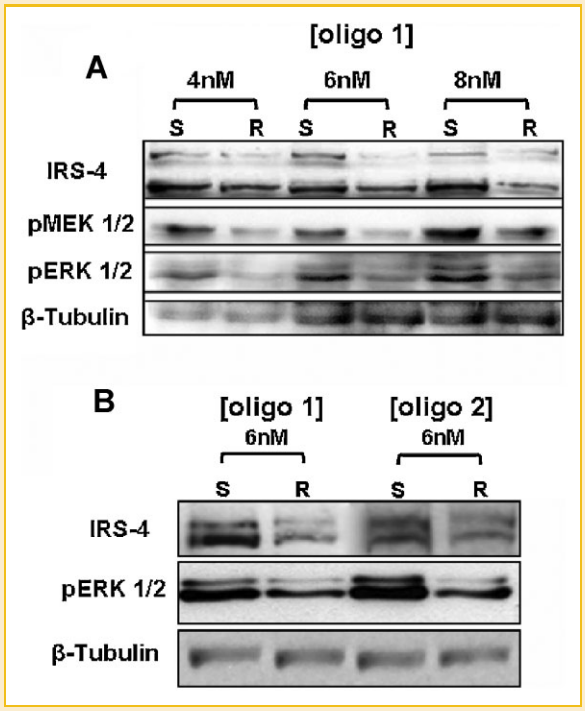

Fig. 2. A: Dose effect of IRS-4 siRNA (oligo 1, R) or scramble (S) oligos on IRS-4, pMEK1/2, pERK 1/2 and β-tubulin levels. B: Effect of IRS-4 siRNA (oligo 1, R), IRS-4 siRNA (oligo 2, R) or scramble (S) oligos on IRS-4, pERK 1/2 and β-tubulin levels. Representative Western blots are shown.

silencing conditions, we increased siRNA (oligo 1) or scramble oligo concentrations in HepG2 cells (Fig. 2A). Similar effects were observed in the range of 6–8 nM of oligo. To verify the specificity of IRS-4 knockdown on HepG2 phenotype we used another siRNA sequence (oligo 2). We observed similar effects in IRS-4 and pERK levels using the two oligos at 6 nM concentration (Fig. 2B). In subsequent experiments, cells were maintained at 37°C for 72 h with IRS-4 siRNA (oligo 1, R) or scrambled (S) oligos at 6 nM concentration.

Our data indicate that the siRNA is both effective and specific. Interestingly, pMEK and pERK decreased dramatically in R cells, confirming the involvement of IRS-4 in ERK regulation, as previously described [Cuevas et al., 2007].

EFFECT OF ACTINOMYCIN D AND/OR TNF-α-TREATMENT ON HepG2 CELL DEATH BIOMARKERS

Following previous dose- and time-dependent results on the effects of the drugs in HepG2 cells [Yamanaka et al., 2000], we evaluated the effects of 18 h of treatment with actinomycin D (0.8 μM) or actinomycin D (0.8 μM) plus TNF-α (35 pM) on: (i) Annexin V bound to cell surface phosphatidylserine (early marker of apoptosis) (Fig. 3A), (ii) JC-1 probe aggregation (mitochondrial membrane potential biomarker) (Fig. 3A), and (iii) DNA laddering (late marker of apoptosis) (Fig. 3B). Mitochondrial intact cells could be discriminated by the presence of JC-1 aggregates (R1, Region 1) from dead cells by the presence of JC-1 monomers (R2, Region 2). As can be seen in Figure 3A, mitochondrial-dependent cell death increased from 15% or 32% in the presence of FBS or actinomycin D, respectively, to 74% in cells exposed to actinomycin D and TNF-α. Treatment with TNF-α (35 pM) alone produced a minor effect on cell death, as measured by flow cytometry (data not shown). Figure 3A shows that in live cells (Region R1), Annexin V-staining (Q2 quadrant) increased from 27% or 33% in FBS- or actinomycin D-treated cells, respectively, to 65% in cells exposed to actinomycin D plus TNF-α. In mitochondrial-damaged cells (Region R2), the amount of Annexin V-positive cells increased (Q4-1 quadrant) from 38% or 69% in cells treated with FBS or actinomycin D, respectively, to 84% in cells incubated with actinomycin D plus TNF-α. For each treatment, percentages were calculated from the corresponding R1 or R2 areas, indicating cells with or without an unaffected mitochondrial membrane potential, respectively. Consistently, actinomycin D treatment led to a significant increase in DNA fragmentation as compared with FBS-treated cells (Fig. 3B). The effect of actinomycin D on DNA fragmentation was amplified by TNF-α in a dose-dependent manner (Fig. 3B). In agreement with flow cytometric studies (data not shown), the TNF-α (35 pM) treatment did not produce any relevant effect on DNA fragmentation as compared with FBS-treated cells (Fig. 3B). Collectively, these data indicate that actinomycin D sensitizes HepG2 cells to TNF-α treatment through mitochondrial damage and that TNF-α subsequently increases apoptosis in a dose-dependent manner.

EFFECT OF IRS-4 DEPLETION ON HepG2 INDUCED APOPTOSIS

Actinomycin D plus TNF-α treatment during 18 h decreased the cells ability to reduce MTT in a TNF-α dose-dependent manner (Fig. 4A). Control (oligofectamine) and S cells showed similar MTT activity at all the actinomycin D and/or TNF-α concentrations tested. In all the cases, however, R cell MTT activity was decreased by about 30–70% over the activity observed in S cells (Fig. 4A). To assess whether the decrease in the MTT reducing capacity of R cells was due to an enhanced apoptosis, we examined the cell cycle using propidium iodide staining and flow cytometry. In the absence of apoptotic stimuli, the sub-G_1 population did not change in response to the IRS-4 levels (Fig. 4B, upper panels). However, in the presence of actinomycin D (0.8 μM) and TNF-α (35 pM) during 18 h, the sub-G1 population in R cells was twofold that observed in S cells. (Fig. 4B, lower panels). To ascertain whether the sensitization to Act D plus TNF-α in IRS-4 depleted cells is limited to HepG2 cell line, we also used HuH 7 and Chang IRS-4-expresing cells. Figure 4C shows that oligo 1 (6 nM) is able to decrease the levels of IRS-4 in the three lines tested and also to sensitize the cells treated with Act D (0.8 μM) and TNF-α (35 pM) during 18 h.

Figure 5A shows that actinomycin D sensitizes HepG2 cells acting at the mitochondria and inducing the release of cytochrome *c* and subsequent procaspase 3 and PARP proteolysis. When TNF-α was added to the treatment, mitochondrial cytochrome *c* release was unaffected as compared with actinomycin D treatment alone. After IRS-4 silencing, HepG2 cells were more sensitive to Act D than control cells (Fig. 5A). The release of cytochrome *c* and subsequent proteolysis of procaspase 3 and PARP (Fig. 5A) increased in R cells with respect to S cells. Focal adhesion kinase (FAK) proteolysis increased in IRS-4-depleted cells with respect to control cells after actinomycin D and TNF-α treatment (Fig. 5B). In summary, these

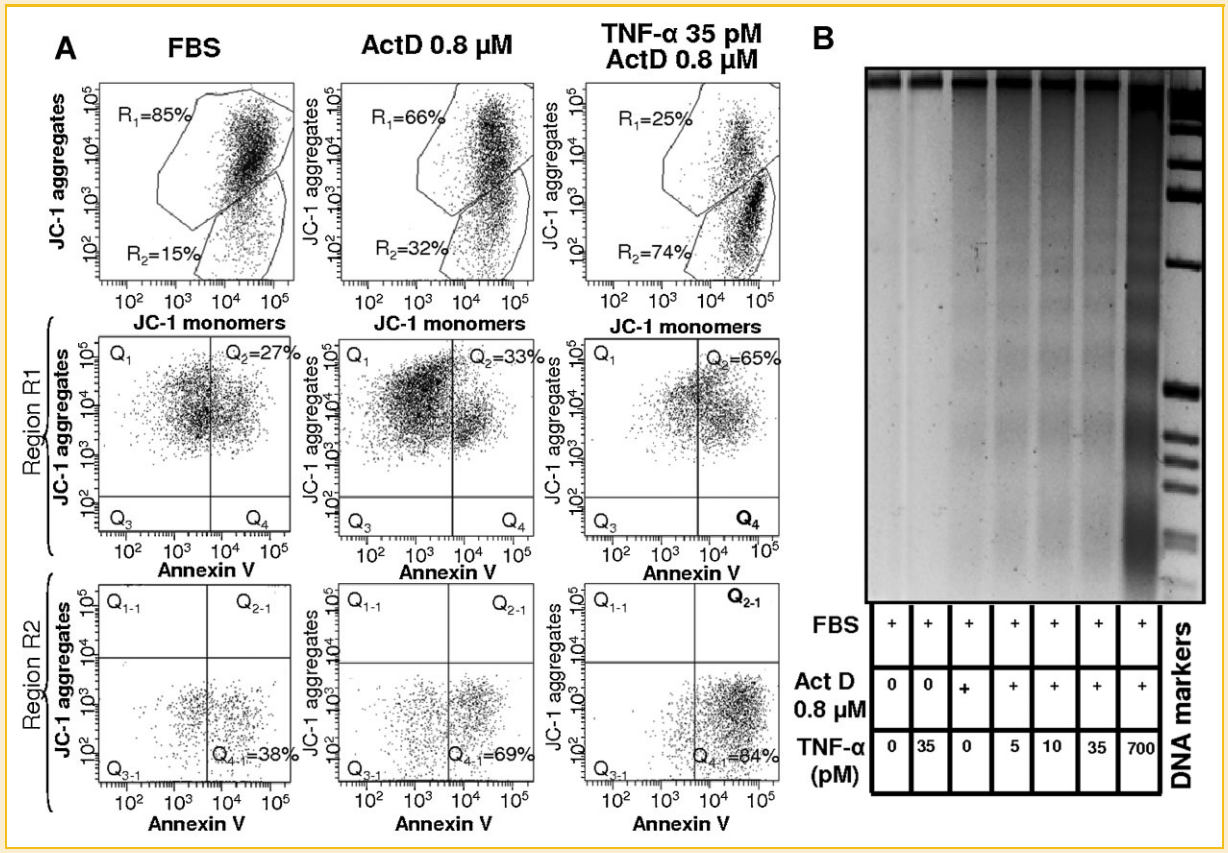

Fig. 3. Effect of actinomycin D or TNF-α plus actinomycin D treatments during 18 h on HepG2 cell death in the presence of FBS (5%). A: Effects of actinomycin D (0.8 μM) or TNF-α (35 pM) plus actinomycin D (0.8 μM) treatment on mitochondrial-dependent apoptosis. B: Dose effect of TNF-α on DNA laddering in HepG2 cells in the absence or presence of actinomycin D (0.8 μM) for 18 h. FBS (5%)-treated cells were used as control. Representative experiments of five experiments performed in duplicate are shown.

results demonstrate that IRS-4 provides a survival signal to HepG2 cells that protects from actinomycin D and/or TNF-α-induced apoptosis.

SIGNALING CASCADES INVOLVED IN HepG2 CELL APOPTOSIS INDUCED BY ACTINOMYCIN D AND/OR TNF-α. EFFECT OF IRS-4 DEPLETION

We observed a significant decrease in the MTT reducing capacity induced by the proapoptotic agents, which was partially reversed in cells cotreated with the JNK inhibitor SP600125 (Fig. 6A) and *N*-acetyl-L-cysteine (NAC) (Fig. 6C). Similarly, we noted that apoptotic DNA laddering induced by TNF-α plus actinomycin D was diminished in the presence of both the JNK inhibitor (SP600125) and NAC (*N*-acetyl-L-cysteine) (Fig. 6B,D).

Time-course studies revealed that TNF-α induces a JNK activation peak after 15 min of treatment, whereas actinomycin D alone slowly activates c-jun phosphorylation (Fig. 7A). In contrast, actinomycin D/TNF-α cotreatment induced sustained JNK activation (Fig. 7A), stressing the importance of double treatment in increasing apoptosis. Figure 7A shows that IRS-4 depletion did not increase JNK activation induced by actinomycin D/TNF-α combination. Increasing the TNF-α dose in the presence of actinomycin D during 1h of treatment stimulated JNK phosphorylation in a dose-dependent manner (Fig. 7B). However, IRS-4 depletion failed to modify pJNK levels, corroborating our previous results. In a similar set of experiments, we observed that neither actinomycin D plus TNF-α treatment nor IRS-4 depletion produced significant effects on pAKT (Ser473) (Fig. 7B).

The IKK/IκB-α/NF-κB system is an essential pathway that regulates HepG2 cell apoptosis. As previously described [Ruan and Pownall, 2009], IκB-α proteolysis is a very good marker of IKK activity. Increasing TNF-α in the presence of actinomycin D stimulated IκB-α proteolysis in a dose-dependent manner. However, this event was independent of IRS-4 levels (Fig. 7B).

EFFECT OF TREATMENT WITH ACTINOMYCIN D AND TNF-α ON GLUTATHIONE (GSH) AND γ-GLUTAMYLCYSTEINE SYNTHETASE (GCS) LEVELS IN HepG2 CELLS DEPLETED OF IRS-4

In R cells, a dramatic GSH depletion can be observed after actinomycin D treatment, which is intensified in the presence of TNF-α. This effect is similar to that found in L-buthionine-(S,R)-sulfoximine (BSO) treated cells (Fig. 7C). BSO is a competitive inhibitor of γ-glutamylcysteine synthetase (GCS). Thus, S cells are more resistant to oxidative stress induced by actinomycin D than R cells; S cell GSH levels diminish only in the presence of both agents. To corroborate these findings, we estimated the levels of γ-glutamylcysteine synthetase (GCS), the enzyme that limits GSH

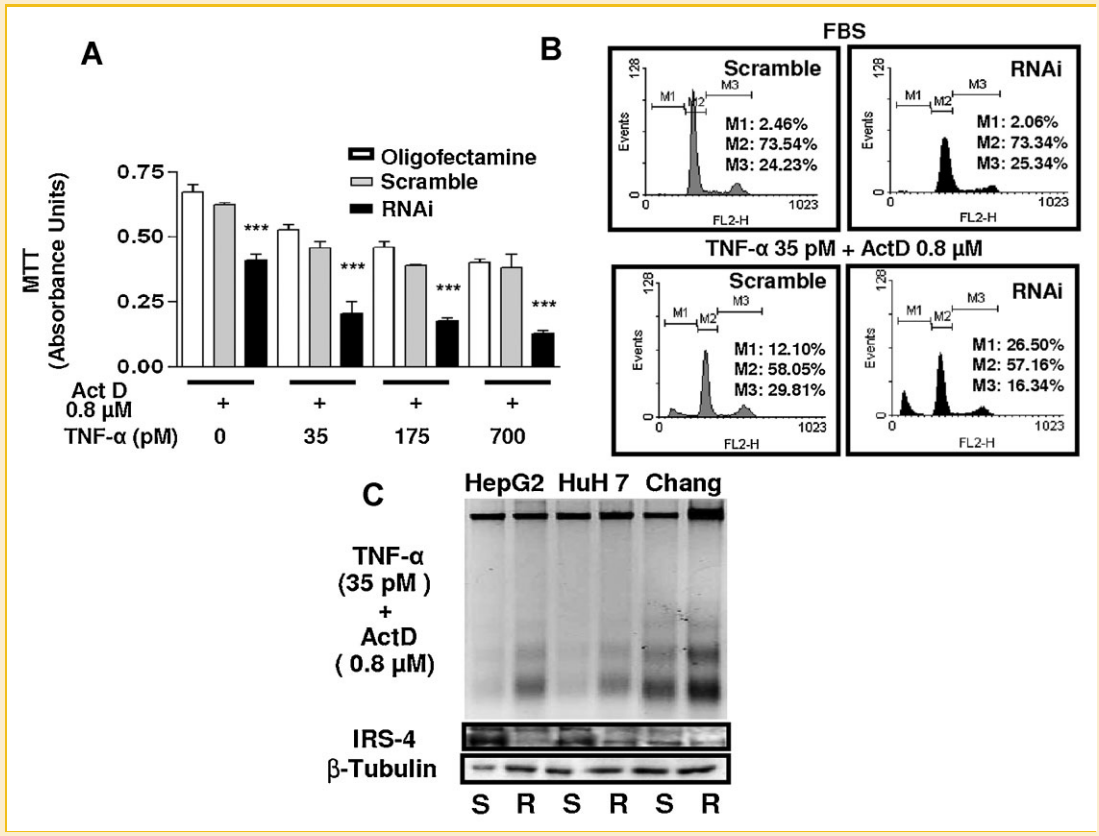

Fig. 4. Effect of IRS-4 depletion on HepG2 cell death biomarkers after TNF-α, actinomycin D or TNF-α plus actinomycin D treatment during 18 h. A: MTT reducing ability of cells transfected with oligofectamine, scramble oligos (Scramble) or IRS-4 siRNA oligos (oligo1, RNAi) and treated with different concentrations of TNF-α and/or actinomycin D. Values represent the means ± SEM of three experiments performed in triplicate. Differences between treated cells with Scramble and RNAi were statistically analyzed by ANOVA (***$P < 0.001$). B: Cell cycle analysis by flow cytometry performed in HepG2 cells transfected with IRS-4 siRNA (oligo 1) or scramble oligos in the presence of FBS (5%) and TNF-α (35pM) plus actinomycin D (0.8 µM) or FBS (5%) alone. A representative experiment of four experiments performed in duplicate is shown. C: Effect of TNF-α (35 pM) plus actinomycin D (0.8 µM) in hepatocarcinoma cell lines transfected with IRS-4 siRNA (oligo 1, R) or scramble (S) oligos on DNA laddering. A representative experiment of two experiments performed in duplicate is shown.

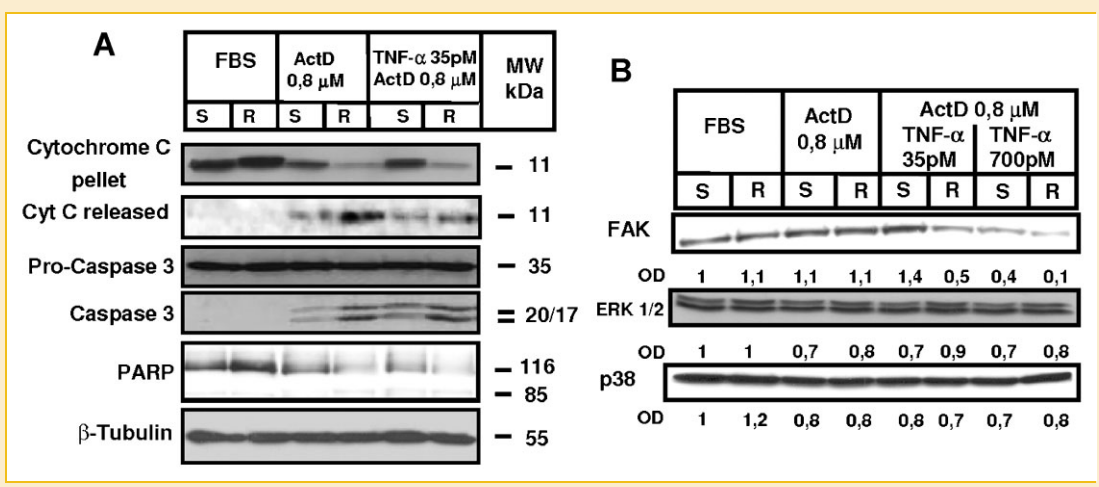

Fig. 5. Effect of actinomycin D with or without TNF-α (18 h of treatment) in the presence of 5% FBS on: cytochrome c release, caspase 3 proteolysis, PARP fragmentation and β-tubulin levels (A) and on FAK, ERK 1/2 and p38 levels (B) in HepG2 cells transfected with IRS-4 siRNA (oligo 1, R) or scramble (S) oligos. Representative experiments of three performed in duplicate are shown. OD, optical density.

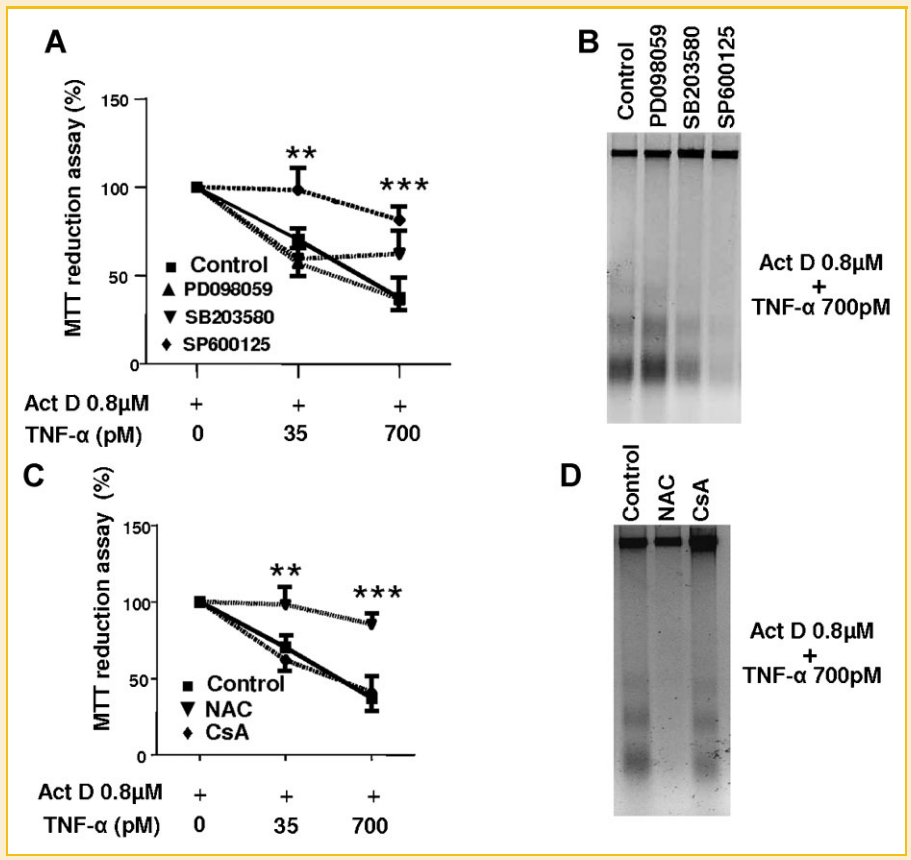

Fig. 6. Effect on MTT reducing ability (A,C) and DNA fragmentation (B,D) of PD098059 (MEK inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), NAC (N-acetyl cysteine) or CsA (cyclosporin A), in HepG2 cells treated with actinomycin D (0.8 μM) in the presence or absence of different concentrations of TNF-α during 18 h. Values represent means ± SEM of three experiments performed in triplicate. Differences versus the corresponding controls were statistically analyzed by ANOVA ($^{***}P < 0.001$ and $^{**}P < 0.01$).

synthesis. In our model, R cells display a decrease in GCS levels (Fig. 7D), in accordance with the data on GSH levels.

DISCUSSION

This study unveils an antiapoptotic role of IRS-4 in hepatocarcinoma cells treated with actinomycin D and TNF-α. Using HepG2 as model, we suggest that IRS-4 inhibits cytochrome *c* release from the mitochondria (Fig. 8) and can thus regulate caspase 3 activation and the proteolysis of the subsequent substrates, PARP, FAK or GCS. Our results extend the role attributed to IRS-4 in liver regeneration [Escribano et al., 2003] and HepG2 cell proliferation [Cuevas et al., 2007] to a new role involved in the resistance of hepatocarcinoma cells to actinomycin D alone or in combination with TNF-α. Cultured HepG2 cells, used as model of HCC, are very resistant to TNF-α, Fas and TRAIL ligands [Suzuki et al., 1998; Yamanaka et al., 2000; Matsui et al., 2002]. It has been established, however, that actinomycin D sensitizes the cells to TNF-α [Yamanaka et al., 2000; Meurette et al., 2005]. The results presented here indicate that actinomycin D provokes cytochrome *c* release from the mitochondria, procaspase-3 proteolysis and PARP fragmentation, driving HepG2 cells to a loss of mitochondrial membrane potential, Annexin V staining and DNA nucleosomal fragmentation, which are classical hallmarks of mitochondrial-dependent apoptosis. The addition of TNF-α to Act D treatment provokes a sustained JNK activation, leading to an increase in apoptosis that could be inhibited by SP600125. Our data indicate that IRS-4 protects the mitochondria from actinomycin D-induced cytochrome *c* depletion, consequently, an increase in procaspase 3 and PARP fragmentation is observed in IRS-4 silenced cells. Interestingly, when TNF-α was combined with Act D other caspase-3 substrates (FAK and GCS) decreased suggesting their fragmentation. In IRS-4-depleted cells, FAK and GCS levels decreased as compared with control cells, which could explain the increased apoptosis observed under these conditions (Fig. 8). Recently, FAK cleavage has been considered an early marker of oxidative stress-induced apoptosis [Mian et al., 2008].

At present, is unknown the signaling pathway involved in HepG2 cell apoptosis induced by IRS-4 depletion. The underlying mechanism seems to be independent of the AKT, IKK/NF-κB and JNK pathways. The role of GSH in Act D plus TNF-α induced-apoptosis is supported by the fact that *N*-acetyl-L-cysteine (NAC) (a glutathione replenisher) inhibits completely cell death. Studies by Pierce et al., (2000) reveal that the combination of TNF-α and Act D causes apoptosis because Act D blocks the transcription of genes

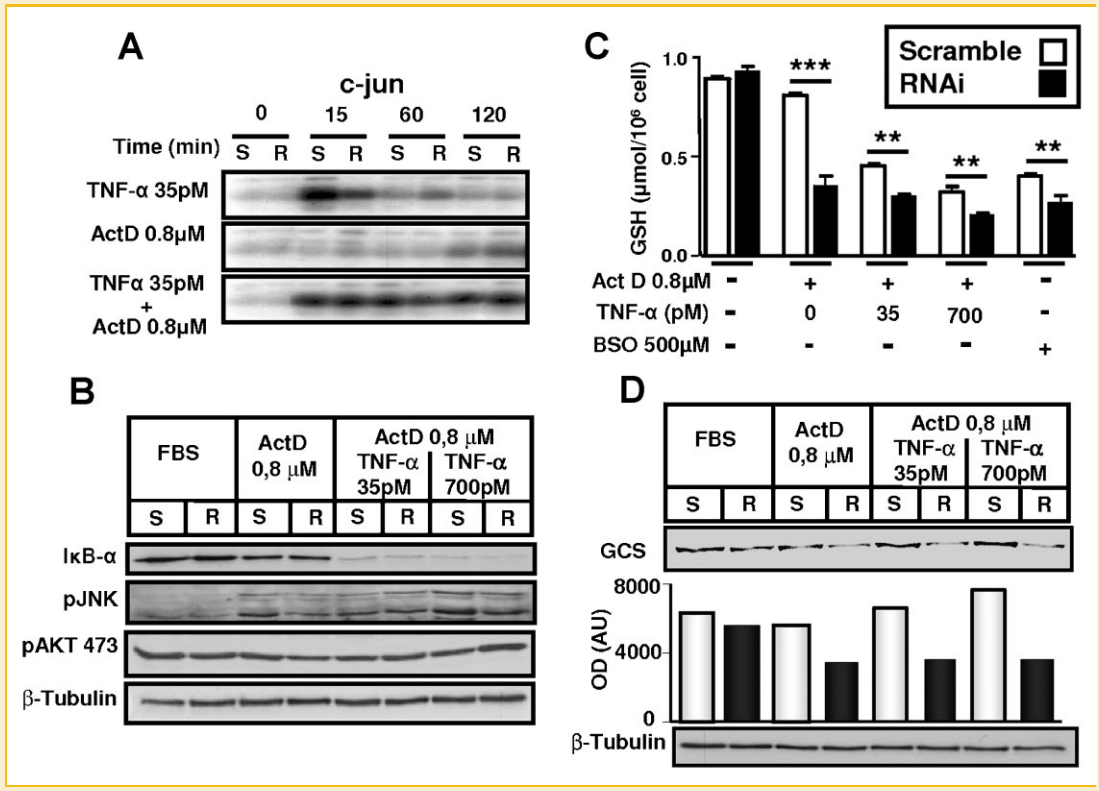

Fig. 7. Effect of IRS-4 depletion (in the presence of FBS 5%) on different signaling pathways in transfected HepG2 cells treated with TNF-α plus actinomycin D. A: Time-course of TNF-α and/or actinomycin D treatment on JNK activity (monitoring phopho-c-jun). B: Dose-dependent effect of TNF-α and/or actinomycin D (1 h of treatment) on pJNK, pAKT (Ser473) and IκB-α levels. C: GSH content of transfected HepG2 cells treated during 18 h with TNF-α and/or actinomycin D or BSO (L-buthionine-(S, R)-sulfoximine) at the indicated concentrations in the presence of FBS (5%). Differences between Scramble and RNAi (oligo 1) transfected cells were statistically analyzed by ANOVA ($^{***}P < 0.001$ and $^{**}P < 0.01$). D: GCS (γ-glutamylcysteine synthetase) levels detected in transfected HepG2 cells treated during 18 h with TNF-α and/or actinomycin D at the indicated concentrations in the presence of FBS (5%). Cells were transfected with IRS-4 siRNA (oligo 1, R) or scramble (S) oligos. Representative experiments of three performed in duplicate are shown. OD, optical density.

required for antioxidant defences [Pierce et al., 2000]. Our results suggest that IRS-4 depletion intensify this effect; in fact, we observed important changes in the antioxidant system, because R cells show decreased GCS and GSH levels in comparison to S cells. Our previous results suggest that IRS-4 stimulates MEK1-2/ERK1-2 cascade in a PKC-dependent manner in HepG2 cells [Cuevas et al., 2007], and by this way could be explained the observed GCS/GSH decrease in IRS-4 depleted cells, since ERK activation leads to increase in mRNA and GCS protein expression and regulation of GSH levels [Morales et al., 1997]. Corroborating this model, a previous result shows that GSH-depleting drugs sensitize hepatoma cells to TNF-α and TRAIL induced apoptosis [Meurette et al., 2005]. Another possible mechanism involved in the antiapoptotic effect of IRS-4 could be related with it nuclear translocation observed in present report. This way has been proposed for IRS-1 which after nuclear translocation is associated with Rad51, an enzymatic component of homologous recombination directed DNA repair (HRR), and could improve DNA repair fidelity and possibly counteracts drug-induced DNA damage [Urbanska et al., 2009].

Given that hepatocarcinoma systemic therapies have failed due to a high resistance to systemic chemotherapy and radiotherapy [Llovet et al., 2003], new therapeutic strategies are necessary to treat this very common malignancy. The antitumor effects of TNF-α can be exploited therapeutically in different human cancers; however, it clinical use is hindered by it severe dose-limiting toxicity [Lejeune and Rüegg 2006]. In order to increase the therapeutic index, several approaches have been pursued, such as regional administration of high TNF-α doses [Grünhagen et al., 2006] or in combination with chemotherapeutic agents (melphalan, actinomycin D) [Cornett et al., 2006; Meany et al., 2008]. Recent clinical studies demonstrate that high response and survival rates can be achieved in the treatment of liver tumors with isolated hepatic perfusion of high doses of chemotherapeutics agents with or without TNFα [Nakamoto et al., 2000; Rothbarth et al., 2006].

In conclusion, we propose (Fig. 8) that IRS-4 is necessary in the homeostasis of GSH, when IRS-4 falls in presence of proapoptotic factors the GSH levels decrease producing the activation of caspase 3 which in turn degrades PARP (involved in DNA reparation), FAK (involved in cell adhesion) and GCS (involved in GSH homeostasis) creating a vicious circle that leads the cell to apoptosis. Moreover, our results provide the rationale for targeting liver tumors with anti-IRS-4 RNAi as a strategy for increasing the therapeutic index of Act D, TNF-α or the combined treatment.

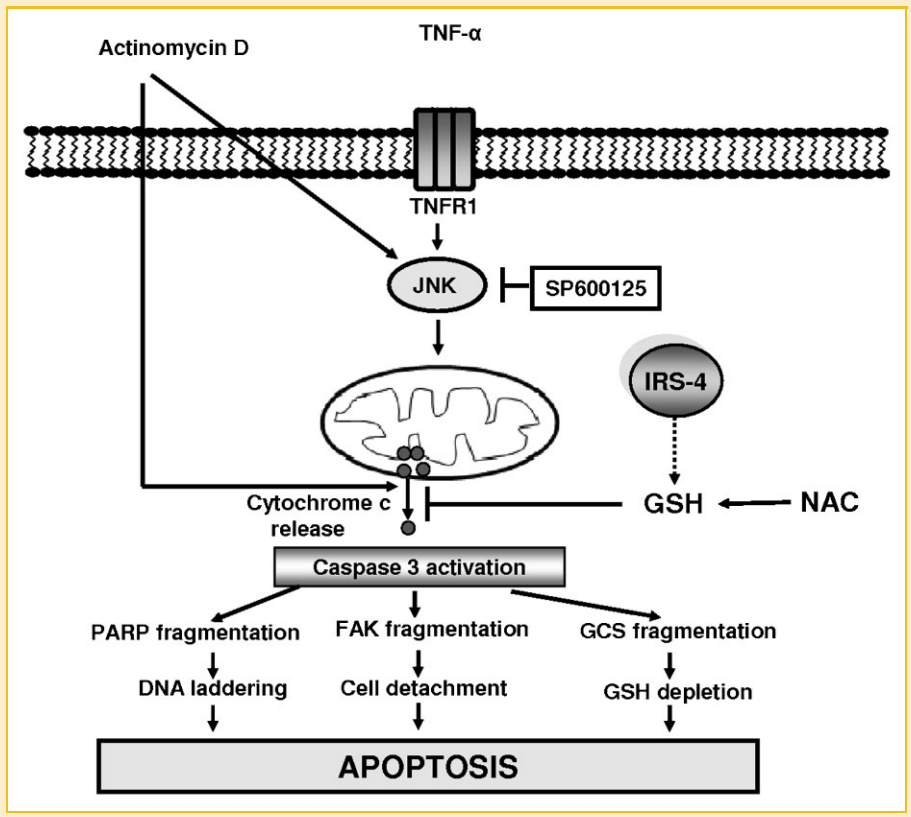

Fig. 8. Role of IRS-4 in conferring HepG2 cancer cells resistance to apoptosis induced by TNF-α plus actinomycin D.

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