

Regulation of the IRF-1 tumour modifier during the response to genotoxic stress involves an ATM-dependent signalling pathway

Jessica Pamment¹, Eleanor Ramsay¹, Michael Kelleher¹, David Dornan¹ and Kathryn L Ball^{*1}

¹Cancer Research UK Laboratories, University of Dundee Medical School, Dundee DD1 9SY, UK

The mechanism by which genotoxic stress induces IRF-1 and the signalling components upstream of this anti-oncogenic transcription factor during the response to DNA damage are not known. We demonstrate that IRF-1 and the tumour suppressor protein p53 are coordinately up-regulated during the response to DNA damage in an ATM-dependent manner. Induction of IRF-1 protein by either ionizing radiation (IR) or etoposide occurs through a concerted mechanism involving increased IRF-1 expression/synthesis and an increase in the half-life of the IRF-1 protein. A striking defect in the induction of both IRF-1 mRNA and IRF-1 protein was observed in ATM deficient cells. Although ATM deficient cells failed to increase IRF-1 in response to genotoxic stress, the induction of IRF-1 in response to viral mimetics remained intact. Re-expression of the ATM kinase in AT cells restored the DNA damage inducibility of IRF-1, whilst the PI-3 kinase inhibitor wortmannin inhibited IRF-1 induction by DNA damage in ATM-positive cells. The data highlight a role for the ATM kinase in orchestrating the coordinated induction and transcriptional cooperation of IRF-1 and p53 to regulate *p21* expression. Thus, IRF-1 is controlled by two distinct signalling pathways; a JAK/STAT-signalling pathway in viral infected cells and an ATM-signalling pathway in DNA damaged cells.

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Introduction

Interferon regulatory factor-1 (IRF-1) is the founding member of a family of interferon (IFN) responsive transcription factors. IRF-1 was first described as a mediator of the interferon response that bound to the virus-inducible ‘enhancer-like’ elements of the IFN- β gene (Fujita *et al.*, 1988; Miyamoto *et al.*, 1988; Taniguchi, 1989). More recently, it has been recognized that activation of IRF-1 leads to the expression of

genes involved in a number of different cellular processes. In addition to the anti-viral response, these include: regulation of the cell cycle (Stevens and Yu-Lee, 1992, 1994) and apoptosis (Kirchhoff and Hauser, 1999; Tamura *et al.*, 1995); development of the T cell immune response (Matsuyama *et al.*, 1993); susceptibility to transformation by oncogenes (Tanaka *et al.*, 1994), and the response to genotoxic agents (Prost *et al.*, 1998; Tanaka *et al.*, 1996). Furthermore, deletion or point mutation of the *IRF-1* gene (Eason *et al.*, 1999; Willman *et al.*, 1993), and exon skipping of IRF-1 mRNA (Harada *et al.*, 1994) have been linked to the development of human haemopoietic malignancies, such as leukaemia and myelodysplastic syndrome (Boulwood *et al.*, 1993; Green *et al.*, 1999; Willman *et al.*, 1993), as well as, solid phase tumours of the gastro-intestinal tract (Ogasawara *et al.*, 1996; Tamura *et al.*, 1996). In conjunction with a recent study showing that IRF-1 can modulate tumour susceptibility in the presence of oncogenic lesions (Nozawa *et al.*, 1999), the above research provides evidence that IRF-1 has tumour suppressor/modifier activity.

The spectrum of IRF-1 responsive genes is dependent on a number of factors including stimulus, cell type and stage of development (Taniguchi *et al.*, 1998). IRF-1 activation has been most extensively studied in haemopoietic cells during the response to viral attack or IFN- γ treatment. Induction of *IRF-1* gene expression following stimulation by IFN- γ is dependent on the JAK/STAT signalling pathway and genes downstream of IRF-1 in this pathway include, IFN- β (Fujita *et al.*, 1989a; Miyamoto *et al.*, 1988), RNA dependent protein kinase (Beretta *et al.*, 1996; Kirchhoff *et al.*, 1995) and 2-5A synthetase (Coccia *et al.*, 1999; Wang and Floyd-Smith, 1998).

Although the role of IRF-1 in the response to viral attack is now well established there is less known about the role of this transcription factor in response to other environmental stresses, such as DNA damage. Mouse embryonic fibroblasts (MEFs) and hepatocytes that are deficient in IRF-1 are compromised in their ability to undergo growth arrest (Tanaka *et al.*, 1996) and to repair damaged DNA (Prost *et al.*, 1998), respectively. Furthermore, maximal induction of the cyclin dependent kinase inhibitor, p21 (WAF1/CIP1), following exposure of MEFs to ionizing radiation (IR), appears to be dependent on both p53 and IRF-1 (Tanaka *et al.*, 1996). This has led to the suggestion that IRF-1

*Correspondence: KL Ball; E-mail: k.l.ball@dundee.ac.uk
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cooperates with p53 in the G1-checkpoint pathway and is upstream of *p21* (Taniguchi *et al.*, 1997). Although these reports show a role for IRF-1 in DNA damage-induced pathways, the mechanism(s) leading to IRF-1 activation in response to genotoxic stress remains unknown.

The current study reports on the mechanism by which IRF-1 is induced by IR and etoposide. In cells exposed to genotoxic stress increased IRF-1 protein levels were accompanied by changes in IRF-1 mRNA levels and protein synthesis, as well as post-translation changes in the rate of IRF-1 protein degradation. Evidence is presented that the ATM kinase is a component of the signalling pathway(s) that leads to changes in IRF-1 expression following exposure to genotoxic stress. Thus, IRF-1 is regulated by a concerted mechanism in response to genotoxic stress and a role for the ATM kinase in coordinating IRF-1 and p53 dependent events has been defined.

Results

Coordination and cooperation of the IRF-1 and p53 proteins during the response to genotoxic stress

Genetic evidence suggests that IRF-1 and p53 converge to suppress tumour development (Nozawa *et al.*, 1999). In addition these two anti-oncogenic transcription factors have been proposed to cooperate in the regulation of gene expression following exposure of MEFs to IR (Tanaka *et al.*, 1996). However, the mechanism by which IRF-1 is induced by genotoxic stress and whether common upstream signalling elements coordinate the activation of IRF-1 with p53 are not known. Before addressing these questions we sought to verify that IRF-1 and p53 could cooperate in human cells. The ectopic expression of p53 and/or IRF-1 in HCT116 (p53^{-/-}) cells was used in conjunction with a *p21* reporter construct to measure transcriptional activity. Consistent with previous results (Tanaka *et al.*, 1996), p53 was an efficient activator of *p21* expression (Figure 1), when the *p21* promoter (-2320 to +1) was linked to a luciferase reporter, whereas IRF-1 was a relatively weak activator (Figure 1; 1 μ g IRF-1 encoding DNA was found by titration to be optimal for *p21* reporter activity-data not shown). When IRF-1 and p53 were co-expressed their effect on reporter readout was synergistic rather than additive (Figure 1a; upper panel). A more striking effect was observed when changes in the level of endogenous *p21* protein were used as a measure of transcriptional activity (Figure 1a; immunoblot). Although p53, by itself, was able to partially activate endogenous *p21* expression, consistent with p53 being required for the expression of *p21* in undamaged proliferating cells (Tang *et al.*, 1998), co-expression of IRF-1 with p53 gave a dramatic induction of *p21* protein levels, despite the fact that IRF-1 by itself did not induce measurable increases in *p21* protein. Together these data suggest that IRF-1 and p53 can cooperate to promote *p21* gene expression and are

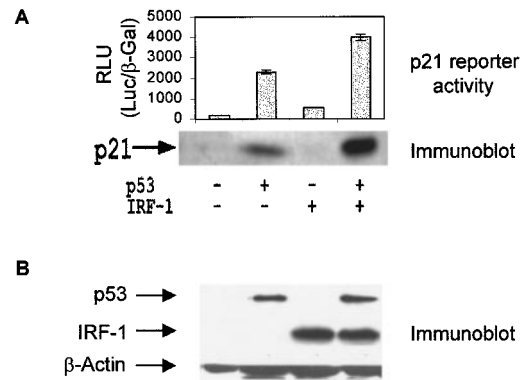


Figure 1 IRF-1 and p53 cooperate to induce *p21* expression. HCT116 (p53^{-/-}) cells were transfected with 1 μ g pcDNA-p53 and/or 1 μ g pcDNA3-IRF-1 (as detailed) plus 1 μ g pCMV- β Gal and 1 μ g *p21*-Luc. The amount of total plasmid DNA added per transfection was normalized using vector DNA with no insert. (a) Reporter activity is given as relative light units expressed as the ratio of *p21*-Luc/ β -Gal. Endogenous promoter activity was determined by direct immunoblot developed using AB-1 following separation on a 15% SDS-polyacrylamide gel (50 μ g total protein/lane). (b) The levels of transfected p53 and IRF-1 expressed (25 μ g total protein/lane) were quantified by 10% SDS-PAGE/immunoblot, detected using DO-1 and C-20, respectively, β -actin levels were determined using AC-15. The data are mean \pm s.d. for data from three independent sets of experiments carried out in triplicate

consistent with studies using MEFs in which *p21* induction and cell cycle arrest were dependent on the presence of both the *IRF-1* and *p53* genes (Taniguchi *et al.*, 1997).

As IRF-1 and p53 can cooperate in a synergistic manner to promote *p21* gene expression we determined whether induction of these two transcription factors might be coordinated during the cellular response to genotoxic stress. Previous studies have shown that IRF-1 is activated in cells exposed to high doses of IR (20 Gy) (Tanaka *et al.*, 1996) at 1 h post-treatment. In the current study IR (5 Gy) and the topoisomerase II inhibitor etoposide (Baguley, 1991) were used and were compared to the double stranded RNA, polyI:polyC, which mimics viral infection by inducing IFN- γ expression (Tamura-Nishimura and Sasakawa, 1990) and up-regulating the *IRF-1* gene via the JAK/STAT pathway (Fujita *et al.*, 1989b).

The induction of IRF-1 protein with time in response to IR (5 Gy; Figure 2a) and etoposide (10 μ M; Figure 2b) were analysed along with p53 protein levels. These studies were carried out using the human melanoma cell line A375, which has wt p53 and induces *p21* protein in response to DNA damage (Blaydes *et al.*, 2000). IRF-1 protein levels peaked 2–4 h post-treatment with IR (Figure 2a) and between 4–6 h with etoposide (Figure 2b). PolyI:polyC induction of IRF-1 occurred at 2 h and was sustained (Figure 2c). There was a striking similarity in the kinetics of IRF-1 and p53 protein induction in response to either IR or etoposide (Figure 2a,b) and the data were consistent with previous studies showing that the response of p53 to DNA strand breaks is rapid

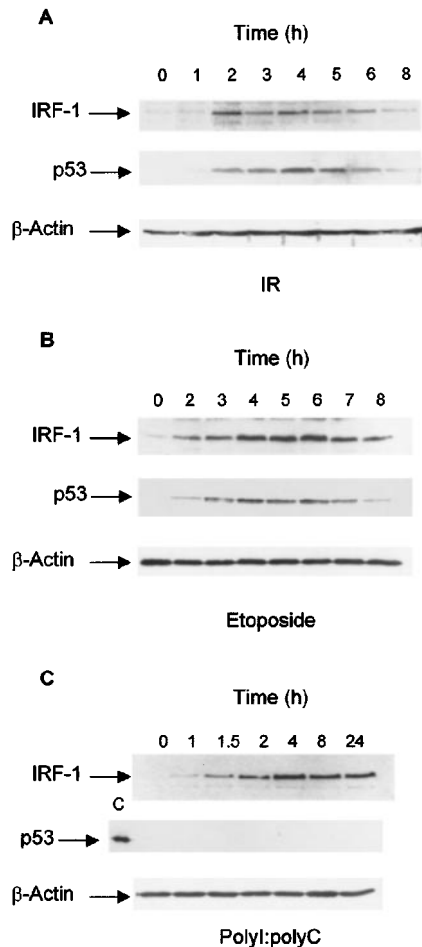


Figure 2 Induction of IRF-1 protein in response to DNA damage. A375 cells were treated with (a) 5 Gy IR, (b) 10 μ M etoposide or (c) 50 μ g/ml polyI:polyC and samples collected at the times given. Cell lysates (50 μ g/lane) were analysed by 10% SDS-PAGE/immunoblot, IRF-1 and p53 were detected using C-20 and 19.1, respectively. The IRF-1 blots were subsequently reprobed using AC-15 to determine the levels of β -actin as a control. The data are representative of at least five separate experiments

but transient (Lu and Lane, 1993). Increases in IRF-1 and p53 protein were completely uncoupled by polyI:polyC treatment, which had no measurable effect on p53 protein levels (Figure 2c).

Regulation of IRF-1 mRNA and protein synthesis by DNA damaging agents

IRF-1 gene expression and rate of protein synthesis are known to be up-regulated by viral infection or IFN- γ via the JAK/STAT pathway, however induction of IRF-1 by IR is independent of JAK/STAT (Tanaka et al., 1996). As we are interested in the signalling pathways that regulate IRF-1 protein levels during the response to DNA damage we first wished to establish whether increases in IRF-1 seen in the presence of low doses of genotoxic agents could be accounted for by changes in gene expression (Figure

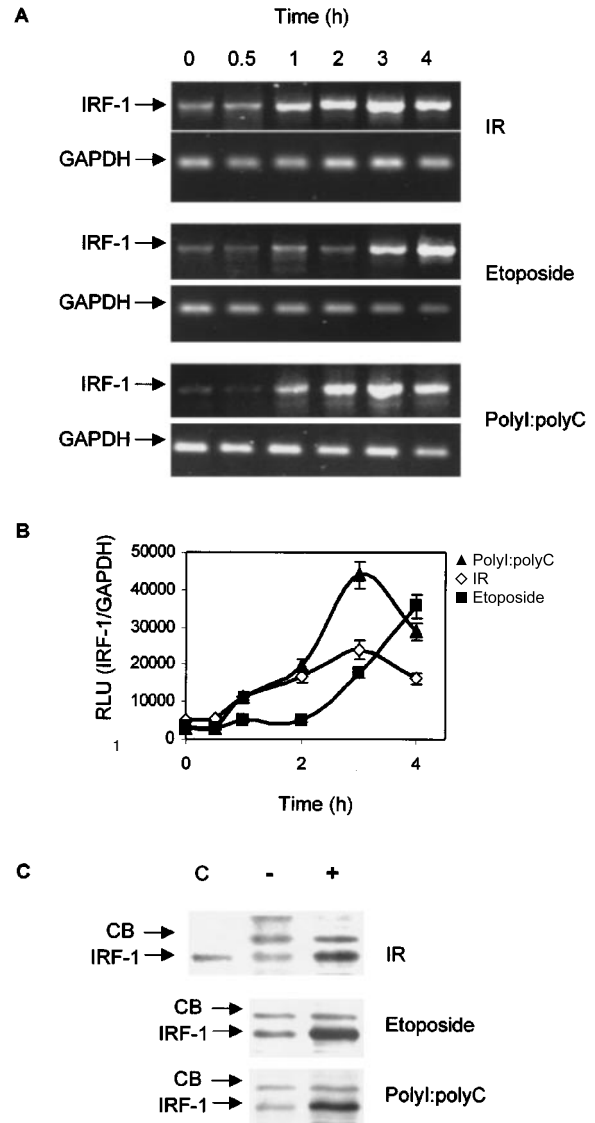


Figure 3 IRF-1 expression and synthesis in DNA damaged cells (a) A375 cells were treated with IR (5 Gy), etoposide (10 μ M) or polyI:polyC (50 μ g/ml), at the indicated time points cells were collected and mRNA was prepared. IRF-1 and GAPDH mRNA levels were determined by semi-quantitative RT-PCR and analysed on 1% or 1.2% agarose gels, respectively. (b) The data from (a) was quantified using a GeneGenius bioimager plus Syngene analysis software and are represented in relative light units for the mean + s.e. of at least two separate time-courses/treatment analysed in triplicate. (c) A375 cells were untreated (-) or treated (+) with IR (5 Gy), etoposide (10 μ M) or polyI:polyC (50 μ g/ml) for a total of 2, 4, and 2 h respectively prior to pulse labelling with 35 S-Met/Cys. Cells were harvested and IRF-1 was immunoprecipitated from lysates using C-20. The immunoprecipitates were analysed by 10% SDS-PAGE, the gels treated with Amplify (Amersham Pharmacia) and the labelled protein was detected by autoradiography. Lane C, is an IRF-1 control of 35 S-labelled protein translated *in vitro*, CB is a contaminating band used as a loading control. The results are representative of at least two individual experiments

3a,b). As expected mRNA analysis revealed that polyI:polyC treatment gave rise to an increase in

IRF-1 mRNA at 1–2 h corresponding to the increases in IRF-1 protein (Figure 3a,b). Interestingly, IRF-1 mRNA levels were also elevated in response to IR and etoposide with kinetics that were again consistent with the increases seen in IRF-1 protein levels.

To determine whether the increases in IRF-1 mRNA resulted in a concomitant increase in protein synthesis pulse-labelling experiments were used to tag newly synthesized IRF-1 protein. Increases in the amount of labelled IRF-1 protein were readily detected, relative to background bands, when the cells were incubated with polyI:polyC, IR or etoposide prior to addition of radiolabel (Figure 3c). Thus, at least in part, the increases in IRF-1 protein detected in cells exposed to either viral mimetics or genotoxic agents can be accounted for by up-regulation of *IRF-1* gene expression and *de novo* protein synthesis. This distinguishes IRF-1 from p53 whose activation in damaged cells is controlled by post-translational events (Lakin and Jackson, 1999).

DNA-damaging agents but not polyI:polyC increase the half-life of IRF-1 protein

Many regulatory proteins have a relatively rapid rate of turnover which ensures that they can respond quickly to small perturbations in either their rate of synthesis or rate of degradation to reach a new steady state level. To determine whether IRF-1 protein levels are regulated solely by increases in gene expression and protein synthesis, or if regulation of IRF-1 protein degradation contributes to induction of the protein in cells by either DNA damage or viral mimetics, we first determined its half-life in untreated A375 cells.

The half-life of IRF-1 protein in untreated cells was calculated to be 38 min (Figure 4a,b) and when the cells were pre-treated with polyI:polyC for 2 h prior to the addition of cyclohexamide, the half-life remained essentially unchanged at 35 min (Figure 4a,b; similar results were obtained when the cells were pre-treated with polyI:polyC for 1.5 or 4 h – not shown). Thus, polyI:polyC has no effect on the rate of IRF-1 degradation. Strikingly, when cells were pre-treated with either IR or etoposide, and cyclohexamide added 2 and 4 h after treatment respectively, the half-life of IRF-1 protein was significantly increased (Figure 4a,b). The $t_{0.5}$ of IRF-1 in the presence of IR and etoposide was calculated to be 114 and 88 min respectively. Thus, both IR and etoposide affect the rate of IRF-1 protein turnover, by inhibiting its degradation. The short half-life of IRF-1 allows it to respond rapidly to lesions in DNA and also ensures that the response is readily reversible.

The ATM kinase is involved in signalling the presence of genotoxic stress to IRF-1

The ATM kinase plays a critical role in the regulation of p53, and certain other transcription factors, as a direct consequence of DNA damage and in particular DNA strand breaks. We therefore sought to determine

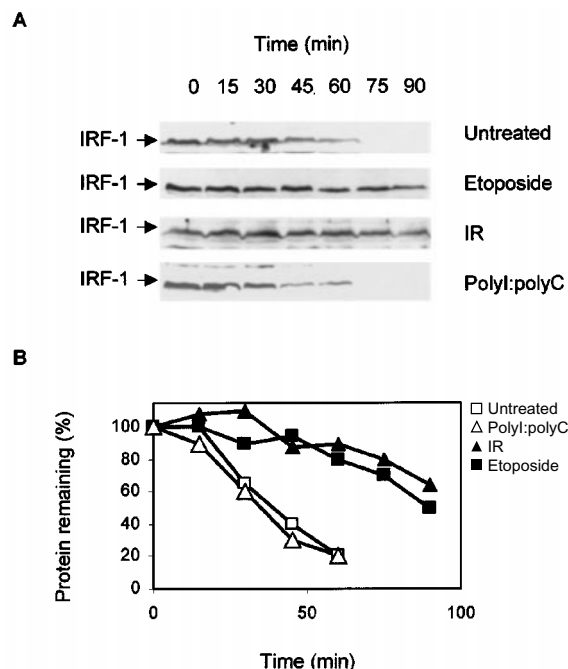


Figure 4 The effect of genotoxic drugs on IRF-1 degradation. (a) Immunoblot analysis of A375 cells either untreated or pre-treated with etoposide (10 μ M) for 4 h, IR (5 Gy) for 2 h or polyI:polyC (50 μ g/ml) for 2 h, prior to the addition of cycloheximide (30 μ g/ml). Cells were harvested at the times shown and analysed (100 μ g/lane) by 10% SDS-PAGE/immunoblot, IRF-1 was detected using the polyclonal serum C-20 and the data are representative of four separate experiments/condition. (b) The intensity of the bands shown in (a) were quantified using a GeneGnome bioimager, and plotted as % remaining protein against time

whether the ATM kinase might also be a component of the upstream pathway leading to the regulation of IRF-1. Fibroblasts derived from patients with ataxia telangiectasia (AT), that are defective in the ATM signalling pathway (Lavin and Shiloh, 1997), were analysed to determine whether the response of IRF-1 to DNA damaging agents in these cells was compromised.

Initial studies demonstrated that, in common with other stress regulated proteins such as p53 and p21 (Gatei *et al.*, 2001), IRF-1 protein levels were noticeably elevated in AT fibroblasts compared to untransformed NHFs, although the levels were comparable to those found in A375 cells (Figure 5a). When the BM0026 AT fibroblasts, acquired as a growing culture from the European Collection of Cell Cultures and used at low passage number, were analysed the induction of IRF-1 seen in response to IR and etoposide treatment of A375 cells was noticeably absent (Figure 5b). However, the pathway leading to IRF-1 up-regulation in response to polyI:polyC was intact as there was a clear induction of IRF-1 protein with comparable kinetics to those seen in A375 cells (Figures 5b and 2c). As it is possible that the regulation of IRF-1 in fibroblasts is different to

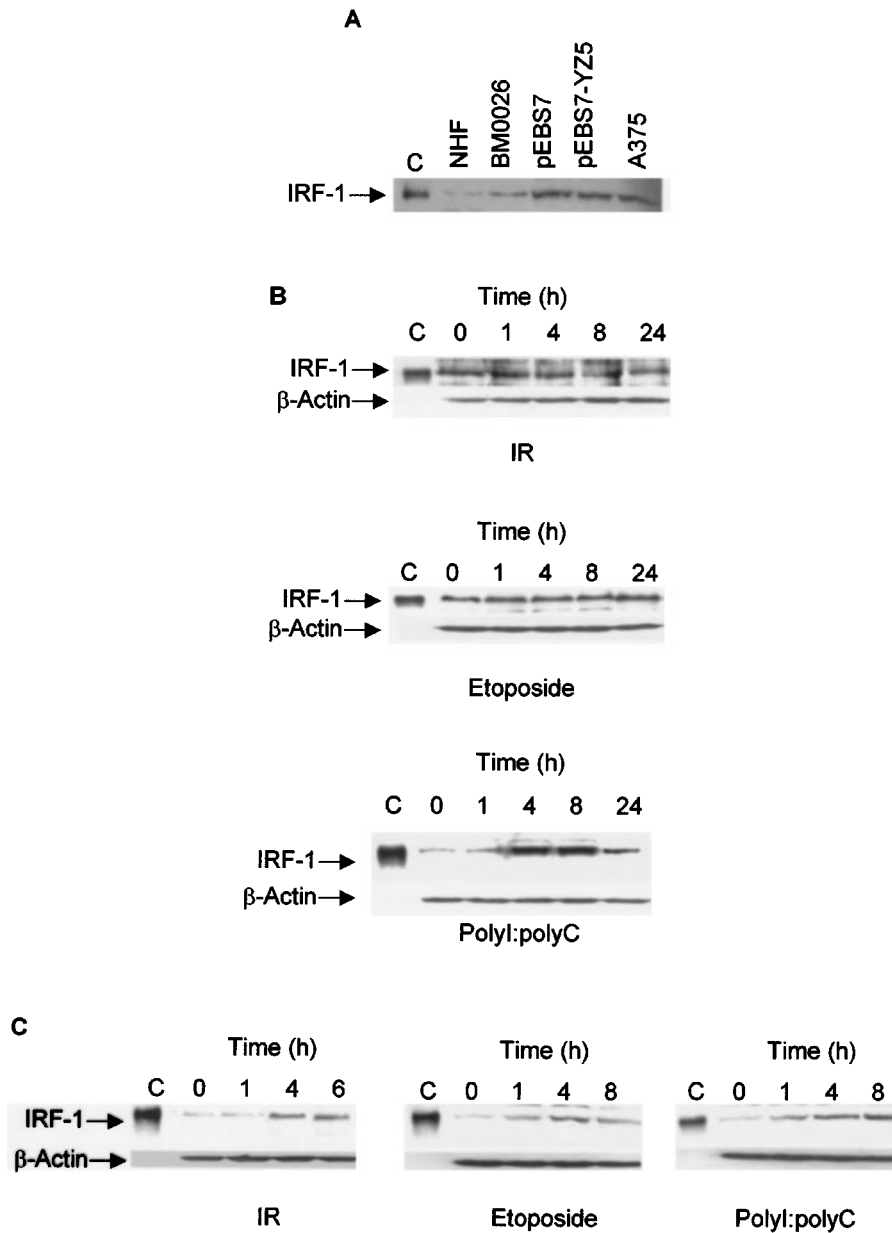


Figure 5 IRF-1 is not induced by genotoxic stress in AT fibroblasts. (a) Immunoblot analysis of IRF-1 protein in crude lysates (100 μ g/lane) from NHF, BM0026 AT fibroblasts, AT221JE-T fibroblasts stably transfected with vector alone (pEBS7) or ATM (pEBS7-YZ5), and A375 cells. IRF-1 was detected using C-20, lane C, is a control of *Sf9* expressed IRF-1 protein. (b) Immunoblot analysis of IRF-1 protein in passage 4 AT fibroblasts (BM0026) treated with IR (5 Gy), etoposide (10 μ M) or polyI:polyC (50 μ g/ml) and samples collected at 0, 1, 4, 8, and 24 h post-treatment. IRF-1 was detected using C-20 and the blots were reprobed for β -actin using AC-15. Lane C, is an IRF-1 control of *Sf9* expressed protein the data are representative of three experiments. (c) Immunoblot analysis of NHFs treated with IR (5 Gy), etoposide (10 μ M) or polyI:polyC (50 μ g/ml), collected at the times shown and separated by 10% SDS-PAGE (100 μ g/lane). IRF-1 and β -actin were detected using C-20 and AC-15, respectively. Lane C, is an IRF-1 protein control and the data are representative of three experiments

that seen in other cell types, the response of IRF-1 to genotoxic stress in untransformed human fibroblasts was determined. Normal human fibroblasts (NHF), cultured from a biopsy and verified to be cytogenetically normal, were used at low passage number (passage 5 or 6). Figure 5c shows that IRF-1 protein levels increased in response to both IR and etoposide and that the time-course of induction was similar to

that seen in A375 cells. Thus, NHFs induce IRF-1 in response to genotoxic agents suggesting that the defect seen in the AT fibroblasts may be specific for the ATM kinase.

To determine which step(s) in the induction of IRF-1 is target by ATM-dependent signals following exposure to genotoxic stress we analysed IRF-1 mRNA levels and IRF-1 protein half-life in the BM0026 ATM-

defective fibroblasts. When ATM-defective cells were treated with IR no significant increase in the amount of IRF-1 mRNA was detected, whereas treatment of these cells with polyI:polyC gave an increase in IRF-1 mRNA with kinetics similar to those observed in A375 cells (Figures 6a and 3a). The half-life of the IRF-1 protein in control undamaged AT cells was increased compared to that of the protein in A375 cells (64 min compared to 38 min), however the half-life remained essentially unchanged in IR treated cells (Figure 6b). Thus, ATM is required for the increased expression of IRF-1 following exposure to DNA damaging agents and is also likely to play a role in determining the rate at which the protein is degraded.

Expression of ATM in AT fibroblasts restored induction of IRF-1 by genotoxic stress

As one of the characteristics of cells from AT patients is their inherent genomic instability (Shiloh and Kastan, 2001) it is possible that the defect in IRF-1 induction in response to DNA damaging agents in AT fibroblasts was due to a secondary effect of the ATM

lesion. In light of this we sought additional evidence that ATM was directly involved in the regulation of IRF-1. Two approaches were taken: firstly the IRF-1 response in ATM-defective cells stably expressing ATM was examined, and second, A375 cells were treated with the PI-3 Kinase inhibitor wortmannin to determine whether damage induced increases in IRF-1 could be overridden by this compound.

To overcome the technical difficulties associated with the transient expression of ATM in AT fibroblasts we used an AT fibroblast line (AT22IJE-T) stably expressing ATM (Ziv *et al.*, 1997). When IRF-1 protein levels were determined in the AT22IJE-T/pEBS7 cells (harbouring a carrier plasmid in the absence of the cDNA for ATM) a similar pattern to that seen in the BM0026 cells was observed (Figure 7a). Thus, polyI:polyC gave an induction of IRF-1 at 4 h and no increase in IRF-1 was observed in cells treated with either IR or etoposide (Figure 7a). The expression of ATM in the AT22IJE-T cells (AT22IJE-T/pEBS7-YZ5) gave a reproducible and statistically significant increase in IRF-1 protein in response to IR and etoposide of 3.3-fold and 2.9-fold, respectively, which is a similar increase to that seen in NHFs

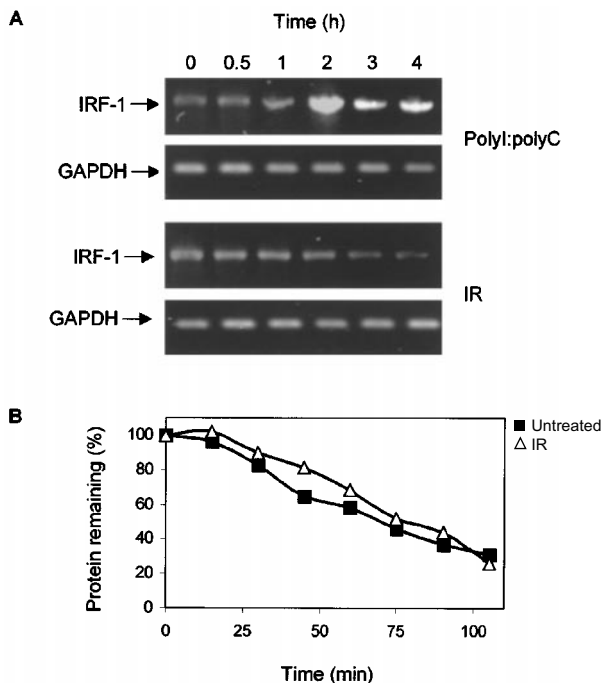


Figure 6 IRF-1 expression and degradation in AT-fibroblasts. BM0026 cells were treated with IR (5 Gy) or polyI:polyC (50 µg/ml), at the indicated time points cells were collected and mRNA was prepared. IRF-1 and GAPDH mRNA levels were determined by semi-quantitative RT-PCR analysed on 1% or 1.2% agarose gels, respectively. The data are representative of at least two independent time-courses analysed in triplicate. (b) BM0026 cells, either untreated or pre-treated with IR (5 Gy) for 2 h prior to the addition of cycloheximide (30 µg/ml), were harvested at 15 min intervals and analysed by 10% SDS-PAGE/immunoblot, IRF-1 was detected using C-20 and chemiluminescence was quantified directly from the membrane using a GeneGnome bioimager. The data are plotted as per cent remaining protein against time

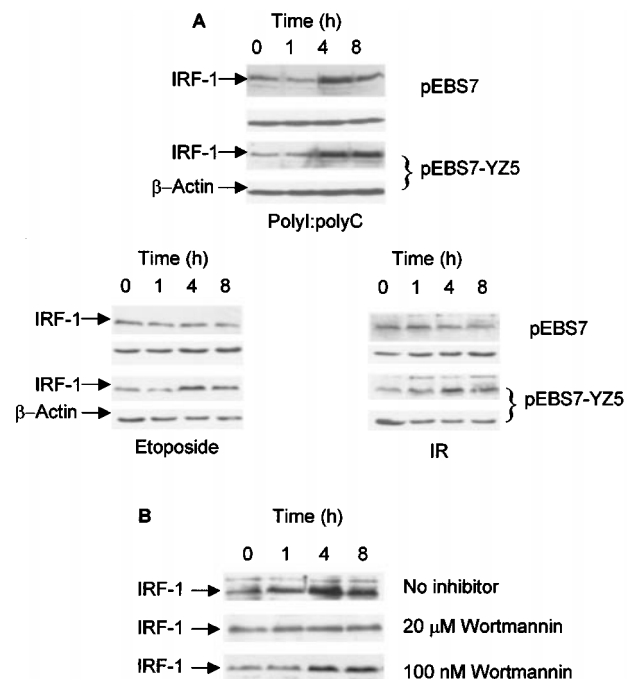


Figure 7 Modulation of ATM activity can regulate IRF-1 protein levels. (a) Immunoblot of AT fibroblasts (AT22IJE-T) stably transfected with empty vector (pEBS7) or the ATM vector (pEBS7-YZ5; Ziv *et al.*, 1997). The cells were treated with polyI:polyC (50 µg/ml), etoposide (10 µg) or IR (5 Gy) and lysates were separated by 10% SDS-PAGE (100 µg/lane). IRF-1 was detected using C-20 and the blots were stripped and re-probed for β-actin using AC-15. (b) Immunoblot of A375 cell lysates (50 µg/lane) either untreated or pre-treated with wortmannin (20 µM or 100 nM) for 10 min prior to the addition of etoposide (10 µM). Samples were taken at 0, 1, 4 and 8 h and IRF-1 was detected using C-20. The data are representative of three individual experiments

(Figure 5c). Thus, the introduction of ATM into ATM-defective cells is sufficient to restore, at least in part, the induction of IRF-1 normally seen in response to genotoxic stress.

Finally, the effects of the fungal toxin wortmannin, a specific inhibitor of the PI-3 Kinase family of enzymes that includes ATM, ATR and DNA-PK, on IRF-1 induction was determined. At 20 μ M wortmannin inhibits ATM and DNA-PK in intact cells, but is only a poor inhibitor of ATR (Sarkaria *et al.*, 1998). When A375 cells were treated with 20 μ M wortmannin, prior to the addition of etoposide, they failed to induce IRF-1 protein, suggesting that the signalling pathway that targets IRF-1 in damaged cells contains a member of the PI-3 Kinase family (Figure 7b). Interestingly, no inhibition of IRF-1 induction was seen when A375 cells were incubated with 100 nM wortmannin, at this concentration wortmannin inhibits the classical PI-3 Kinases but does not inhibit ATM or DNA-PK. Taken together these data provide evidence that ATM is a component of the upstream signalling pathway leading to the induction of IRF-1 during the response to genotoxic stress.

Discussion

Eukaryotic cells respond to DNA damage by activating damage checkpoint pathways, which arrest cell cycle progression and alter induced gene expression to allow for repair and /or apoptosis. Our understanding of how signalling networks are activated to coordinate the cellular respond to genotoxic stress has grown rapidly in the past few years. A number of stress activated kinases have been identified which target gene expression through the actions of downstream factors such as p53 and NF- κ B. Although the IRF-1 transcription factor has previously been associated primarily with the IFN response (Taniguchi *et al.*, 2001) it has also been proposed that IRF-1 is activated in response to IR (Tanaka *et al.*, 1996; Taniguchi *et al.*, 1998). The results presented here suggest a role for the IRF-1 pathway in regulating cell growth not only as a consequence of exposure to IR but also in response to DNA strand breaks caused by the inhibition of topoisomerase activity. We have demonstrated that IRF-1 induction is regulated by ATM-dependent signalling events, which impact on both IRF-1 expression and post-translational regulation. As the accumulation of p53 protein in IR treated cells is also dependent, at least in part, on ATM activity our data provide an explanation for how p53 and IRF-1 are coordinatly up-regulated during the response to DNA damage (Figure 2) allowing them to cooperate to regulate p21 expression (Figure 1) and cell growth (Tanaka *et al.*, 1996).

IRF-1 induction was observed in A375 cells and normal human fibroblasts in response to both IR and etoposide, induction occurred within 4 h of treatment and was mediated by changes in both protein synthesis and post-translational changes in the rate of IRF-1

protein degradation. Although both IR and etoposide induced *de novo* synthesis of IRF-1 and inhibited its degradation, it was noticeable that there were quantitative differences. Thus effects on protein turnover made a greater contribution to the induction of IRF-1 protein, relative to protein synthesis, in response to IR. Conversely, increased expression and protein synthesis played a more significant role during the response to etoposide. No significant increase in IRF-1 protein levels was observed in AT fibroblasts following treatment with either IR or etoposide and the reasons for this were a failure to up-regulate *IRF-1* expression coupled with a lack of effect on the half-life of the existing IRF-1 protein. Interestingly, when we used AT cell lines we were able to completely uncouple induction of IRF-1 by damaging agents from induction by viral mimetic, suggesting that these two pathways function independently from each other.

In common with other stress responsive proteins, such as p53, GADD45, LMP2 and p21 (Canman *et al.*, 1994; Gatei *et al.*, 2001; Siddoo-Atwal *et al.*, 1996), elevated basal levels of IRF-1 protein were detected in AT fibroblasts compared to control cells (Figure 5a). Paradoxically, this suggests that ATM may play a negative role in the regulation of IRF-1 protein levels in proliferating cells and this is supported by the observation that the half-life of IRF-1 is increased in AT cells compared to A375 cells (Figures 4 and 6b). Indeed, recent reports show that ATM protein levels and activity increase in response to mitogenic stimulation suggesting that the kinase functions during cell growth, as well as the response to DNA damage (Fukao *et al.*, 1999; Yang and Kastan, 2000). However, as well as having increased levels of some stress related proteins ATM-defective cells also show altered activity of stress regulated enzymes such as JNK and Cdc2 (Paules *et al.*, 1995; Sung *et al.*, 1998). Such global attenuation of stress responsive pathways suggests that ATM-defective cells are under continuous stress, perhaps due to high basal levels of double strand DNA breaks and increased levels of reactive oxygen species (Gatei *et al.*, 2001), which would argue against ATM having a specific effect on IRF-1 protein levels in cycling cells. In the current study although higher basal levels of IRF-1 protein may contribute to its lack of induction in damaged AT cells they are unlikely to fully explain the defect in the IRF-1 response. Firstly, IRF-1 protein levels are not maximal in the ATM-defective cells as treatment with poly-I:polyC leads to a significant increase in both the BM0026 and AT22IJE-T cells. Second, whereas we were unable to detect any significant increase in IRF-1 protein in BM0026 cells treated with either IR or etoposide, p53 protein levels, which are also elevated in these cells, increased in response to both these stimuli although as expected induction was delayed and reduced when compared to cells treated with cisplatin (data not shown). Finally, the expression of ATM in the AT22IJE-T cells, in which the highest basal levels of IRF-1 protein were detected, partially restored the ability of these cells to induce IRF-1 following treatment with IR or etoposide (Figure 7).

A number of transcription factors can be placed downstream of ATM, either as ATM substrates or as substrates of downstream signalling components. The best characterized of the pathways leading to induction of gene expression through the activation of specific transcriptional regulators is ATM-dependent modulation of p53. Activation of ATM in response to DNA damage leads to either direct phosphorylation of p53 (Banin *et al.*, 1998), or stimulation of Chk2-dependent phosphorylation (Chehab *et al.*, 1999), within the N-terminal domain inhibiting Mdm2 binding and promoting binding of the coactivator p300 (Dornan and Hupp, 2001; Lambert *et al.*, 1998). Mdm2 is a negative regulator of p53 transcriptional activity (Momand *et al.*, 1992; Wu *et al.*, 1993) and a component of the E3 ubiquitin ligase that targets p53 for degradation in proliferating cells (Fang *et al.*, 2000; Honda and Yasuda, 2000). Thus ATM-dependent phosphorylation contributes to the induction of p53 protein by inhibiting its degradation and also promotes its activity as a transcription factor through the recruitment of the p300 coactivator. ATM has also been implicated in the regulation of E2F1 during the response to etoposide and adriamycin; in this case ATM-dependent phosphorylation of E2F1 decreases its rate of degradation promoting E2F1-mediated apoptosis (Lin *et al.*, 2001).

ATM can regulate transcription through a more indirect route. For example ATM is required, in some cell types, for the activation of NF- κ B in response to IR (Li *et al.*, 2001; Piret *et al.*, 1999). Although the molecular mechanism by which ATM leads to activation of NF- κ B remains unclear I κ B-kinase is required to transduce the signal and both activation of the kinase and degradation of the NF- κ B inhibitor protein, I κ B, are impaired in ATM deficient cells (Li *et al.*, 2001). Regulation of c-jun by ATM involves modulation of upstream kinases/phosphatases and similar to our findings for IRF-1 (Figure 6), regulation of c-jun mRNA levels. Thus, induction of c-jun mRNA is defective in AT- compared to normal-lymphoblasts, whilst the response of c-jun to certain growth factors is enhanced in ATM-defective cells (Sikpi *et al.*, 1999).

Based on information of the consensus phosphorylation site for ATM (Kim *et al.*, 1999) we would not expect IRF-1 to be an efficient substrate for this enzyme. Thus, it is likely that ATM modulates the rate of IRF-1 degradation indirectly through the actions of downstream kinase (Chehab *et al.*, 1999; Li *et al.*, 2001) and/or phosphatase (Bar-Shira *et al.*, 2002) signalling components or via modification of another protein that impacts on the rate of IRF-1 degradation. For example, the phosphorylation of c-jun in response to IR is compromised in AT-cells (Lee *et al.*, 1991, 1998) and studies have shown that the activities of at least two members of the pathway regulating c-jun activity are defective in the absence of ATM, although c-jun itself does not appear to be phosphorylated by the ATM kinase. Another example, is the phosphorylation of MDM2 by ATM, so that in addition to phosphorylating p53 ATM also phosphorylated its

negative regulator and this appears to contribute to inactivation of MDM2 as a component of the p53 ubiquitin ligase complex (Maya *et al.*, 2001). Although post-translational modification of IRF-1 is known to modulate its activity as a DNA-binding protein and transcription factor the cellular components in these regulatory pathways remain to be identified (Lin and Hiscott, 1999; Sharf *et al.*, 1997; Watanabe *et al.*, 1991). Similarly although IRF-1 degradation is mediated via the proteasomes nothing is currently known about the pathway leading to its ubiquitination or about the factors that may influence this process (Nakagawa and Yokosawa, 2000).

Our results highlight a role for ATM in the regulation of IRF-1 during the response to genotoxic stress and uncouple induction of IRF-1 by DNA damaging agents from induction by viral mimetics. Thus, ATM appears to provide the key to explaining how p53 and IRF-1 can be coordinately regulated in a stress specific manner to cooperate in activation of the *p21* promoter. However, p53 is unlikely to provide the link between ATM and induced transcription of IRF-1 as Tanaka *et al.* (1996) showed that p53^{-/-} MEFs were unimpaired in their ability to induce IRF-1 protein following exposure to IR. Identification of the transcription factor responsible for regulating *IRF-1* expression during the response to genotoxic stress will therefore be of great interest.

Materials and methods

Cell cultures

A375 human melanoma cells and NHFs were cultured in Dulbecco's Minimal Essential Media (DMEM, Gibco-BRL) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin, at 37°C and 10% CO₂. HCT116 p53^{-/-} cells were a kind gift from B Vogelstein (Johns Hopkins University School of Medicine) and were maintained in McCoy's 5A medium (Gibco-BRL) supplemented with 10% (v/v) FBS and incubated at 37°C with an atmosphere of 10% CO₂. AT22IJE-T/pEBS7 and AT22IJE-T/pEBS7-YZ5 cells were grown as previously described (Ziv *et al.*, 1997). BM0026 ATM-defective cells were grown in DMEM supplemented with 20% (v/v) FBS and 1% (v/v) penicillin/streptomycin, at 37°C.

Chemicals and antibodies

The anti-IRF-1 serum (C-20; Santa Cruz), the p21 Mab AB-1 (Oncogene Research Products) and the anti-p53 Mab 19.1 were all used at a final concentration of 1 μ g/ml. A stock solution of 10 mM etoposide (Sigma) was prepared in DMSO. PolyI:polyC and cycloheximide (Calbiochem) were dissolved in 0.5 M HEPES, pH 7.0 at 50 mg/ml and ethanol at 250 mg/ml, respectively. Wortmannin was obtained from Calbiochem. The final concentration at which these agents were used is described in the figure legends. Cells treated with IR were exposed to 5 Gy of γ -radiation.

Immunoblots

Cells were lysed in NP-40 buffer (50 mM Tris, pH 8.0, 1% (v/v) NP40, 150 mM NaCl, 5 mM EDTA, 25 mM NaF, 2 mM

DTT, 400 µg/ml Pefabloc, 2 µg/ml pepstatin, 4 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml soya bean trypsin inhibitor, 1.2 mM benzamide) incubated on ice for 15 min and centrifuged at 14000 g for 15 min, the supernatants were analysed by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose. The immunoblots were blocked in 5% milk-PBS+0.1% Tween (PBST) for 1 h and incubated with the primary antibody diluted in 5% milk-PBST for 2 h at RT or overnight at 4°C. Swine anti-rabbit or rabbit anti-mouse secondary antibodies (Dako) were used at 1:2000 in 5% milk-PBST for 1 h. The immunoblots were washed exhaustively in between each step with PBST and antibody binding was detected by enhanced chemiluminescence.

Luciferase reporter assay

HCT116 p53^{-/-} cells were seeded into 60 mm dishes the day before transfection. Cells were transfected with 1 µg of p21-Luc and 1 µg of pCMV-βGal using lipofectamine 2000 (Gibco-BRL), as indicated in the manufacturer's handbook and harvested 24 h later. The luciferase and β-Gal assays were carried out as indicated in the manufacturer's handbook (Promega) and the signals were detected using a Fluoroskan Ascent F1 (Labsystems) and Dynatech plate reader at 414 nm, respectively.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from cell pellets using TRIzol® (1 ml/60 mm plate) to lyse the cells. Chloroform was added (0.2 ml/sample) to separate the aqueous and organic phases, followed by precipitation of the RNA with isopropanol (0.5 ml/sample) and the quality of the RNA was determined by analysis on a 1.4% agarose-formaldehyde gel. First strand cDNA was synthesized from 1 µg of total RNA, using 1.5 µM oligo (dT) primer (Promega), 0.2 mM each of dNTP (Roche), 10 U Rnase inhibitor (Promega), 10 mM DTT (Promega), Omniscript reverse transcriptase (Qiagen) and RT buffer (Qiagen) in a final volume of 20 µl. For each PCR reaction 2 µl of the cDNA reaction was added to 0.2 mM each of dNTP, 2 U Hot Star Taq polymerase (Qiagen) and gene specific PCR primers (50 pmol of each) in the buffer provided (Qiagen). The primers used were as follows (5' to 3') CTACGGTGCACAGGGAATGG and CATGCCATC-ACTCGGATGC for IRF-1; GTCAGTGGTGGACCTG-ACCT and ACCTGGTGTCTAGTGTAGCC for GAPDH.

For semi-quantitative RT-PCR the number of amplification cycles was determined separately for both IRF-1 and GAPDH using mRNA from both A375 and BM0026 cells.

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Standard curves were constructed for cycle number verse signal (RLU) and all subsequent experiments were carried out in the linear range. The PCR products were compared to reactions carried out with plasmid DNA containing authenticated IRF-1 and GAPDH cDNAs. The number of cycles used were 32 cycles for IRF-1 and 30 cycles for GAPDH, at least two independent time-courses were analysed for each condition and RT-PCR reactions were carried out and analysed in triplicate.

Metabolic labelling and immunoprecipitation

A375 cells were incubated in 10 ml methionine/cysteine-free media (ICN CELLECT® met/cys deficient media) with 10% (v/v) dialysed FBS (Gibco-BRL) supplemented with [³⁵S]-methionine; [³⁵S]-L-cysteine (ICN, TRAN³⁵S-label) at 200 µCi/7 cm² flask. The cells were incubated at 37°C and 5% CO₂ for 30 min. IR (5 Gy), etoposide (10 µM) and polyI:polyC (50 µg/ml) were added as detailed in the figure legend. ³⁵S-labelled cell pellets were lysed in ice-cold RIPA buffer (400 µl) (10 mM Tris pH 7.2, 150 mM NaCl, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 1 mM DTT, 5 mM NaF, 2 µg/ml pepstatin, 4 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml soya bean trypsin inhibitor, 1.2 mM benzamide). The lysates were left on ice for 15 min and centrifuged at 14000 g for 15 min at 4°C. The supernatant was removed and incubated (200 µl) with 2 µg of anti-IRF-1 IgG (C-20) for 3 h at 4°C, protein G-Sepharose beads (20 µl) were added and the incubation continued for a further 2 h. The protein G-Sepharose beads had been pre-incubated with 3% (w/v) BSA in RIPA buffer before addition to the supernatant. The beads were washed three times with RIPA buffer and once with PBS. Bound proteins were analysed by the addition of SDS-PAGE sample buffer and electrophoresis using a 10% SDS-polyacrylamide gel (Laemmli, 1970). The gels were subsequently fixed (50% (v/v) methanol, 10% (v/v) acetic acid) and treated with Amplify (Amersham) prior to drying and autoradiography.

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