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p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding

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The tumour suppressor protein p53 is expressed at very low levels in normal cells but accumulates in response to DNA damaging agents such as u.v. irradiation. This increase is accompanied by transcriptional upregulation of the expression of a number of proteins including Mdm2 which can in turn inhibit p53 dependent transcriptional activation, creating a feedback loop resulting in down-regulation of p53 activity. Mutant p53 proteins are however frequently detected at constitutively high levels in many tumours and tumour cell lines, indeed this phenomenon has been used in several studies to diagnose p53 mutation in patient tumours. We show here that expression of mouse mutant p53 in tumour cell lines of this type results in high levels of both the endogenous p53 protein and the exogenously expressed mutant mouse protein, whereas the human tumour line MCF7 does not exhibit high levels of either endogenous human or exogenously expressed mouse mutant p53 unless stabilisation is induced by DNA damage. This suggests that the stability of mutant p53 is not intrinsic to mutant p53 protein structure but may vary in different cell backgrounds. We present evidence that p53 protein stability in tumour cell lines is determined by association with the Mdm2 tumour suppressor protein, and that p53 mutants which are unable to bind Mdm2 are stable in MCF7 cells. We propose that tumour lines which express high levels of transcriptionally inactive mutant p53 are unable to induce the expression of the Mdm2 protein which would normally provide a feedback mechanism down-regulating p53 protein levels in the absence of DNA damage signals. MCF7 cells however express a transcriptionally active p53 and retain the feedback regulation of p53 protein levels by Mdm2.

Keywords: p53 tumour suppressor protein; Mdm2 tumour suppressor protein; protein stability; DNA damage

Introduction

In normal cells the half-life of wild-type p53 protein is very short (in the order of 5–20 min) consistent with its rapid turnover, and it is usually undetectable by standard immunohistochemistry and immunocytochemistry. Several previous studies have shown that DNA damaging agents induce stabilisation and transient accumulation of wild-type p53 which leads to an arrest of the cell cycle at the G_1/S boundary (Kuerbitz *et al*., 1992), presumably to allow for repair of DNA lesions. The arrest is probably largely achieved by the p53 mediated induction of transcription of the Waf1/Cip1 gene (el Deiry *et al*., 1994; Harper *et al*., 1993). The product of this gene, the p21 protein is an inhibitor of cyclin-dependent protein kinases which are necessary for cell cycle progression. The mechanism of the DNA damage dependent induction of p53 protein itself is little understood but at least in part it must require protection against proteolysis. Inhibitors of cellular transcription and translation do not block accumulation in normal cells which suggests that the increase is not provided by newly synthesised protein, so stabilisation may be regulated by post-translational modification of the protein (Fritsche *et al*., 1993). Phosphorylation at serines 15 and 315 of human p53 have been shown to affect stability (Fiscella *et al*., 1993; Lin and Desiderio, 1993), and u.v. irradiation is known to induce phosphorylation of p53 by MAP kinase and a novel c-Jun kinase (Milne *et al*., 1994, 1995). Such modifications could induce a change in protein conformation which reduces the susceptibility to degradation, and there is evidence that ubiquitin dependent degradation mediated by E6 *in vitro* is preferential for the 1620^+ wild-type conformation of p53 (Medcalf and Milner, 1993). Similarly p53 may undergo a conformational change when bound to DNA (Halazonetis *et al*., 1993; Halazonetis and Kandil, 1993) and it appears that DNA binding may reduce E6 binding and subsequent proteolysis *in vitro*, while E6 binding inhibits DNA binding under similar conditions (Molinari and Milner, 1995). Indeed Reed *et al*. (1995) have suggested that wild-type p53 may sense DNA damage directly by increased binding to DNA with strand breaks, so that DNA damage itself may lead directly to stabilisation. However p53 is also induced by non-genotoxic stress such as hypoxia and heat shock (Graeber *et al*., 1994). In fact cells expressing E6 show increased degradation of p53 and cannot accumulate the protein even after DNA damage, but heat or hypoxia does induce accumulation in these cells (Kessis *et al*., 1993). This suggests that alternative pathways for stabilising p53 exist which may be particularly important in large solid tumours where hypoxia in the centre of the tumour mass has been shown to induce cell death (Graeber *et al*., 1996). This might be the mechanism for selection of mutant p53 cell clones which survive hypoxic stress and eventually colonise the tumour leading to malignancy. A novel mechanism which may relate to p53 induction has been described by Mosner *et al*. (1995). They have demonstrated that wild-type p53 protein can inhibit the translation of its own messenger RNA by binding to a

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putative stem loop structure in the 5′ untranslated region. They postulate that release from this negative feed-back mechanism e.g. by damage induced nuclear transport of p53, might allow a rapid increase in p53 levels.

Loss of p53 function through mutation in the p53 gene (usually accompanied by loss of the remaining allele) occurs in about half of human malignancies (Harris and Hollstein, 1993). p53 function can also be lost through other mechanisms including interaction with host proteins e.g. Mdm2 (Finlay, 1993), and viral proteins e.g. papillomavirus E6 (Kessis *et al*., 1993); adenovirus E1b (Sarnow *et al*., 1982); SV40 large T (Lane and Crawford, 1979), so inhibition of the tumour suppressor activity of p53 may be a feature of most human cancers (Zambetti and Levine, 1993). p53 mutation in tumours is frequently associated with constitutively elevated levels of protein (Bartek *et al*., 1991; Rodrigues *et al*., 1990) which often correlates with poor prognosis (Thor *et al*., 1992; Martin *et al*., 1992) and the transition to malignancy (Bennett *et al*., 1992). In these types of tumour the mechanism leading to accumulation of the protein is an increase in stability so that the half-life of the protein is increased to several hours in tumour cell lines (Oren *et al*., 1981; Reihsaus *et al*., 1990). An understanding of both the mechanism that underlies the accumulation of mutant p53 and its biological consequences may lead to new therapeutic approaches. There are however some situations where constitutively high levels of apparently wild-type p53 have been observed particularly in colon adenomas (Tominaga *et al*., 1993; Williams *et al*., 1993), cells transformed by Ras and Myc oncogenes (Lu and Lane, 1993), cells transformed by DNA viruses (Sarnow *et al*., 1982), and the F9 teratocarcinoma line (Oren *et al*., 1982). The mechanism of accumulation is again unknown but it is clear that mutation of the p53 gene is not always sufficient or even necessary to lead to accumulation of the protein. This can be demonstrated by transfecting mutant p53 genes into human cancer cell lines. The temperature sensitive mutant mouse p53 Val135 accumulates to high levels in the T47D breast tumour line which expresses high levels of endogenous human mutant p53, while mouse p53 Val135 is barely detectable in the MCF7 breast tumour line which shows low levels of expression of endogenous wild-type p53 (Vojtesek and Lane, 1993). A parallel situation can be found in Li-Fraumeni Syndrome patients who have a germ-line mutation in one of their p53 alleles. Even so their normal cells express undetectably low levels of p53 protein, and only in the tumours of these individuals where expression of the remaining wild-type allele is lost are high levels of p53 observed (Srivastava *et al*., 1990; Malkin *et al*., 1990). Originally it was thought that the types of mutation inherited were very restricted, perhaps reflecting a class of mutant proteins which were intrinsically unstable, but we now know that a whole range of mutations can be found associated with the syndrome (reviewed in Malkin, 1994).

To investigate the behaviour of mutant p53 in tumour cell lines we have expressed a number of mouse mutant p53 proteins in human tumour lines and demonstrate that they are expressed at a low level in MCF7 cells in the absence of DNA damage. These observations support the concept that cellular environment can regulate the expression of mutant p53. We show that p53 levels are low in the presence of Mdm2 protein, and that those p53 mutants which are unable to bind Mdm2 either because of mutations in the binding domain, or loss of oligomerisation, are stable in MCF7 cells. p53 dependent expression of Mdm2 may therefore provide a feedback loop with the dual role of repressing p53 dependent activation of transcription, and reducing the overall p53 protein level.

Results

Mutant Mop53 accumulates to high levels in A431 and MDA468 but not in MCF7 cells

A431, MDA468, and MCF7 cells were stably transformed with CMV vectors expressing mouse mutant p53. Pools of transfectants were fixed and stained with monoclonal antibody DO-1 to detect endogenous human p53 or with PAb242 or PAb248 to detect mouse p53 expression. Western blotting and Elisa were also performed on cell lysates with the same antibodies. A413 CMVHis169 and MDA468 CMVHis169 cells showed strong nuclear staining of all cells with DO-1 and with PAb248 (A431 CMVHis169 Figure 1a −u.v. panels, but results were similar with four different mutant proteins in both A431 and MDA468). Elisa of A431 CMVHis169 (Figure 2 top two panels) and Western blots (Figure 3c and d) confirm high levels of both endogenous human p53, and mouse His169 p53 expressed from the plasmid in A431 and MDA468.

MCF7 transfectants however show strong nuclear staining with DO-1 and Pab248 in less than 5% of cells while the rest of the cells show very weak heterogeneous staining (Figure 1b −u.v. panels) indicating that both proteins are unstable in the majority of cells. The small number of MCF7 cells which show strong nuclear staining may be exhibiting elevated p53 levels as a result of a response to 'normal' growth conditions e.g. damage induced by abberrant replication. Occasional p53 positive cells are seen even in sections of normal tissues and might reflect events which occur rather more frequently in rapidly dividing immortal tumour lines like MCF7. To determine if high levels of endogenous MCF7 and mutant mouse p53 co-localise in these cells, double immunofluorescence staining was carried out on un-irradiated MCF7 CMVHis169 cells using anti-human p53 mouse monoclonal antibody DO-1 and anti-mouse p53 rabbit serum CM-5 (which was first preadsorbed with pure human p53 and normal mouse serum to remove cross-reaction with human p53). DO-1 and CM-5 staining co-localised in the occasional positive MCF7 CMVHis169 cells (results not shown) indicating that both proteins are simultaneously stabilised in these cells.

The majority of tumour cell lines expressing endogenous mutant p53 mutant proteins show similar characteristics to A431 and MDA468 in that they express high levels of endogenous p53 detectable by strong nuclear staining with specific antibodies. We show that A431 and MDA468 also support expression of high levels of the exogenous mouse mutant p53

expressed from transfected plasmids. Point mutations in p53 often produce proteins which are conformationally altered, and it had been assumed that the mutant proteins have a longer half life perhaps because they are not degraded as rapidly as wild-type p53. MCF7 tumour cells however express apparently wild-type endogenous p53 (Casey *et al*., 1991) which is transcriptionally active (results not shown). MCF7 cells

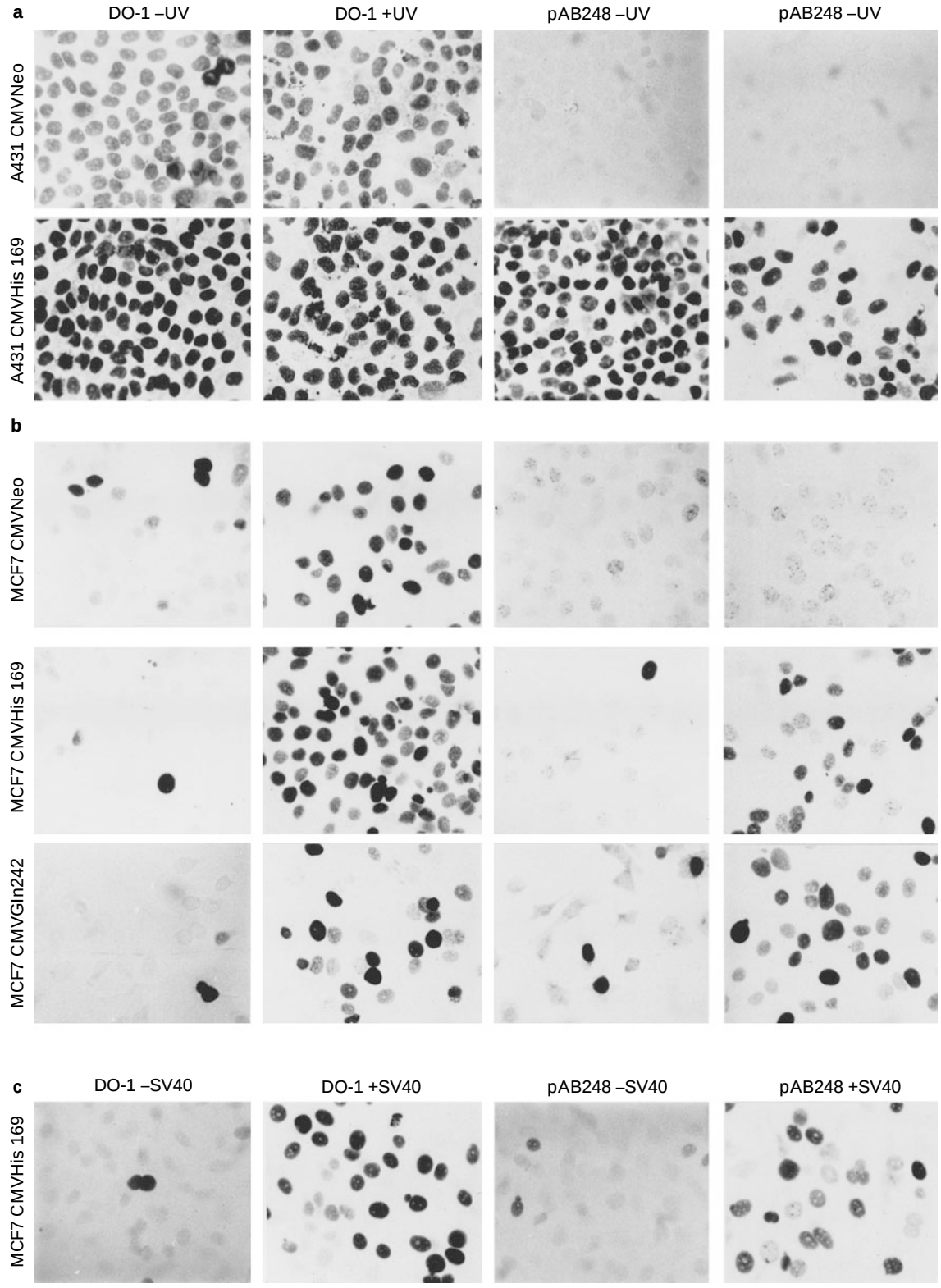

Figure 1 Mutant mouse p53 stability varies in different tumour cell backgrounds. Detection of mouse p53 protein in stably transfected cell lines shows that the mutant mouse p53 is present at constitutively high levels in the A431 human tumour line but not in MCF7 cells, although high levels of the protein are induced by DNA damage (u.v. irradiation) or SV40 virus infection of MCF7. (**a**) A431 CMVNeo and A431 CMVHis169 stained with DO-1 to detect endogenous human p53 or with PAb248 to detect mouse p53 expressed from the plasmid with or without UV treatment as indicated. (**b**) MCF7 CMVNeo, MCF7 CMVHis169 and MCF7 CMVGln242 stained with DO-1 or PAb248 with or without u.v. treatment as indicated. (**c**) MCF7 CMVHis169 either infected with SV40 virus or mock infected as indicated and stained with DO-1 or PAb248

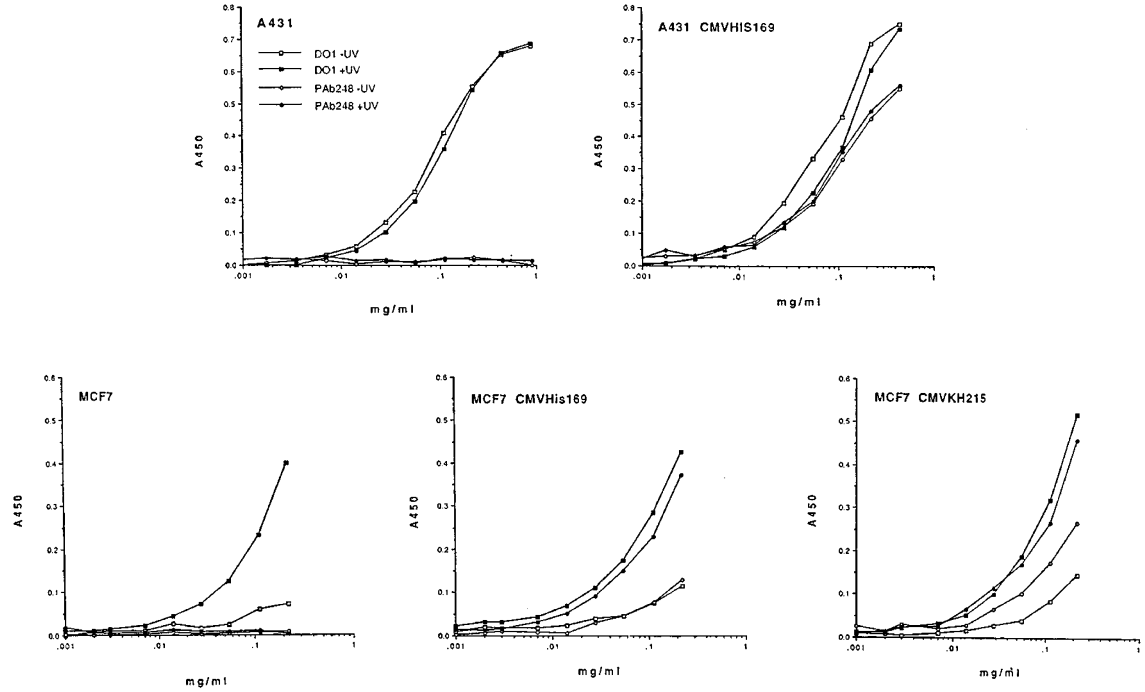

Figure 2 Two site ELISA assay of cell lysates of A431, A431 CMVHis169, MCF7, MCF7 CMVHis169 and MCF7 CMVKH215 with or without u.v. treatment. ELISA plates were coated with DO-1 or with PAb248 to capture endogenous human p53, or mouse p53 expressed from the plasmid, respectively. Bound p53 was detected with rabbit polyclonal anti-p53 antibody CM-1. The X axis represents cell lysate concentration (mg/ml), the key to identification is shown in the first panel

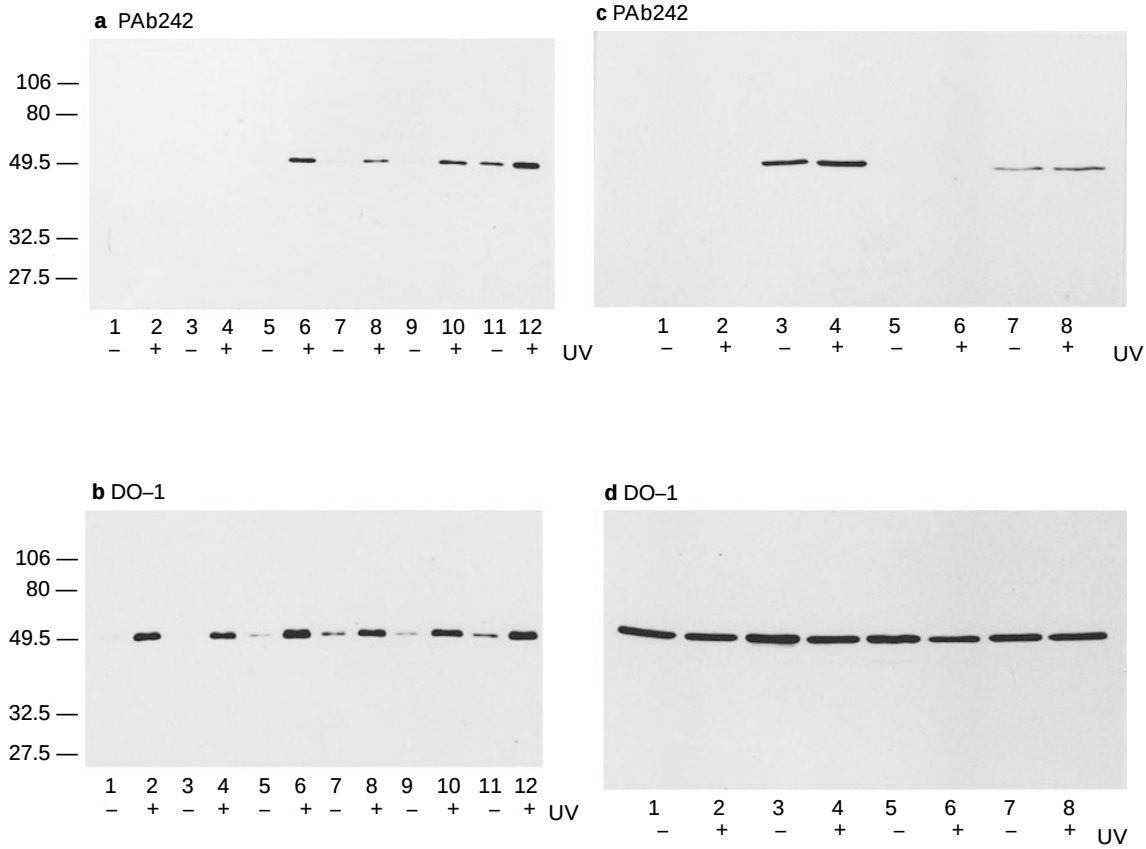

Figure 3 Western blot of cell lysates with or without u.v. treatment as indicated below the lanes. (**a** and **b**) show; MCF7 (lanes 1 and 2), MCF7 CMVNeo (lanes 3 and 4), MCF7 CMVHis169 (lanes 5 and 6), MCF7 CMVGln242 (lanes 7 and 8), MCF7 CMVVal135 (lanes 9 and 10), MCF7 CMVKH215 (lanes 11 and 12). (**c** and **d**) show A431 (lanes 1 and 2), A431 CMVHis169 (lanes 3 and 4), MDA468 (lanes 5 and 6), MDA468 CMVHis169 (lanes 7 and 8). Endogenous human p53 was detected with DO-1 and mouse p53 expressed from the plasmid was detected with PAb242 as indicated

differ from the class of tumour lines described above in that they express only low levels of endogenous p53 and in addition seem unable to support high levels of expression of introduced mouse mutant p53 as described above. This suggests that the environment of the cell rather than conformational changes in the protein due to mutation of the p53 gene determine p53 stability.

u.v. irradiation leads to accumulation of mutant Mop53 in MCF7 cells

DNA damage in normal cells caused by u.v. or ionising radiation and other agents including mitomycin C results in increased levels of p53 as a result of post-translational stabilisation of the protein by an unknown mechanism. u.v. irradiation of MCF7 cells also results in a dramatic increase in p53 detected by DO-1 cell staining (Figure 1b +u.v. panels), Elisa assay (Figure 2 bottom three panels) or Western blotting (Figure 3a and b), therefore the mechanism of DNA damage-dependent p53 stabilisation is still intact in these cells. Similarly the introduced mouse mutant p53s are also induced by u.v. irradiation in MCF7, proving that the cells are indeed expressing the introduced mouse p53, although both of the p53 proteins are barely detectable before the DNA damage response. The high levels of both the human and mouse p53 proteins expressed in A431 or MDA468 cells are not affected by u.v. irradiation induced DNA damage (Figure 1a +u.v. panels, Figure 2 top 2 panels and Figure 3c and d).

This indicates that these point mutations do not result in intrinsically stable Mop53, but that stable p53 expression in tumour lines such as A431 and MDA468 is the result of the cell environment. It is possible that most tumour lines may have aquired secondary mutations in pathways that either target p53 for rapid degradation, or which result in constitutive activation of the stabilisation mechanism which is a normal response to DNA damage, in which case MCF7 cells retain these mechanisms of p53 regulation and behave somewhat like non-tumour cells in this respect. u.v. irradiation of A431 or MDA468 expressing His169 p53 does not result in any further increase in mouse or human p53 which may have reached the maximum steady state level of expression. The expression of p53 in the MCF7 lines however shows some variation between mutants (Figure 2) where under un-induced conditions some mouse p53 mutants (e.g. KH215) are more stable than others (e.g. His169). This may indicate that the effect of a mutation on protein conformation may at least in part determine some intrinsic susceptibility to degradation.

Infection of MCF7 cells with SV40 leads to accumulation of p53 via association with T antigen

Stabilisation of wild-type p53 is also achieved by association with a number of viral proteins including SV40 large T antigen. This is thought to result in p53 inactivation and may be a necessary step in permitting viral replication in the cell without triggering a DNA damage response resulting in cell death or growth arrest. MCF7 cells expressing His169 mouse p53 were infected with SV40 T antigen to demonstrate stabilisation of both mutant and endogenous p53 by association with T-antigen (Figure 1c). Stabilisation by this route is therefore unrelated to MCF7 cell background and confirms that the protein is expressed by the plasmid. Since T antigen fails to bind many mutant proteins, the stabilisation of the mouse protein may be achieved here by oligomerisation with the wild-type p53 which can associate with T antigen.

Instability of mouse p53 in MCF7 is not due to inhibition of translation

(Mosner *et al*., 1995) have demonstrated the inhibition of translation of p53 mRNA by wild-type p53 protein. The functionally wild-type p53 expressed by MCF7 cells could therefore affect translation of the cDNA expressed by our CMV plasmids, although Mosner *et al*. suggest that this process requires nucleotides −216 to −108 in exon 1 of the 5′ untranslated region (5′UTR) to form a putative stem loop, and our plasmids only include nucleotides up to −135. However to ensure that a translation inhibition mechanism is not affecting our results we created a plasmid CMV5'ΔUTRHis169 from which we had removed all of the 5′UTR (Figure 4a). The MCF7 CMV5′ΔUTRHis169 cells (Figure 4b lanes c and d) behave like MCF7 CMVH169 (Figure 4b lanes a and b) i.e the protein is expressed at low levels until induced by u.v. irradiation. These results indicate that changes in translation or transcription are not responsible for increased mutant mouse p53 levels in response to DNA damage but that this is likely to be a consequence of altered protein stability.

Truncated Mop53 proteins are stabilised in MCF7 cells

MCF7 cells were stably transfected with plasmids expressing a series of truncated mouse p53 proteins (Figure 5a). Deletion of sequences from the N-terminus (NΔ37 and NΔ95) or the C-terminus (CΔ103) resulted in increased levels of the Mop53 protein in the absence of DNA damage (Figure 5b). The CΔ30 and CΔ61 deletions retain a degree of wild-type tumour suppressor activity which prevents the selection of stably transfected MCF7 cells (data not shown), but when expressed as double mutants His169CΔ30 and His169CΔ61 they were also expressed at high levels in the absence of DNA damage.

These results suggest that sequences in the N- and C-termini of p53 determine stability in MCF7. To define these further, we examined the stability of a deletion (p53ΔSS) of amino acids 327–341 which disrupts oligomerisation and results in monomeric Mop53 protein (Shaulian *et al*., 1993, Figure 5c). Val135ΔSS and His169ΔSS proteins were both more stable in MCF7 indicating that oligomerisation is required for down regulation of protein levels. A truncated Mop53 including amino acids 1–11 and 299–387 (p53DD, Shaulian *et al*., 1995) which contain the oligomerisation domain, co-expressed with His169Mop53 also stabilised both the Mop53 and the endogenous Hup53 (Figure 5c). p53DD can form mixed tetramers with full length p53 and may disrupt or 'poison' complexes increasing their stability. It is noteworthy that truncation mutants retaining the oligomerisation domain e.g NΔ95 which are expressed

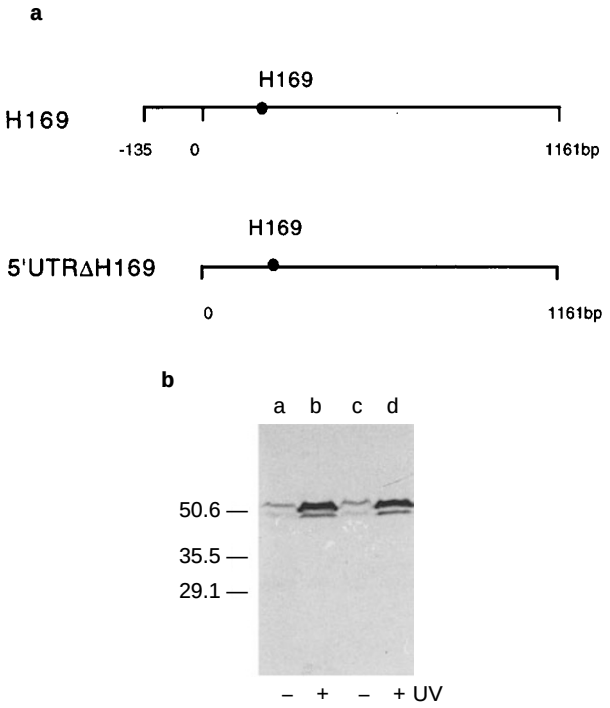

Figure 4 Induction of high levels of protein in MCF7 cells is not dependent on the presence of the 5′ untranslated region. (**a**) Diagram indicating the extent of mouse p53 cDNAs in the CMV expression plasmids. pCMV 5′UTRΔHis169 contains a cDNA which only includes the protein coding region, while all the other pCMV mouse p53 constructs used also include 135 bases of the 5′ untranslated region. (**b**) Western blot with a mixture of two rabbit polyclonal anti-p53 antibodies CM-1 and CM-5 to detect both p53s simultaneously. Lanes a, b, MCF7 CMVHis169, lanes c, d, MCF7 5′UTRΔHis169 with or without u.v. treatment as indicated. The upper band is human p53, the lower is mouse p53

at high levels in MCF7, also increase levels of the endogenous p53 in the absence of DNA damage whereas monomeric truncations e.g. CΔ103 or His169ΔSS do not increase endogenous p53 (Figure 5b and c). The stability of the His169Δ30 protein which retains its ability to oligomerise suggest that sequences at the extreme C-terminus may also be required for efficient degradation.

A Mop53 mutant which is unable to bind mdm2 is stabilised in MCF7

Since deletion of N terminal sequences of Mop53 resulted in high levels of protein, we stably transformed MCF7 cells with a plasmid expressing the Mop53 Gln22,Ser23 mutant which is unable to bind Mdm2 protein (Lin *et al*., 1994). Again this protein was stable in MCF7 (Figure 5d). The stabilisation of Gln22,Ser23 and also NΔ37 and NΔ95 indicate that the interaction of p53 with Mdm2 is required for the normal down regulation of p53 levels in MCF7 cells. Mdm2 binding is also thought to require oligomerised p53 (Marston *et al*., 1995).

Disruption of p53 – Mdm2 interaction in MCF7 CMVHis169 mouse p53 cells stabilises p53

MCF7 CMVHis169 cells were micro-injected with a monoclonal antibody 3G5 which binds to Mdm2 and disrupts its binding to p53 (Blaydes *et al*., 1997). Cells co-stained with anti mouse IgG and CM-1 anti-Human p53 antiserum or CM-5 anti-Mouse p53 antiserum, showed strong nuclear staining for both the human and the mouse p53 protein only in the presence of 3G5

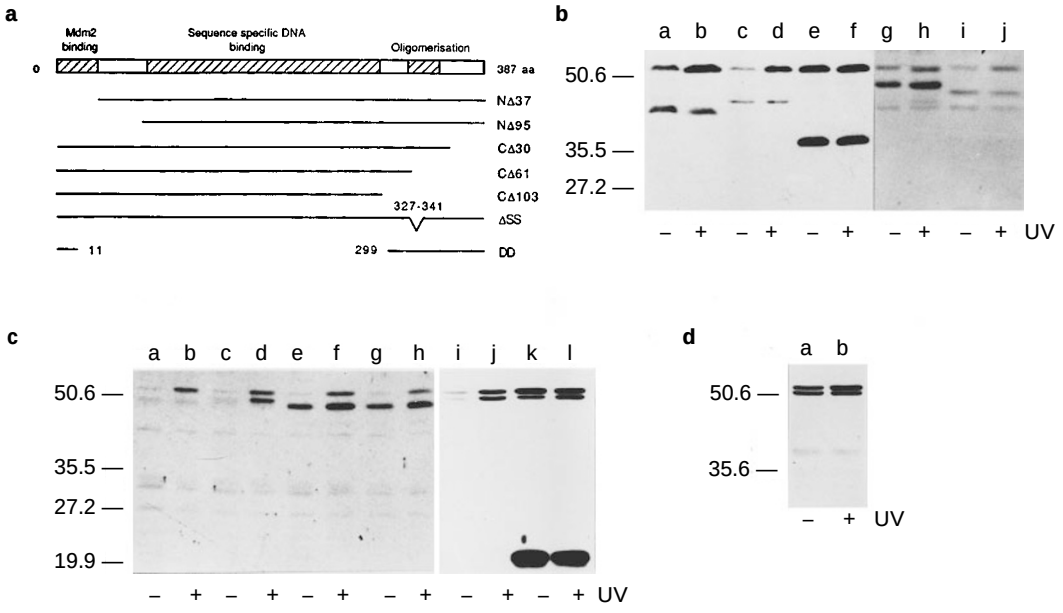

Figure 5 Truncation mutants of Mop53 are stabilised in MCF7 cells. (**a**) Diagram of the truncated p53 proteins expressed in MCF7 stable lines. (**b**) Western blot with a mixture of two rabbit polyclonal anti-p53 antibodies CM-1 and CM-5 to detect both p53s simultaneously. Lanes a, b, MCF7 CMVCΔ103, lanes c, d, MCF7 CMVNΔ37, lanes e, f, MCF7 CMVNΔ95, lanes g, h, MCF7 CMVHis169CΔ30, lanes i, j, MCF7 CMVHis169CD61. (**c**) Lanes a, b, MCF7 CMVNeo, lanes c, d, MCF7 CMVHis169, lanes e, f, MCF7 CMVHis169ΔSS, lanes g, h, MCF7 CMVVal135ΔSS, lanes i, j, MCF7 CMVHis169, and lanes k, l, MCF7 CMVHis169 co-expressed with pCDNAhygrop53DD. (**d**) Lanes a, b MCF7 CMVGln22,Ser23. All with or without u.v. treatment as indicated. The upper band is human p53, the lower is mouse p53

antibody (Figure 6a, b and c, d). In control experiments where cells were injected with the anti-Mdm2 antibody 4B2, nuclear staining for p53 did not coincide with 4B2 staining (Figure 6c and d). This supports a role for Mdm2 in the down-regulation of p53 protein expression in MCF7 cells.

Expression of Mdm2 in A431 cells reduces p53 stability in A431 cells

A431 cells which express endogenous His273 mutant human p53 were micro-injected with a plasmid expressing full length mouse Mdm2 (pMdmX2) or a plasmid expressing a truncation mutant Mdm2 unable to bind to p53 (pMdmΔXM) (Barak *et al*., 1994). Cells co-stained with CM1 anti-Hup53 antiserum and 2A10 (an antibody specific for the C-terminus of Mdm2) showed loss of nuclear p53 staining in cells where full length Mdm2 is expressed (Figure 7a and b). In control experiments the truncated Mdm2 protein did not abrogate p53 expression (Figure 7c and d). These results indicate that the presence of Mdm2 protein is responsible for the regulation of p53 levels in tumour cell lines.

Discussion

In tissue culture cells (Maltzman and Czyzyk, 1984) and in whole animal models (Midgley *et al*., 1995) p53 protein levels are normally very low but rise in response to DNA damage. This in turn can lead to cell cycle arrest or cell death by apoptosis. These responses are dependent on the activation of p53 in response to the genotoxic insult, indeed the pathways by which p53 detects and responds to DNA damage as well as the downstream response to p53 activation may well reveal other potential oncogenes and tumour suppressors. Although many insights have been gained into the cellular responses to p53 activation i.e.

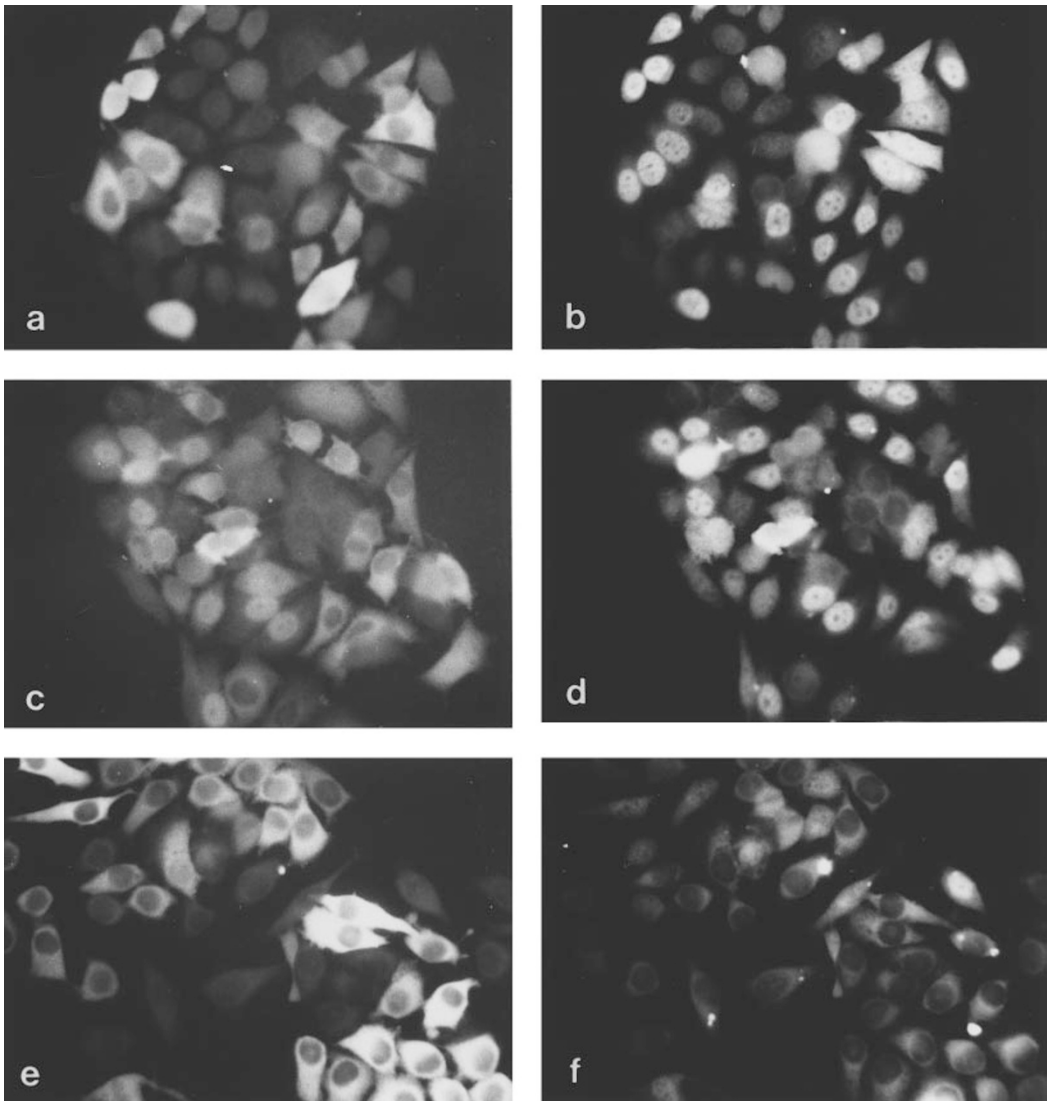

Figure 6 p53 protein levels in MCF7 are elevated when the interaction between p53 and Mdm2 is disrupted. MCF7 CMVHis169 cells were microinjected with mouse monoclonal antibodies 3G5 (**a**, **b**, **c**, **d**) or 4B2 (**e**, **f**). Cells were fixed and co-stained with CM-1 (**a**, **b** and **e**, **f**) or CM-5 (preadsorbed with pure human p53 to remove residual cross reactivity) (**c**, **d**) followed by FITC conjugated rabbit anti-mouse IgG (**a**, **c**, **e**) and Texas Red conjugated swine anti-rabbit IgG (**b**, **d**, **f**). Fluorescence microscopy shows nuclear p53 staining with CM-1 (**b**) or CM-5 (**d**) only in the presence of 3G5 (**a**, **b**), but no nuclear staining with CM-1 (**f**) was visible in the presence of 4B2 (**e**)

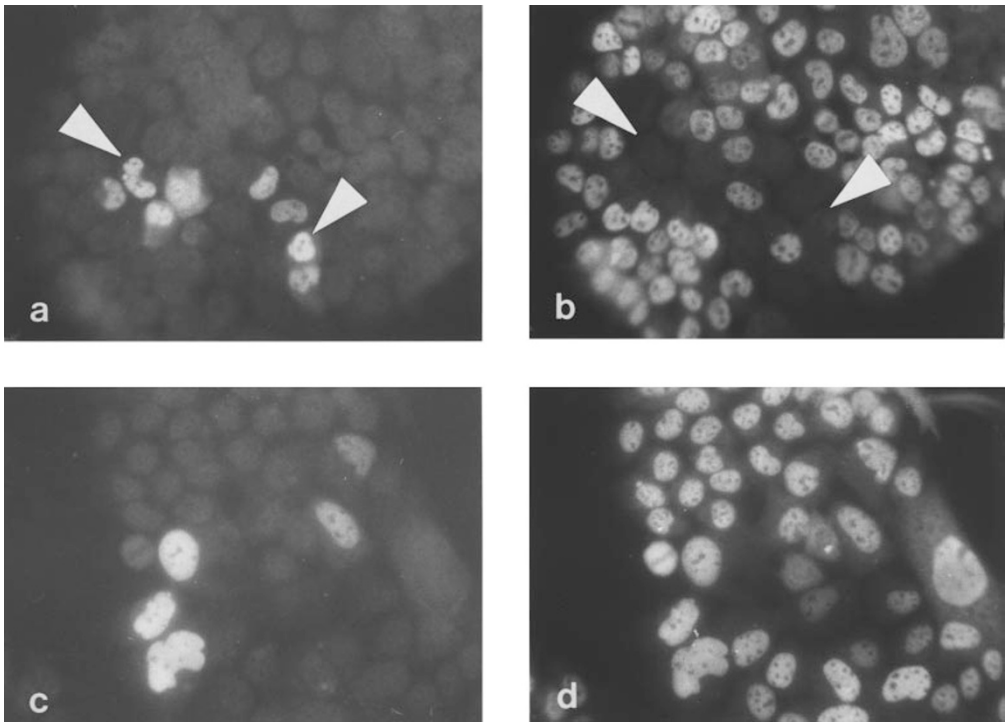

Figure 7 p53 expression is abrogated in A431 cells expressing Mdm2. A431 cells were microinjected with pMdm2X2 (**a**, **b**) or pMdm2ΔXM (**c**, **d**). Cells were fixed and co-stained with rabbit anti-p53 antiserum CM-1 and mouse monoclonal 2A10 anti Mdm2 antibody followed by FITC conjugated rabbit anti-mouse IgG (**a**, **c**) and Texas Red conjugated swine anti-rabbit IgG (**b**, **d**). Cells expressing Mdm2 showed no nuclear p53 staining with CM-1 (**a**, **b**) while mutant Mdm2ΔXM expression did not affect p53 staining (**c**, **d**)

switching on of p53 responsive genes which regulate cell cycle or apoptosis, little is understood about the mechanisms by which p53 detects DNA damage leading to its activation. The elevation of p53 protein levels in response to DNA damage occurs by a post-translational mechanism that increases the stability of the protein. Constitutively high levels of apparently wild-type p53 have been observed in some colon cancer lines (Williams *et al*., 1993; Tominaga *et al*., 1993). In contrast, some lines derived from Bloom's Syndrome patients are unable to stabilise their p53 in response to damage unless they are first fused to another cell type (Lu and Lane, 1993). This would suggest that these cells may lack a component required for DNA damage detection by p53, which can be supplemented by a fusion partner.

Perhaps most strikingly, many tumours and tumour cell lines express constitutively high levels of p53, and this is usually associated with mutation in the p53 gene. It had been assumed that mutant p53 is more stable perhaps because its altered conformation makes the protein less susceptible to degradation. It is also possible that many tumour cells aquire secondary mutations that could either reduce the rate of degradation of p53 protein, or might turn on a pathway which normally stabilises p53 in response to the cellular environment. This report demonstrates however that mutation of p53 itself is not sufficient to cause accumulation. Although most tumour lines are in the same class as A431 and show high levels of both endogenous and introduced mutant p53 protein expression which is not further increased by DNA damaging agents, MCF7 tumour cells express relatively low levels of p53 until DNA damaging agents induce the stabilisation response. Effectively the exogenously expressed mouse p53 mutants in this study are only stable in circumstances when the cell's endogenous p53 is stable. This is suggestive of the situation in the cells of Li–Fraumeni patients whose normal cells express both a wild-type and an inherited mutant allele of p53 but only show very low levels of p53 protein (Malkin *et al*., 1990). High levels of p53 are only seen in the tumours of these patients where wild-type p53 expression is lost.

Co-existence of mutant and wild-type p53 is rarely seen in human tumours, suggesting that a single mutant allele cannot confer a fully dominant negative phenotype *in vivo* (Slingerland and Benchimol, 1991) and a growth advantage could be gained by mutating or deleting the second allele. In most tumour lines the strong selection against expression of wild-type p53 expression results in cells which express only mutant protein which is often present at high levels in the nucleus. When we introduced mouse mutant p53s into cell lines of this type we found that these proteins are also expressed at high levels. The MCF7 breast tumour line however is slightly unusual in that the endogenous p53 protein behaves like wild-type, has transcriptional activity (results not shown) and is present at low levels in most of the cells. In this tumour line mouse mutant protein is unstable along with the endogenous human p53. The results described here suggest that sequences of p53 protein required for Mdm2 interaction and homo-oligomerisation are required for down-regulation of p53 protein in MCF7 cells since deletion of these sequences stabilises p53 in the absence of DNA damage. In

addition disruption of the p53-Mdm2 interaction in MCF7 cells also stablises p53, while Mdm2 expression in A431 cells ablates the expression of endogenous mutant p53. These observations are supported by two recent reports demonstrating that Mdm2 can promote destabilisation of p53. Haupt *et al*. (1997) cotransfected plasmids expressing p53 and Mdm2 into p53 null cells and observed that Mdm2 overexpression did not reduce p53 mRNA or the rate of p53 protein synthesis, but did reduce the p53 protein level presumably by affecting protein stability. We show more directly here that disrupting the p53-Mdm2 interaction by microinjection of 3G5 antibody increases p53 protein level in MCF7 cells. Kubbutat *et al*. (1997) show that expression of endogenous Mdm2 correlates with low levels of p53, and that the proteosome inhibitor lactacystin elevates p53 and Mdm2 levels indicating that stability is determined by susceptibility to the ubiquitin dependent degradation pathway. MCF7 cells express full length Mdm2 protein which is bound to p53 as shown by co-immunoprecipitation experiments (data not shown) while similar experiments in A431 cells fail to show detectable Mdm2 expression and therefore no p53-Mdm2 complexes are detected. We postulate that tumour cell lines like A431 which do not express active p53 cannot induce Mdm2 protein which would normally act as a feedback control on p53 protein levels. MCF7 cells however retain a transcriptionally active form of p53 and can therefore express Mdm2 which in turn targets p53 for rapid turnover. The ability of Mdm2 to down-regulate expression of mutant p53 in tumour lines could explain the strong selection for loss of the remaining wild-type allele in tumours which have acquired point mutations in p53. Low levels of mutant p53 expressed in the presence of Mdm2 may not be sufficient to block the transcription activation activity of the remaining wild-type protein, hence the importance of losing all active p53 expression in order to escape DNA damage induced surveillance. Several human tumours including sarcomas express wild-type p53 but have amplified Hdm2 suggesting that wild-type p53 activity can be overcome in some circumstances by elevated levels of Hdm2 protein (Oliner *et al*., 1992; Blaydes *et al*., 1997). The DNA damage response in normal cells may therefore involve the disruption of this feedback control resulting in rising levels of transcriptionally active p53 protein. Interestingly the upregulation after DNA damage of Mop53 expressed in MCF7 cells was not observed in transient transfection experiments. Instead the transiently expressed exogenous mouse proteins were constitutively high while the endogenous protein was unaffected and remained low, increasing only after DNA damage (results not shown). This suggests that the transiently expressed protein is not recognised by the regulatory system, possibly because the transfection procedure itself prevents post-translational modification of the exogenous protein. It may therefore be difficult to study the DNA damage response in transient transfection experiments, so MCF7 lines stably transformed with mouse p53 expressing plasmids could provide a valuable model of u.v. induced p53 stabilisation.

The requirement of the oligomerisation domain for regulation of p53 levels and Mdm2 binding is reflected in observations that two families with increased susceptibility to develop tumours show elevated expression of p53 in their normal tissues. Recently these patients were shown to have inherited a p53 allele with mutation in the oligomerisation domain (Barnes *et al*., 1992; Lomax *et al*., 1997; Varley *et al*., 1996) It will be interesting to determine whether these mutant proteins are able to escape Mdm2 mediated regulation of p53 levels. We cannot however rule out the possibility that the truncations of p53 described here may remove a site on the protein required for binding of ubiquitinising proteins such as a cellular equivalent of the E6 ubiquitin ligase, or for other steps involved in degradation. Indeed the stability of the His169CΔ30 mouse p53 which retains the oligomerisation domain suggests that the extreme C-terminal domain may also be required for efficient degradation. *In vitro* experiments using E6 mediated degradation of similar truncated p53 proteins have given conflicting reports of the effects of C-terminal sequences and oligomerisation on susceptibility to degradation (Medcalf and Milner, 1993; Marston *et al*., 1995). This may now be explained in part by the presence of Mdm2 protein in some reticulocyte extracts (Hall and Milner, 1997).

Patterns of p53 expression in tumours are of considerable interest to clinicians. Although 50% of tumours show high levels of p53 protein which are clearly associated with p53 mutation, the remaining tumours do not overexpress p53. The observations in this report are therefore of great assistance in interpreting the results of immunohistochemical staining for p53 in human tumours and offer a clear explaination as to why strongly staining tumours have only mutant p53. In this model p53 accumulation occurs whenever Mdm2 is absent i.e. when p53 is not active as a transcription factor. In tumour cells that still contain wild-type p53, agents that disrupt the p53-Mdm2 complex would mimic the effect of radiation or chemotherapy by activating the p53 pathway without causing unnecessary DNA damage. Conversely maintaining the interaction between Mdm2 and p53 in the presence of DNA damage should confer radioresistance to normal cells. These effects might be used to enhance the therapeutic index of conventional anticancer treatments.

Materials and methods

Cell culture

The A431 human vulval carcinoma line and MDA468 breast tumour line (which both express mutant p53 with a mutation of arginine to histidine at amino acid 273) and the MCF7 breast tumour line (which expresses a functionally wild-type p53) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% foetal calf serum.

Antibodies

Monoclonal antibody (Mab) DO-1 (Vojtesek *et al*., 1992) recognises only human p53. PAb 242, and PAb248 (Yewdell *et al*., 1986; Lane *et al*., 1996) recognise only mouse p53. CM-1 is a rabbit polyclonal raised against human p53 expressed in *E. coli*, (Midgley *et al*., 1992) and CM-5 is a rabbit polyclonal raised against mouse p53 expressed in *E. coli* (Midgley *et al*., 1995). MAbs 3G5, 4B2 and 2A10 (Chen *et al*., 1993) recognise Mdm2 protein. 3G5

has been shown to block the interaction of p53 with Mdm2 (Blaydes *et al*., 1997).

Plasmids

Plasmid pCMVNeo-*Bam* (Baker *et al*., 1990) was used for expression of mouse p53 mutant proteins in cell lines. The mouse p53 temperature sensitive Val135 (Michalovitz *et al*., 1990) and the linker insertion KH215 (Finlay *et al*., 1988) mutations were inserted directly into the *Bam*HI site of pCMVNeo. Other mutations were created by oligonucleotide directed mutagenesis using the 'Sculptor' kit supplied by Amersham. The synthetic oligonucleotides used were: TCGTGAGACACTGCCCCCA (His169), GCATGAACCAGCGACCTA (Gln 242), TTCTCCGAAGACTGGATGGACGATCTGTTG (deletion of N terminal 37 amino acids, NΔ37), TCCGAAGACTGGATGACTTACCAGGGCAAC (NΔ95), TCTGGAGACAGCAGGTGACTGCCTCTGCAT (CΔ30), TTCACCCTCAAGATCTGACTGCCTCTGCAT (CΔ61), GAAGAAAATTTCCGCTGACTGCCTCTGCAT (CΔ103), ACATTTTCAGGCCAATCGAAACTACTTCTT (Gln22,Ser23). Mutations were confirmed by DNA sequencing and the mutated cDNAs were transferred to plasmid pCMVNeo-*Bam*. The plasmids are referred to throughout as pCMVVal135, pCMVKH215, pCMVHis169, pCMVGln242 pCMVNΔ37 etc. The p53ΔSS deletion was recreated by deleting a SacII-StuI fragment (Shaulian *et al*., 1992) which effectively substitutes a single proline for residues 327–341. The deletion of all 5′ untranslated sequences (5′UTRΔ) was created by transferring an end-filled *Sty*I restriction fragment including the 5′ 251 base pairs of mouse p53 to *Eco*RV cut pBluescript. The remaining 3′ part of the cDNA was then inserted as an *Xho*I fragment so that the complete coding region lacking the 5′UTR could be transferred to pCMVNeo-*Bam* as a *Bam*HI-*Bgl*II fragment. The p53DD fragment which includes amino acids 1–11 and 299–387 (Shaulian *et al*., 1995) was inserted into pCNA3hygromycin (a gift from S Duff). pMdm2X2 which expresses full length Mdm2, and pMdm2ΔXM expressing truncated Mdm2 deficient in p53 binding (Barak *et al*., 1994), were gifts from M Oren.

Transfection

5×10^5 cells grown in 60 mM tissue culture dishes were transfected with 8.8 mg plasmid DNA as described in Sambrook *et al*., (1989). Following exposure to DNA/calcium phosphate precipitate in the presence of 1 mM chloroquine for 4 h, the cells were subjected to a 15% glycerol shock for 1 min. Cells were then incubated for 24 h at 37°C before trypsinisation and replating at 1:10 dilution in medium containing 1 mg/ml geneticin (Gibco) and 150 μg/ml hygromycin as appropriate. Colonies arising after 2–3 weeks were pooled and grown up for further analysis.

u.v. irradiation and SV40 infection

After removing the medium, plates of cells were subjected to 50 J/M^2 u.v. light in a Spectrolinker XL-1500 u.v. crosslinker (Spectronics Corporation). Medium was readded and the cells incubated at 37°C for 8 h before cells were fixed for staining, or cell lysates were prepared.

Cells were infected with SV40 virus (10^9 p.f.u./ml) for 3 h at 37°C. The virus was replaced with fresh medium and the cells incubated for 48 h before being fixed for cell staining.

Immunocytochemistry

Cells grown on 35 mm plastic tissue culture dishes were fixed for 5 min in cold methanol:acetone (1:1 by volume) and incubated overnight at 4°C with monoclonal antibody supernatant and washed in PBS before adding peroxidase-conjugated rabbit anti-mouse IgG (Dako) diluted 1:100. After incubation for 1 h at room temperature peroxidase activity was detected with 3′,3-diaminobenzidine in 0.03% nickel sulphate.

Microinjection into cells

Cells grown on 35 mm tissue culture plates were injected with protein A purified monoclonal antibodies (1.3 mg/ml) or plasmid DNA (100 μg/ml) using the Eppendorf 5242 Microinjector, and 5170 Micromanipulator. After a 24 h incubation, cells were fixed for 5 min in cold 1:1 methanol:acetone and incubated for 1 h with CM-1 or CM-5 serum diluted 1:1000, or monoclonal antibodies diluted to 1 μg/ml, washed in PBS and incubated for 1 h with FITC conjugated rabbit anti-mouse IgG (1:100, Sigma) and Texas Red conjugated swine anti-rabbit IgG (1:200, Amersham).

ELISA assay

Two-site immunoassays were performed using mouse monoclonal antibodies DO-1 or PAb 248 (30 μg/ml) to coat the plates and polyclonal rabbit antiserum CM-1 to detect captured p53 as described in (Vojtesek *et al*., 1992). Cells were lysed in 150 mM NaCl, 50 mM Tris.HCl pH 8.0, 5 mM EDTA, 1% NP40, 1 mM phenylmethylsulphonyl fluoride for 30 min on ice and the cell extract cleared by centrifugation at 14 000 r.p.m. for 20 min at 4°C. Total protein concentration of lysates was determined by Bradford assay and lysates were adjusted to the same concentration. 50 μl of twofold dilution series of lysates were added to wells and incubated for 3 h at 4°C. After washing p53 was detected using CM-1 diluted 1:1000, followed by peroxidase-conjugated swine anti-rabbit IgG (Dako) diluted 1:1000. Peroxidase activity was detected with tetramethylbenzidine at OD_{450} in an ELISA plate reader.

Gel electrophoresis and immunoblotting

Total cell lysate (10 μg) prepared as for ELISA assay was separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane using a Biorad Mini Trans-blot Electrophoretic Transfer cell for 1 h, 150 mA, at room temperature in 25 mM Tris·HCl, 190 mM glycine, 20% methanol. Prestained molecular weight markers (Biorad) were run in parallel. Blots were blocked in 5% dried milk, 0.1% Tween 20 in PBS for 1 h at room temperature then incubated 1 h at room temperature with monoclonal antibodies DO-1 or PAb248 (ascites), or rabbit anti-serum CM-1 or CM-5 all diluted 1:1000. Blots were washed in PBS, 0.1% Tween 20 then incubated for 1 h with peroxidase-conjugated swine anti-rabbit IgG (Dako) diluted 1:2000, and protein bands were detected by Enhanced Chemiluminescence using reagents supplied by Amersham.

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References

Baker SJ, Markowitz S, Fearon ER, Willson JK and Vogelstein B. (1990). *Science*, **249,** 912–915.

Barak Y, Gottlieb E, Juven-Gershon T and Oren M. (1994). *Genes and Dev*., **8,** 1739–1749.

Barnes DM, Hanby AM, Gillett CE, Mohammed S, Hodgson S, Bobrow LG, Leigh IM, Purkis T, MacGeoch C, Spurr NK *et al*. (1992). *Lancet*, **340,** 259–263.

Bartek J, Bartkova J, Vojtesek B, Staskova Z, Lukas J, Rejthar A, Kovarik J, Midgley CA, Gannon JV and Lane DP. (1991). *Oncogene*, **6,** 1699–1703.

Bennett WP, Colby TV, Travis WD, Borkowski A, Jones RT, Lane DP, Metcalf RA, Samet JM, Takeshima Y, Gu JR *et al*. (1993). *Cancer Res*., **53,** 4817–4822.

Blaydes JP, Gire V, Rowson JM and Wynford-Thomas D. (1997). *Oncogene*, 1859–1868.

Casey G, Lo Hsueh M, Lopez ME, Vogelstein B and Stanbridge EJ. (1991). *Oncogene*, **6,** 1791–1797.

Deppert W. (1994). *Semin. Cancer Biol*., **5,** 187–202.

Chen J, Marechal V and Levine AJ. (1993). *Mol. Cell. Biol*., **13,** 4107–4114.

el Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y *et al*. (1994). *Cancer Res*., **54,** 1169–1174.

Finlay CA. (1993). *Mol. Cell. Biol*., **13,** 301–306.

Finlay CA, Hinds PW, Tan TH, Eliyahu D, Oren M and Levine AJ. (1988). *Mol. Cell. Biol*., **8,** 531–539.

Fiscella M, Ullrich SJ, Zambrano N, Shields MT, Lin D, Lees Miller SP, Anderson CW, Mercer WE and Appella E. (1993). *Oncogene*, **8,** 1519–1528.

Fritsche M, Haessler C and Brandner G. (1993). *Oncogene*, **8,** 307–318.

Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW and Giaccia AJ. (1996). *Nature*, **379,** 88–91.

Graeber TG, Peterson JF, Tsai M, Monica K, Fornace Jr, AJ and Giaccia AJ. (1994). *Mol. Cell. Biol*., **14,** 6264–6277.

Halazonetis TD, Davis LJ and Kandil AN. (1993). *EMBO J*., **12,** 1021–1028.

Halazonetis TD and Kandil AN. (1993). *EMBO J*., **12,** 5057–5064.

Hall AR and Milner J. (1997). *Oncogene*, **14,** 1371–1376.

Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SK. (1993). *Cell*, **75,** 805–816.

Harris CC and Hollstein M. (1993). *N. Engl. J. Med*., **329,** 1318–1327.

Haupt Y, Maya R, Kazaz A and Oren M. (1997). *Nature*, **387,** 296–299.

Kessis TD, Slebos RJ, Nelson WG, Kastan MB, Plunkett BS, Han SM, Lorincz AT, Hedrick L and Cho KR. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 3988–3992.

Kubbutat MHG, Jones S and Vousden KH. (1997). *Nature*, **387,** 299–303.

Kuerbitz SJ, Plunkett BS, Walsh WV and Kastan MB. (1992). *Proc. Natl. Acad. Sci. USA*, **89,** 7491–7495.

Lane DP and Crawford LV. (1979). *Nature*, **278,** 261–263.

Lane DP, Stephen CW, Midgley CA, Sparks A, Hupp TR, Daniels DA, Greaves R, Reid A, Vojtesek B and Picksley SM. (1996). *Oncogene*, **12,** 2461–2466.

Lin J, Chen J, Elenbass B and Levine AJ. (1994). *Genes Dev*., **8,** 1235–1246.

Lin WC and Desiderio S. (1993). *Science*, **260,** 953–959.

Linzer DIH and Levine AJ. (1979). *Cell*, **17,** 43–52.

Lomax ME, Barnes DM, Gilchrist R, Picksley SM, Varley JM and Camplejohn RS. (1997). *Oncogene*, **14,** 1868–1874.

Lu X and Lane DP. (1993). *Cell*, **75,** 765–778.

Malkin D. (1994). *Biochim. Biophys. Acta*, **1198,** 197–213.

Malkin D, Li FP, Strong LC, Fraumeni Jr, JF, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA *et al*. (1990). *Science*, **250,** 1233–1238.

Maltzman W and Czyzyk L. (1984). *Mol. Cell. Biol*., **4,** 1689–1694.

Marston NJ, Jenkins JR and Vousden KH. (1995). *Oncogene*, **10,** 1709–1715.

Martin HM, Filipe MI, Morris RW, Lane DP and Silvestre F. (1992). *Int. J. Cancer*, **50,** 859–862.

Medcalf EA and Milner J. (1993). *Oncogene*, **8,** 2847–2851.

Michalovitz D, Halevy O and Oren M. (1990). *Cell,* **62,** 671–680.

Midgley CA, Fisher CJ, Bartek J, Vojtesek B, Lane D and Barnes DM. (1992). *J. Cell. Sci*., **101,** 183–189.

Midgley CA, Owens B, Lane DP and Hall PA. (1995). *J. Cell. Sci*., **108,** 1843–1848.

Milne DM, Campbell DG, Caudwell FB and Meek DW. (1994). *J. Biol. Chem*., **269,** 9253–9260.

Milne DM, Campbell LE, Campbell DG and Meek DW. (1995). *J. Biol. Chem*., **270,** 5511–5518.

Molinari M and Milner J. (1995). *Oncogene*, **10,** 1849–1854.

Mosner J, Mummenbrauer T, Bauer C, Sczakiel G, Grosse F and Deppert W. (1995). *EMBO J*., **14,** 4442–4449.

Oliner JD, Kinzler KW, Meltzer PS, George DL and Vogelstein B. (1992). *Nature*, **358,** 80–83.

Oren M, Maltzman M and Levine AJ. (1981). *Mol. Cell. Biol*., **1,** 101–110.

Oren M, Reich NC and Levine AJ. (1982). *Mol. Cell. Biol*., **2,** 443–449.

Reed M, Woelker B, Wang P, Wang Y, Anderson ME and Tegtmeyer P. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 9455–9459.

Reihsaus E, Kohler M, Kraiss S, Oren M and Montenarh M. (1990). *Oncogene*, **5,** 137–145.

Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV and Lane DP. (1990). *Proc. Natl. Acad. Sci. USA*, **87,** 7555–7559.

Sambrook J, Fritsch EF and Maniatis T. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York.

Sarnow P, Ho YS, Williams J and Levine AJ. (1982). *Cell*, **28,** 387–394.

Shaulian E, Haviv I, Shaul Y and Oren M. (1995). *Oncogene*, **10,** 671–680.

Shaulian E, Zauberman A, Ginsberg D and Oren M. (1992). *Mol. Cell. Biol*., **12,** 5581–5592.

Shaulian E, Zauberman A, Milner J, Davies EA and Oren M. (1993). *EMBO J*., **12,** 2789–2797.

Slingerland JM and Benchimol S. (1991). *J. Cell. Physiol*., **148,** 391–395. Srivastava S, Zou ZQ, Pirollo K, Blattner W and Chang EH. (1990). *Nature*, **348,** 747–749

Thor AD, Moore II, DH, Edgerton SM, Kawasaki ES, Reihsaus E, Lynch HT, Marcus JN, Schwartz L, Chen LC, Mayall BH *et al*. (1992). *J. Natl. Cancer Inst*., **84,** 845–855.

Tominaga O, Hamelin R, Trouvat V, Salmon RJ, Lesec G, Thomas G and Remvikos Y. (1993). *Oncogene*, **8,** 2653–2658.

Varley JM, McGown G, Thorncroft M, Cochrane S, Morrison P, Woll P, Kelsey AM, Mitchell ELD, Boyle J, Birch JM and Evans DGR. (1996). *Oncogene*, **12,** 2437–2442.

Vojtesek B, Bartek J, Midgley CA and Lane DP. (1992). *J. Immunol. Methods*, **151,** 237–244.

Vojtesek B and Lane DP. (1993). *J. Cell. Sci*., **105,** 607–612.

Williams AC, Browne SJ, Yeudal WA, Paterson IC, Marshall CJ, Lane DP and Paraskeva C. (1993). *Oncogene*, **8,** 3063–3072.

Yewdell JW, Gannon JV and Lane DP. (1986). *J. Virol*., **59,** 444–452.

Zambetti GP and Levine AJ. (1993). *FASEB J*., **7,** 855–865.