The mechanisms of regulation of Hdm2 protein level by serum growth factors

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The abbreviations used are: PI_3 kinase, phosphatidylinositol-3 kinase; Akt, v-akt murine thymoma viral oncogene homologue kinase, MEK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; CDS, coding sequence; FCS, foetal calf serum.

Abstract

Cell cycle progression in response to serum growth factors is dependent on the expression of functional Hdm2 (Mdm2), which inhibits p53-dependent transcription of anti-proliferative genes. In a well characterised non-transformed human fibroblast model, growth factors induce the expression of Hdm2 with rapid kinetics. Here we dissect the mechanistic basis for this critical response. In contrast to previous studies in which components of the growth factor signalling pathways were over-expressed, *hdm2* mRNA expression is not induced with immediate-early kinetics in these cells. Rather, the elevated Hdm2 protein levels which follow growth factor stimulation are primarily a consequence of PI_3 kinase-dependent stabilisation of the Hdm2 protein combined with a global increase in protein synthesis.

1. Introduction

The transcription factor p53, acts at a pivotal point of multiple stress-response pathways, and as such its activity in proliferating cells must be tightly regulated in order to prevent activation of a cell-cycle arrest or apoptotic response. The primary pathway whereby p53 function is restricted in unstressed cells is via interaction with the Hdm2 oncoprotein (Mdm2 in mouse) [1]. Hdm2 suppresses the transcriptional activation function of p53, and also promotes its nuclear export and proteosome-dependent destruction [2]. In absence of Hdm2 function, normal fibroblast cells induce expression of the cdk inhibitor, $p21^{WAF1}$, and undergo a p53-dependent cell

cycle arrest [3], whereas in other cell-types, spontaneous apoptosis may occur [4-6]. Hdm2 / Mdm2 is itself a transcriptional target of p53, as the gene has a promoter, P2, which contains p53 binding sites [7,8] and, in *in vitro* cultured mouse embryo fibroblasts the levels of the *mdm2*-P2 mRNA transcript is highly dependent upon p53 [9]. A second promoter, P1, is constitutively active, though the *mdm2 / hdm2*-P1 mRNA transcript is poorly translated [10].

Levels of Hdm2 / Mdm2 protein are positively regulated by growth factor signalling pathways that promote cell proliferation and survival [11,12]. Several distinct mechanisms underlying this regulation have been demonstrated: Mosner *et al* [11] showed that the increased Mdm2 occurred in the absence of any increase in total *mdm2* mRNA on Northern blots, however expression of the *mdm2* gene was subsequently shown to be induced with immediate early kinetics in response to signalling from chimeric platelet-derived growth factor β receptors [13]. Experimental activation of one of the major growth factor receptor tyrosine kinase-induced pathways, the Ras-Raf-MEK-ERK kinase cascade, induces *mdm2* P2-transcription through activation of AP1 and ETS transcription factor binding to sites in the murine P2-promoter [14]. We have previously shown that this pathway is at least partially conserved in human cells [15,16], and Ras-Raf-MEK-ERK signalling also selectively promotes the export of *hdm2* mRNA from the nucleus to sites of translation in the cytoplasm [16]. Ras-Raf-MEK-ERK signalling is also known to regulate Hdm2 function through the increased transcription of the Hdm2-binding protein $p14^{ARF}$ [17]. Finally, growth factor induced activation of phosphatidylinositol-3 kinase (PI_3 kinase) - Akt/PKB kinase signalling results in phosphorylation of Hdm2 / Mdm2, which

increases the levels of the protein by reducing its rate of proteosome-dependent degradation [18-20].

Given that these mechanisms have been the subject of independent studies, it is difficult to determine their relative contribution to the levels in Hdm2 protein expression in any given situation. To address this, we have taken a well defined experimental model, induction of Hdm2 expression immediately following serum re-stimulation of non-transformed human fibroblasts, and undertaken an investigation of the mechanisms regulating Hdm2 protein levels in these cells.

2. Methods

2.1. Cell culture, reagents, and cell cycle analysis

MRC-5 hTERT cells [21] were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 0.1% or 10% foetal calf serum (FCS) (Autogen Bioclear). The following chemicals were added where indicated: Nutlin-3 (Alexis Biochemicals), MG132 (Sigma), cyclohexamide (Sigma), LY294002 (Calbiochem), U0126 (Promega). For DNA content analysis, cells were fixed in 70% ethanol in PBS before DNA was stained with propidium iodide, and quantified using a FACSCalibur flow cytometer (BD Biosciences). Cells were transfected using Lipofectamine 2000 reagent (Invitrogen).

2.2. mRNA and protein quantification.

Western blotting was performed as described previously [16] using antibodies to Hdm2 (2A9 or 2A10 [22], (2A9 was used unless indicated otherwise)), p53 (DO-1, Serotec), $p21^{WAF-1}$ (Clone SX118, Pharmingen), β-actin (Sigma), AKT, phospho-Ser-473 Akt, ERK1/ERK2 and phospho-Thr202r/Tyr204 ERK1/ERK2 (all from Cell Signalling Technology). mRNA analysis, cell fractionation, polyribosome purification, RNA extraction, and validation of the fractionation by semi-quantitative PCR for small cytoplasmic RNA Y4, was performed as described previously [16]. cDNA synthesis and Taqman quantitative PCR (qPCR) for *hdm2*-P1*, hdm2*-P2*, hdm2*-CDS and *gapdh* was performed using previously described primers and probes [16]. *hdm2* qPCR assays were performed in duplicate, and mean values normalised to *gapdh* levels. n values represent the number of individually treated sets of cells analysed.

3. Results

3.1. Hdm2 is required for cell-cycle progression in hTERT-immortalised normal human fibroblasts.

MRC-5 hTERT is an immortal cell line that has been derived by retroviral transduction of MRC-5, a well characterised human fibroblast strain, with a vector expressing the hTERT component of telomerase [21]. Previous work has shown that serum stimulation of growth arrested MRC-5 cells induces a rapid, and robust accumulation of Hdm2 protein [19]. We therefore first established whether MRC-5 hTERT cells retain normal dependence upon serum growth factors and Hdm2 function for cell cycle progression. Culture of sub-confluent MRC-5 hTERT cells for

24 h in 0.1% serum results in the expected accumulation of cells with a G_1 DNA content (Fig. 1A). Re-stimulation with 10% serum results in cell cycle re-entry and DNA replication after 6-24 h culture. However, if Nutlin-3, a pharmacological inhibitor of the Hdm2 : p53 interaction [6] is added to the medium, G_1 exit is blocked, confirming that these cells remain dependent on Hdm2 function.

Serum starvation resulted in reduced Hdm2 protein levels compared to asynchronous, cells (Fig. 1B). Following serum re-stimulation, Hdm2 protein levels increased rapidly, within 30 min. of stimulation. Hdm2 detectable by the 2A9 monoclonal antibody (upper panel) continued to increase from 30 min up to a peak at approximately 3 h post-stimulation, after which it declined. This induction was confirmed using an antibody to a different epitope on Hdm2 (2A10). In comparison to Hdm2, $p21^{WAF-1}$ showed a more modest, and delayed, increase, with an approximately 2 fold up-regulation observable at 3 h post-stimulation. During this time course, overall levels of p53 remained essentially unchanged, potentially reflecting a combined effect of higher rates of synthesis with increased Hdm2-dependent degradation..

3.2. Effects of serum-stimulation on *hdm2* mRNA synthesis, nuclear export and translation.

We next investigated whether the elevation of Hdm2 protein levels following serum stimulation involves a selective increase in *hdm2*-mRNA expression, using previously validated reverse transcription - qPCR assays which are specific to each *hdm2* transcript [16]. 1 h following serum stimulation there was no significant increase in

either *hdm2*-P1 or *hdm2*-P2 transcript levels in the cells, compared to the *gapdh* housekeeping mRNA (Fig. 2Ai). A qPCR assay directed towards the *hdm2* coding sequence (CDS) also showed no change. This contrasted to when p53-dependent transcription in the cells was activated by Nutlin-3 (Fig. 2Aii), which caused an 11.7 fold increase in *hdm2* P2-transcript levels, and a corresponding 5.2 fold increase in total *hdm2* CDS mRNA.

We therefore performed sub-cellular fractionation analysis [16] to determine whether *hdm2* mRNA is regulated at the post-transcriptional level following serum-stimulation (Fig. 2B). Within the 1 h timescale examined, there was no significant increase in the levels of *hdm2* mRNA transcripts in the cytoplasmic fraction, indicating that serum stimulation does not lead to a selective enhancement of nuclear export of *hdm2* mRNA. Furthermore, when cytoplasmic extracts were subjected to sucrose density centrifugation, there was no serum-induced increase in *hdm2* transcripts in the high molecular weight-polyribosome associated fraction, indicating that rates of *hdm2* translation are not selectively increased.

3.3. Role of PI_3 kinase - Akt-dependent Hdm2 protein stabilisation

Fig. 3A shows that when proteosome-dependent degradation of Hdm2 is inhibited using MG132, Hdm2 protein accumulates as least as rapidly as is does following serum stimulation. Therefore Hdm2 protein is being actively synthesised in serum starved cells, and serum-induced signalling pathways could theoretically cause the observed increase in Hdm2 protein solely by reducing the rates of its degradation.

Treatment of MRC-5 hTERT cells with the PI_3 kinase inhibitor, LY294002, blocked serum-induced activation of Akt (Fig. 3B) and also effectively blocked the serum induced increase in Hdm2 protein levels (Fig. 3B, compare lanes 3 and 4), demonstrating that the increase in Hdm2 protein is PI_3 kinase dependent. Inhibition of PI_3 kinase did not block the increase in Hdm2 protein caused by the MG132 proteosome inhibitor alone (not shown), and, more importantly, Hdm2 protein was still upregulated in the presence of 10% serum and MG132 when LY294002 was added. Therefore LY294002 does not prevent Hdm2 protein synthesis in either the absence or presence of serum growth factors. Together these data suggest that the primary mechanism underlying the selective up-regulation of Hdm2 protein levels following serum stimulation of human fibroblasts is the well documented PI_3 kinase – Akt dependent enhancement of Hdm2 protein stability, with no evidence of any selective increase in *hdm2* mRNA synthesis, export or translation. This conclusion is supported by the analysis of Hdm2 protein stability in these cells (Fig. 3C). Following blockade of protein synthesis by cyclohexamide, Hdm2 protein falls to undetectable levels within 45 min in starved cells, whereas following serum stimulation Hdm2 is still clearly detectable at this time point. In the presence of serum and LY294002, Hdm2 is degraded more rapidly than in serum starved cells.

3.4. MEK activity is required for optimal Hdm2 protein synthesis

The previous experiments do not exclude a role for Ras-Raf-MEK-ERK signalling in optimal growth factor-induced Hdm2 expression, and therefore we investigated the role of this pathway. We found that, when the pharmacological MEK inhibitor U0126 is added at the time of serum re-stimulation, ERK activation is blocked and a modest,

but reproducible, decrease in Hdm2 protein levels is observed (Fig. 4A). U0126 also causes a small reduction in the Hdm2 protein levels induced by MG132, indicating a decrease in the rate of protein synthesis following MEK inhibition. This reduction in Hdm2 synthesis did not occur due to any specific effects on *hdm2* mRNA synthesis or translation, as U0126 did not cause any decrease in *hdm2* mRNA associated with polyribosomes relative to *gapdh* mRNA (Fig. 4B). However, when the amounts of RNA yielded from the different fractions were analysed, we observed a reduction in the proportion of the total cytoplasmic RNA that was associated with polyribosomes from 42% in serum re-stimulated cells to 24% with U0126 treatment. This represents a 42% decrease in global translation rates following MEK inhibition, and would account for the U0126-induced reduction in Hdm2 protein levels compared to the majority of other, more stable, cellular proteins.

4. Discussion

Along with p53, Hdm2 lies at a point of integration of a huge range of both intra- and extra-cellular stimuli, the fine balance of the activities of these two proteins playing a key role in cell proliferation and survival [1]. Hdm2 itself can be regulated at multiple points, both pre-and post-translational, even in response to a single type of stimulus and it is often difficult to establish, in any given situation, which mechanisms are primarily responsible for any observed changes in cellular Hdm2 activity. Here we have shown that, when phenotypically normal human fibroblast cells are stimulated to proliferate by serum growth factors, the rapid increase in Hdm2 protein levels is primarily dependent upon activation of the PI_3 kinase – Akt/PKB signalling pathway, and resultant reduced rates of proteosome-dependent degradation of the Hdm2

protein, which is known to involve the direct phosphorylation of Hdm2 by Akt/PKB [18-20].

A second major growth factor-induced kinase cascade, Ras-Raf-MEK-ERK is, as has previously been shown in different experimental systems by a number of groups including our own [13,14,16], also required for optimal rates of Hdm2 synthesis in these cells. However, this is not due to any selective regulation of *hdm2* mRNA expression or translation, but rather reflects the known requirement for Ras-Raf-MEK-ERK signalling for the global increase in mRNA translation and protein synthesis in response to serum growth factors [23,24]. It is interesting to consider why the previously described mechanisms of specific MEK-dependent regulation of Hdm2 synthesis are not observed in this system. We have shown previously that incubation of breast cancer epithelial cells with U0126 for 7-24 h results in the selective inhibition of nuclear export of *hdm2* mRNA [16], however serum stimulation of fibroblasts has no specific effect on *hdm2* mRNA export. Potentially therefore, *hdm2* regulation at this level occurs in some cell types, and not others, or alternatively the effect may only be observed following prolonged inhibition of MEK activity. Other studies have shown murine *mdm2* to be induced by growth factors receptors and Ras-Raf-MEK-ERK signalling, through immediate early type transcriptional activation of the P2 promoter [13,14], Here we have shown that this does not occur following serum stimulation of human fibroblasts. Whilst we have previously confirmed that human Hdm2-P2 transcription expression is positively influenced by MEK activity in cancer cells [16], we have noted differences in the transcription factor binding elements in the human and murine P2-promoters which could result in the human promoter being less responsive to this pathway [15]. Alternatively, it may be relevant

that previous reports [13,14] only directly demonstrated MEK-dependent regulation of P2 transcription when components of the signalling pathway were either artificially over-expressed, or contained constitutive activating mutations, and therefore, over-stimulation of the growth factor receptor-Ras-Raf-MEK-ERK pathway may be required for Hdm2 P2 promoter activation to occur.

In the broader perspective, Hdm2 over-expression is associated with a wide number of malignancies and Hdm2 is a target for a new class of small molecules with potential a cancer therapeutics [6]. This study on the normal pathways of Hdm2 regulation highlights some of the multiplicity of mechanisms whereby dysregulated intracellular signalling pathways might result in Hdm2 over-expression in tumours.

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Figure Legends

Fig. 1 Role of Hdm2 in serum-stimulated cell-cycle re-entry. MRC-5 hTERT cells were cultured for 24 h in DMEM / 0.1% FCS, then refed with DMEM / 10% FCS before analysis at the indicated time points. (A) Cellular DNA content was

determined by flow cytometric analysis of propidium iodide stained cells. Nutlin–3 (5 μM) was added to the DMEM / 10% FCS where indicated. (B) Hdm2 was detected by western blotting using both 2A9 (top panel) and 2A10 monoclonal antibodies, which recognise different epitopes. Exposure times for the individual blots were varied in order to best observe the changes in Hdm2 protein levels.

Fig. 2 Effect of serum stimulation on *hdm2* mRNA expression, localisation and translation. *Hdm2* mRNA in MRC-5 hTERT cells was analysed using RT - qPCR. *hdm2-P1* (white bars) and *hdm2-P2* (grey bars) transcripts, as well as *hdm2*-CDS containing mRNA (black bars). (Ai) Cells were starved and refed as in Fig. 1A. Total RNA from starved (0 h) and refed (1 h) cells was analysed. Data is mean $\pm$ S.E.M. for 5 individual experiments. (Aii) Asynchronous cells growing in DMEM / 10% FCS were exposed to 5 μM Nutlin-3 (N) or carrier control (C) for 3 h before total RNA and protein was extracted for RT – qPCR and western blot analysis. (B) Cells were cultured as in Ai. Prior to mRNA extraction, cells were fractionated by low salt hypotonic lysis to produce a nuclear pellet, and soluble cytoplasmic fraction (S). The cytoplasmic fraction was further fractionated to isolate polyribosome associated mRNA (PA). Fractionations were validated using semi-quantitative PCR for small cytoplasmic RNA Y4 and *gapdh*. Two different amounts (3 fold dilution) of input cDNA were used for each sample. The graphs show *hdm2* levels at 1 h as a percentage of those in starved cells. n=2.

Fig. 3 PI_3 kinase dependent Hdm2 stabilisation is sufficient to account for the serum-induced Hdm2 induction. (A) Cells were cultured in DMEM / 0.1% FCS for 24 h then cells were refed with medium containing 10% serum and / or 50 μM MG132. (B)

Cells were cultured in DMEM / 0.1% FCS for 24 h then refed using medium containing the indicated combinations of 10% serum, 50 μM MG132 and 10 μM LY294002. Akt activation by phosphorylation at serine 473 was determined following 45 min. exposure of cells to 10% serum and either 10 μM LY294002 or carrier control. (C) Cells in 100 mm culture dishes were transfected for 24 h with 8 μg of the pCMV-mdm2 expression vector (encoding full length human Hdm2), before being cultured in DMEM / 0.1% FCS for 24 h. Cells were refed with medium containing the indicated combination of serum and 10 μM LY294002 or carrier control for 45 min. before 100 μg/ml cycloheximide (CHX) was added to the medium to block new protein synthesis. Cells were lysed at the time points indicated after cyclohexamide addition.

Fig. 4 MEK activity is required for optimal Hdm2 synthesis following serum re-stimulation. Cells were cultured in DMEM / 0.1% FCS for 24 h then treated as indicated (A) Cells were refed with medium containing 10% serum and / or 50 μM MG132 or 25 μM U0126 before blotting for Hdm2. ERK phosphorylation was determined at 45 min post serum re-stimulation. (B) *hdm2* coding sequence containing mRNA was quantified in either total (TOT) or polyribosome associated (PA) RNA fractions from cells 45 min after refeeding. Levels are shown relative to *gapdh* mRNA expression. Open bars = no serum, grey bars = 10% serum, black bars = 10% serum + 25 μM U0126.

Fig. 1

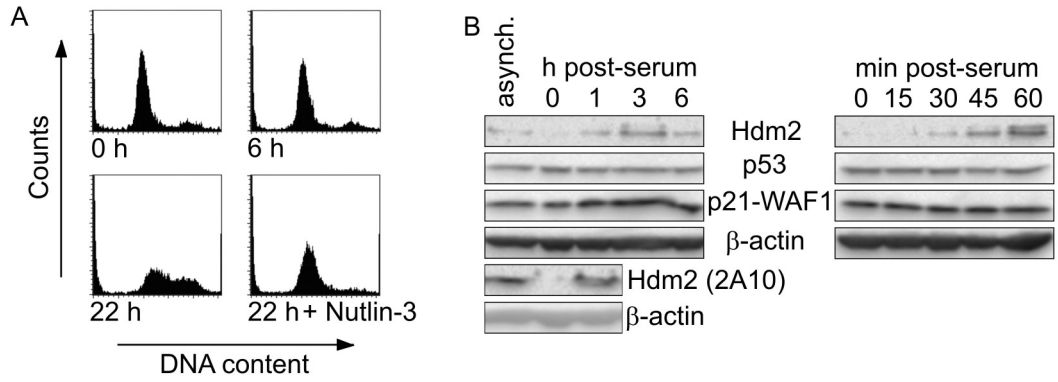
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Fig. 2

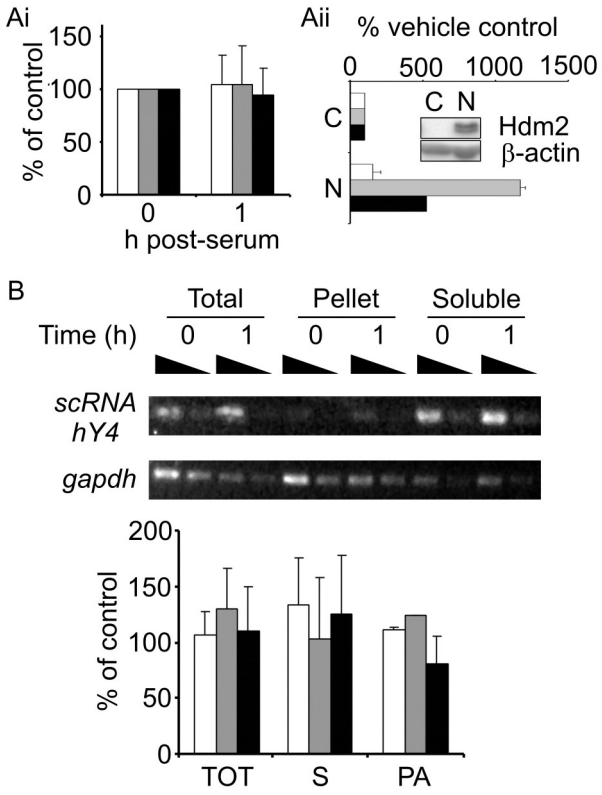
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Fig. 3

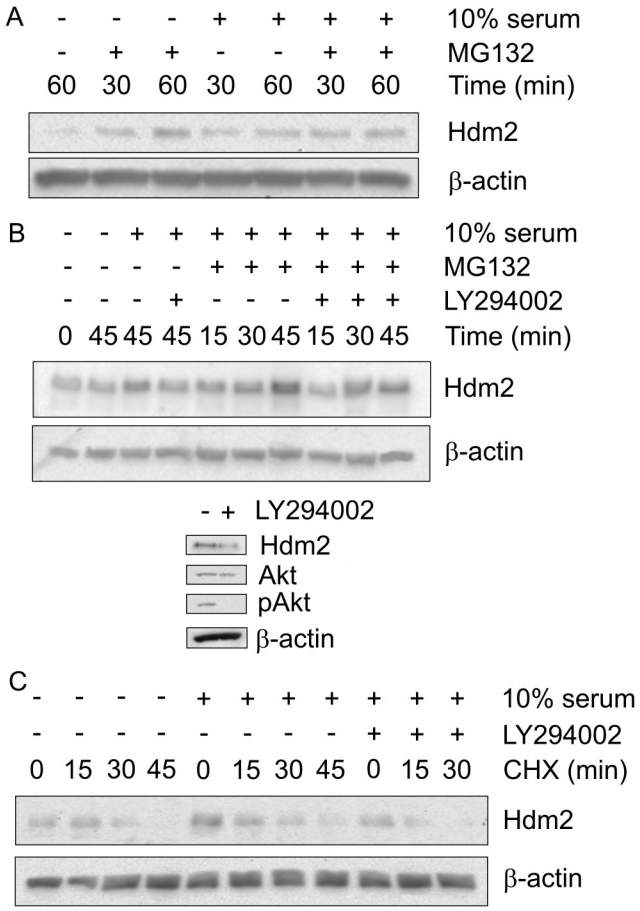
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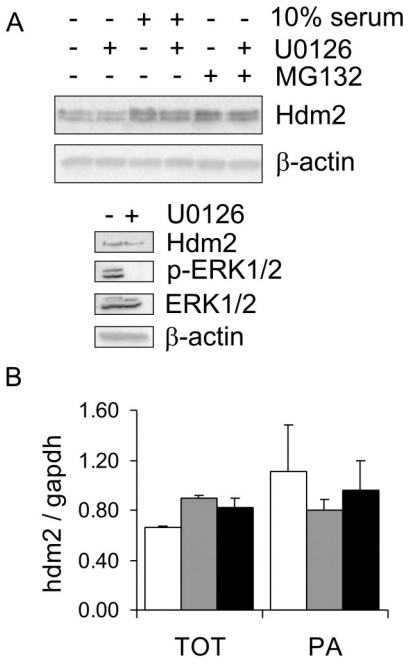
Fig. 4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