FEBS Letters 580 (2006) 300–304

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

Anna Phillips[a], Chris J. Jones[b], Jeremy P. Blaydes[a,*]

[a] *Cancer Sciences Division, Somers Cancer Research Building, School of Medicine, University of Southampton, MP 824, Southampton General Hospital, Southampton SO16 6YD, UK*
[b] *Department of Pathology, School of Medicine, Cardiff University, UK*

Received 23 September 2005; revised 8 December 2005; accepted 8 December 2005

Available online 19 December 2005

Edited by Varda Rotter

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 overexpressed, *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 phosphatidylinositol-3 kinase-dependent stabilisation of the Hdm2 protein combined with a global increase in protein synthesis.**

Keywords: p53; Mdm2; Mitogen-activated protein kinase; v-akt Murine thymoma viral oncogene homologue kinase; Growth factor

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)-v-akt murine thymoma viral oncogene homologue 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).

*Corresponding author. Fax: +44 (0)23 8079 5152.
E-mail address: j.p.blaydes@soton.ac.uk (J.P. Blaydes).

Abbreviations: 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

0014-5793/$32.00
doi:10.1016/j.febslet.2005.12.026

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 were performed as described previously [16]. cDNA synthesis and Taqman quantitative PCR (qPCR) for *hdm2*-P1, *hdm2*-P2, *hdm2*-CDS and *gapdh* were 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 upregulation 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

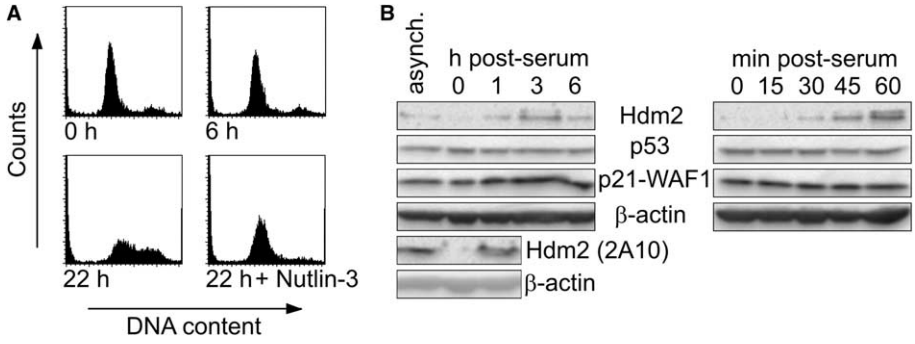

Fig. 1. Role of Hdm2 in serum-stimulated cell-cycle re-entry. MRC-5 hTERT cells were cultured for 24 h in DMEM/0.1% FCS, then refed with DMEM/10% FCS before analysis at the indicated time points. (A) Cellular DNA content was determined by flow cytometric analysis of propidium iodide stained cells. Nutlin-3 (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.