FEBS Letters 536 (2003) 220–224

FEBS 26969

Differences in the ubiquitination of p53 by Mdm2 and the HPV protein E6

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Received 20 December 2002; accepted 8 January 2003

First published online 23 January 2003

Edited by Julio Celis

Abstract The human papillomavirus (HPV) protein E6 can promote the ubiquitination of the p53 tumour suppressor in vitro, providing an explanation for the ability of E6 to induce p53 degradation in vivo and contribute to the potential tumorigenic effect of the virus. Instead, in non-infected cells, p53 levels are primarily destabilised by the ubiquitin E3 ligase activity of the Mdm2 protein. Here we have compared the effects of E6 and Mdm2 on p53 ubiquitination in vivo. We show that whereas in the presence of Mdm2 proteasome inhibitors induce the accumulation of ubiquitinated forms of p53, this does not occur in the presence of E6. Accordingly, we confirm that the effect of E6 and p53 is independent of the six C-terminal lysine residues in p53, which have previously been described to play an important role for effective ubiquitination and degradation of 53 mediated by Mdm2. We also show that other yet unidentified residues in p53 are also susceptible to ubiquitination. These results indicate that E6 does not induce ubiquitination of p53 in the same way as Mdm2 in order to promote its degradation, suggesting important differences between the Mdm2 and E6 effects on p53 degradation.

Key words: E6; Mdm2; Ubiquitin; p53; Proteasome

1. Introduction

The tumour suppressor p53 is activated by several kinds of stress and induces the expression of an ever growing number of proteins that are involved in causing the inhibition of cell proliferation or promoting cell death by apoptosis (reviewed in [1]). In normal non-stressed cells p53 has a very short half-life (5–20 min) due to an autoregulatory feedback loop mechanism in which the Mdm2 protein plays a key role (reviewed in [2]). Wild-type p53 (WTp53) acts as a transcriptional activator of the *Mdm2* gene. In turn, Mdm2, which itself has a very short half-life due its susceptibility to degradation, interacts with p53 and functions as a ubiquitin E3 ligase that promotes the conjugation of ubiquitin to specific lysine residues in p53 [3–7]. This conjugation to ubiquitin serves as a tag that effectively targets p53 for degradation by the proteasome.

Human papillomavirus (HPV) infects the basal cells of different epithelia, including the genital and anal areas, and HPV DNA has been found in 90% of cervical cancers and 50% of vulval cancers [8]. There are over 120 different strains of HPV [9], of which a number are known to be 'high risk' for cervical carcinoma. This is the second most common cause of cancer-related death in women worldwide, in some developing countries accounting for the highest cancer mortality [10]. As the viral DNA integrates into the genome the expression of two viral products, E6 and E7, is enhanced disturbing the normal terminal differentiation process of cervical cells. E7 binds the retinoblastoma gene product whereas E6 mediates the degradation of p53, among other effects. As a result, crucial cell cycle check-points are compromised leading to transformation of the host cells (for review see [11]). E6 is thought to target the tumour suppressor protein p53 for proteasome-mediated degradation through the recruitment of E6-associated protein (E6-AP), a cellular protein with known E3 ligase activity [12]. Through this interaction the E6/E6-AP complex would promote p53 ubiquitination and degradation [13,14] in a process analogous to the Mdm2-mediated p53 degradation in non-infected cells. The similarity between these two pathways of p53 degradation, Mdm2-mediated and E6-induced, has been assumed but never fully investigated.

Here we show clear differences in the levels of p53 ubiquitination obtained with E6 and with Mdm2 which suggest that the degradation of p53 mediated by each of these proteins has different requirements.

2. Materials and methods

2.1. Cells, antibodies and reagents

H1299 cells were cultured in RPMI medium supplemented with 10% foetal calf serum and gentamicin. Human p53 was detected using the mouse monoclonal antibody DO1 [26]. Human and mouse mdm2 were detected using the 4B2 mouse monoclonal antibody [27]. p21 and c-myc were detected using the 118 [28] and 9E10 mouse monoclonal antibodies. Anti β-galactosidase mouse monoclonal antibodies were obtained from Oncogene. Proteasome inhibitor MG132 was obtained from Calbiochem.

2.2. Plasmids

Expression from constructs pcDNA3 E6, pCOC-X2mdm2, pCMVhmdm2, pCMV-p21, pcDNA3c-myc and pcDNA3 β-galactosidase was under the control of the CMV promoter. WTp53 and p53R273H were expressed from pcDNA3 vectors [15] and pcDNA3-p536KR was described by Rodriguez et al. [6].

2.3. Transfection of cells and Western blotting

H1299 cells were transfected using the calcium-phosphate method as described in [15]. After 36 h cells were lysed in Novex loading buffer supplemented with 0.1 M dithiothreitol and proteins were separated on 4–12% Novex polyacrylamide gels, transferred to polyvinylidene difluoride membranes and developed with the relevant antibodies as previously described in [15].

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0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00054-1

2.4. Purification of His-tagged ubiquitin conjugates

Purification of His-tagged ubiquitin-conjugated proteins was as described in [15]. His-ubiquitin-tagged proteins were analysed by Western blot analysis with antibodies against the relevant protein.

3. Results

3.1. Mdm2 and E6 effects on p53 polyubiquitination

In a first experiment we compared the effects of ectopic 'high risk' HPV-16 E6 and Mdm2 on ectopic p53 levels and ubiquitination using the p53- and HPV-negative cell line H1299. In agreement with the current literature, both proteins markedly decreased p53 levels; in both cases the level of protein was recovered by the proteasome inhibitor MG132 (Fig. 1A).

We also analysed the ubiquitinated forms of p53 in this experiment using an in vivo assay that makes it possible to obtain samples enriched in ubiquitinated products [15]. H1299 cells were transfected with expression vectors for p53 and E6 or p53 and Mdm2 together with an expression vector for His_6-tagged ubiquitin. Cells were lysed in strong denaturing conditions to prevent deubiquitination and His_6-tagged complexes were captured with nickel-agarose beads, electrophoresed and blotted with the DO1 antibody against p53. As shown in Fig. 1A, a band corresponding to the molecular weight of non-ubiquitinated p53 could be detected in this assay. This band appeared even in the absence of ectopic

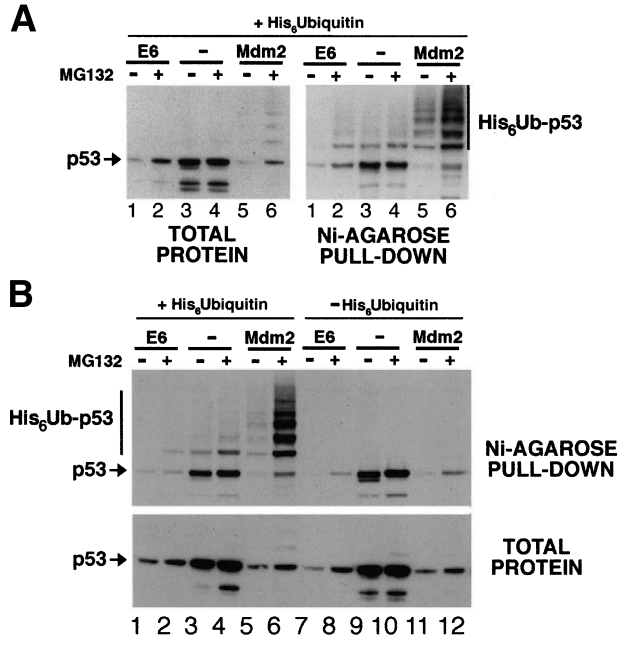

Fig. 1. Comparison of the p53 ubiquitination patterns in the presence of E6 or Mdm2. A: H1299 cells were transfected with 1 μg WTp53 and 2 μg His_6-ubiquitin expression vectors together with 2 μg vectors expressing E6 (lanes 1 and 2), Mdm2 (lanes 5 and 6) or control pcDNA3 vector (lanes 3 and 4). In lanes 2, 4 and 6, cells were treated with 20 μM MG132 for 3 h before harvesting. In the left panel, whole cell extracts were analysed by Western blot using the DO1 antibody against p53. In the right panel, p53 was detected in His_6-ubiquitin-enriched fractions. B: The same experiment was carried out in the presence (lanes 1–6) or absence (lanes 7–12) of His_6-ubiquitin expression vector. In lanes 2, 4, 6, 8, 10 and 12, cells were treated with 20 μM MG132 3 h before harvesting. p53 was detected in total cell extracts (top panel) or His_6-ubiquitin-enriched fractions (lower panel) as above.

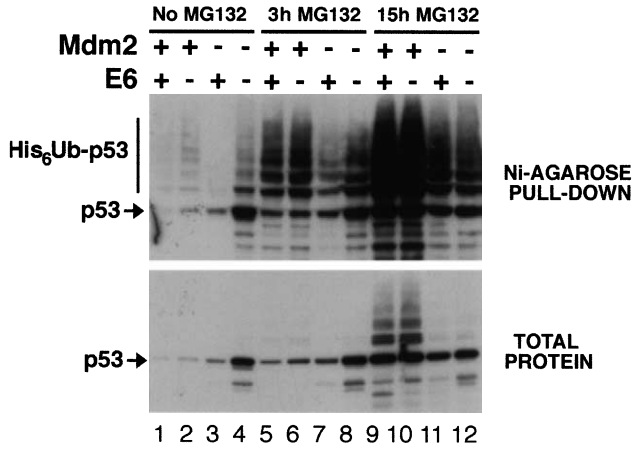

Fig. 2. E6 does not interfere with Mdm2-mediated ubiquitination and increased time of exposure of cells to MG132. H1299 cells were transfected with 1 μg WTp53 and 2 μg His_6-ubiquitin expression vectors together with 2 μg of vectors expressing E6 and Mdm2 (lanes 1, 5 and 9), Mdm2 (lanes 2, 6 and 10), E6 (lanes 3, 7 and 11) or control pcDNA3 vector (lanes 4, 8 and 12). Cells were either left untreated (lanes 1–4), treated with 20 μM MG132 for 3 h (lanes 5–8) or treated with 10 μM MG132 for 15 h (lanes 9–12) before harvesting.

His_6-tagged ubiquitin and therefore could be due to non-specific binding of p53 to the nickel-agarose beads or to the ability of p53 to bind to nickel as reported by others [16,17].

A dramatic increase in the ratio between ubiquitinated forms of p53 and total p53 was detected only in the presence of Mdm2, but not in the presence of E6 (Fig. 1A). The high molecular weight His_6-tagged ubiquitin–p53 complexes detected in the presence of Mdm2 have been suggested to correspond to single ubiquitin residues bound to multiple lysines in p53 (multiple mono-ubiquitinated forms) ([18]; L. Stevenson, personal communication). Whether any of these forms contains polyubiquitin chains bound to p53 (poly-ubiquitinated forms) is still being investigated.

Even when the proteasome inhibitor MG132 was added, the accumulation of His_6-tagged ubiquitin–p53 complexes in the presence of E6 was negligible in comparison with the accumulation of ubiquitinated forms of p53 in the presence of ectopic Mdm2 with or without MG132.

Low levels of mono- and higher order ubiquitin–p53 complexes were also detected in cells not overexpressing Mdm2 or E6. A band similar to the mono-ubiquitinated form was also apparent in ubiquitination assays performed by other authors even in the absence of ectopic Mdm2 [19]. In order to ascertain whether these forms of p53 correspond to ubiquitinated forms of p53, and not to other forms of p53 that could bind to the nickel-agarose beads, we carried out the same procedure used in the ubiquitination assay with cells that were not transfected with the His_6-ubiquitin expression vector. As shown in Fig. 1B, these bands did not appear in this assay, and therefore we conclude that they are likely to correspond to His_6-ubiquitinated forms of p53. Interestingly, the levels of these forms were not increased by MG132 as effectively as the ubiquitinated forms induced by Mdm2.

These forms, together with non-ubiquitinated p53, were decreased by the overexpression of E6 and their levels were restored with MG132 (Fig. 1A,B). This could indicate that although E6 does not induce a strong ubiquitination pattern

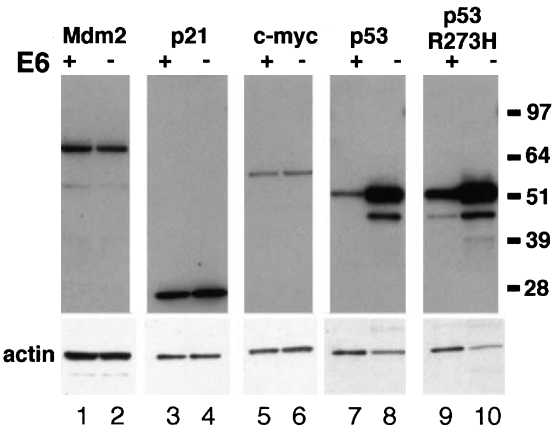

Fig. 3. E6 does not reduce the levels of other proteins susceptible to proteasome degradation. H1299 cells were transfected with 10 μg human Mdm2, 2 μg p21, 5 μg c-myc, 1 μg WTp53 or 5 μg p53R273H expression vector in the presence or absence of 2 μg E6 expression vector. Total cell extracts were analysed by Western blotting using relevant antibodies.

like the one induced by Mdm2, it can also induce the degradation of ubiquitinated forms of p53. Confirming this, E6 induced the disappearance of the ubiquitinated forms produced by the co-expression of Mdm2 (Fig. 2). This result also indicates that E6 does not interfere with the ubiquitination of p53 by Mdm2.

In order to test whether the treatment with MG132 was simply not sufficient to induce the accumulation of ubiquitinated forms of p53 in the presence of E6, we increased the incubation time with MG132 from 3 h to 15 h. As shown in Fig. 2 (lanes 9–12), in the presence of E6 the ratio between the levels of His_6-ubiquitin–p53 complexes and total p53 did not increase significantly above the result obtained in the absence of E6 expression.

To determine whether the degradation of p53 is a specific effect of E6 we tested the effect of E6 expression on the levels of other proteins that are also susceptible to proteasome-mediated degradation, such as Mdm2, p21 and c-myc [15,20–22]. Unlike p53, the levels of these proteins were not significantly decreased by E6 when they were expressed ectopically (Fig. 3). This also supports that E6 is not inhibiting the expression of the p53 from the vector since in this experiment Mdm2, p21 and c-myc expression are driven by the same promoter as p53. The lack of effect of E6 on the levels of Mdm2 also indicates that the E6 pathway does not interfere with the E6-mediated degradation of p53 as mentioned before. Additionally, we also showed that the levels of the transcriptionally inactive mutant of p53 (p53R273H) were also decreased by E6, and therefore the decrease of p53 levels by E6 is not due to the expression of a p53-dependent gene.

3.2. *p53 can be ubiquitinated at sites different from its six C-terminal residues*

The six C-terminal lysine residues in the human p53 sequence (370, 372, 373, 381, 382, and 386) are required for effective Mdm2-mediated ubiquitination and degradation of p53 in vivo [6]. In another study, Nakamura and co-workers [7] showed that mutation of only four of these lysine residues to alanine (372, 373, 381 and 382) was sufficient to observe a decrease in Mdm2-mediated degradation of p53. However, neither of these studies totally excluded the existence of other ubiquitination sites in p53. As shown in Fig. 4, when higher amounts of Mdm2 expression plasmid were used than those described by Rodriguez et al. [6], the p53 mutant in its six lysine C-terminal residues (p536KR) was still susceptible, although to a lower extent than wild-type p53, to Mdm2-mediated degradation. This indicated that residues other than the six C-terminal lysines in p53 are involved in Mdm2-mediated degradation of p53.

Furthermore, when the nickel-agarose pull-down was carried out, we observed that in our conditions, ubiquitinated forms of p53 could still be detected with the p536KR mutant (Fig. 4). Nevertheless, the apparent mobility of these ubiquitinated forms was significantly faster than that of the ubiquitin–p53 complexes obtained with the p53 wild-type protein and ectopic Mdm2. This result shows that although the six C-terminal lysine residues of p53 are necessary for efficient ubiquitination of p53 by Mdm2, other residues in p53 are susceptible to Mdm2-mediated ubiquitination, at least when this protein is expressed at higher levels. Accordingly, in vitro ubiquitination assays showed that the p536KR mutant could still be efficiently ubiquitinated [6]. Interestingly, even in the absence of ectopic Mdm2, a mono-ubiquitinated form(s) of the p536KR mutant was as easily detected as with WTp53, indicating that the appearance of this form is due to the activity of the low levels of endogenous Mdm2 in these cells or to the activity of another ubiquitinating factor.

3.3. *The six C-terminal lysine residues in human p53 are not necessary for E6-mediated degradation of p53*

The results described in Section 3.2 showed that E6 and Mdm2 clearly differ in their effects on p53 ubiquitination patterns. To confirm this difference, we carried out experiments with a p53 mutant that is defective for Mdm2-induced ubiquitination and degradation.

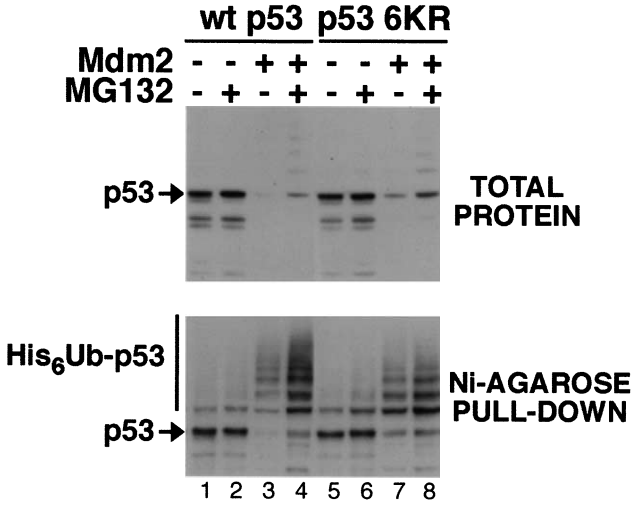

Fig. 4. Ubiquitination of the p536KR mutant. H1299 cells were transfected with 1 μg WTp53 (lanes 1–4) or p536KR (lanes 5–8) expression vectors together with 2 μg control pcDNA3 vector (lanes 1, 2, 5 and 6) or Mdm2 expression vector (lanes 3, 4, 7 and 8). In all lanes, 2 μg of His_6-ubiquitin expression vector was included. In lanes 2, 4, 6 and 8, cells were treated with 20 μM MG132 for 3 h before harvesting. Whole extracts (top panel) or His_6-ubiquitin-enriched fractions (lower panel) were analysed by Western blot using the DO1 antibody against p53.

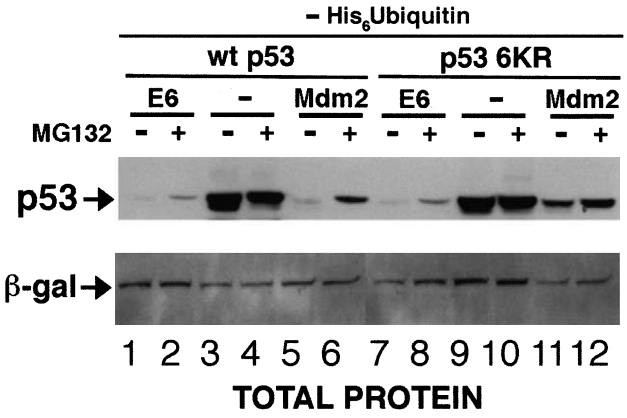

Fig. 5. H1299 cells were transfected with 1 μg WTp53 (lanes 1–6) or p536KR (lanes 7–12) expression vectors together with 2 μg of vectors expressing E6 (lanes 1, 2, 7 and 8), Mdm2 (lanes 5, 6, 11 and 12) or control pcDNA3 vector (lanes 3, 4, 9 and 10). In lanes 2, 4, 6, 8, 10 and 12, cells were treated with 20 μM MG132 for 3 h before harvesting. Whole cell extracts were analysed by Western blot using the DO1 antibody against p53.

When the involvement of the six C-terminal lysine residues in the degradation of p53 in the presence of E6 was analysed, different conclusions were reached by different authors. Whereas Rodriguez et al. [6] suggested that mutation of these lysine residues impaired p53 degradation in HeLa cells, in the other report Nakamura et al. [7] observed that the mutated form of p53 with lysines 372, 373, 381 and 382 replaced by alanine was still susceptible to E6-mediated degradation in H1299 cells. In order to clarify this dilemma, we repeated these experiments using the same cell line (H1299) as Nakamura et al. [7] with the p536KR mutant. As shown in Fig. 5, in agreement with these authors, we observed that the p536KR mutant was as susceptible to E6-induced degradation as the wild-type protein. This leads us to conclude that the six C-terminal lysine residues in p53 are not involved in E6-mediated degradation of p53 in H1299 cells and supports the existence of important differences between the degradation of p53 by E6 and Mdm2 as suggested in the previous experiments described here.

4. Discussion

We have shown that Mdm2 and E6 have strikingly different effects on the ubiquitination patterns of p53 in vivo. These observations may suggest that the degradation of p53 by E6 does not require a ubiquitination event in vivo. Supporting that ubiquitination is not an essential event for p53 degradation, Asher and co-workers have recently shown that the NAD(P)H quinone oxidoreductase (NQO1) inhibitor dicoumarol can induce the degradation of p53 and that this degradation pathway is not associated with accumulation of ubiquitinated forms of p53 [23]. Interestingly, these authors previously showed that NQO1 expression inhibits the degradation of p53 in the presence of E6 but not when Mdm2 is overexpressed. Whether E6 can directly affect the NQO1-regulated pathway for p53 degradation needs further research. This interpretation is in total disagreement with the results obtained in vitro, where E6 clearly induces the ubiquitination of p53 [13,14]. Another explanation that makes it possible to reconcile the results presented here with the observation of E6-induced ubiquitination of p53 in vitro is that E6 is more efficient at inducing poly-ubiquitination of p53 than Mdm2, which as shown by many authors induces the appearance of relatively low molecular weight forms of ubiquitinated p53 that probably correspond to multiple mono-ubiquitinated forms of p53 and not to poly-ubiquitinated forms of the protein. Supporting this interpretation, it is interesting to note that the pattern of ubiquitination obtained in vitro with each of these proteins differs substantially. Whereas Mdm2 induces a ladder of ubiquitination similar to that obtained in vivo, therefore probably corresponding primarily to multiple mono-ubiquitinated forms of p53 [24,6], the ubiquitinated forms of p53 induced by E6 in vitro have a significantly higher molecular mass [13,14]. This suggests the intriguing possibility that E6 promotes the conjugation of polyubiquitin chains that are immediately degraded and also difficult to detect by Western blot techniques. However, no qualitative change in the p53 ubiquitination ladder was detected even when exposure to MG132 was increased and when blots were performed with 7% polyacrylamide gels (data not shown).

Contributing to the difficulties in detecting the high molecular weight polyubiquitin-conjugated p53 forms even in the presence of MG132, one can speculate that proteasome inhibition could activate or allow deubiquitination of p53 by enzymes such as the HAUSP [25].

We also report the detection of ubiquitinated forms of the p536KR mutant that suggest the existence of ubiquitination sites on p53 other than the six C-terminal lysines. The evolution of the contribution of these novel sites to Mdm2-mediated degradation of p53 and whether the E6-induced degradation of p53 is dependent on ubiquitination at all will require the identification of these novel lysine residues.

Acknowledgements: We are grateful to Drs M. Oren, K.A. Levine, X. Lu, R. Stad, S. Hietanen and A.D. Crouch for providing us with expression vectors. We also thank Dr Dimitri Xirodimas for constructive comments. This work was funded by Cancer Research UK. D.P.L. is a Gibb fellow of Cancer Research UK.

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