Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53

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Abstract The tumor suppressor p53 is degraded by the ubiquitin-proteasome system. p53 was polyubiquitinated in the presence of E1, UbcH5 as E2 and MDM2 oncoprotein. A ubiquitin molecule bound MDM2 through sulfhydroxy bond which is characteristic of ubiquitin ligase (E3)-ubiquitin binding. The cysteine residue in the carboxyl terminus of MDM2 was essential for the activity. These data suggest that the MDM2 protein, which is induced by p53, functions as a ubiquitin ligase, E3, in human papillomavirus-uninfected cells which do not have E6 protein.

Key words: MDM2; p53; Ubiquitin ligase; E6AP; HECT domain

1. Introduction

The p53 tumor suppressor gene product exerts antiproliferation effects including growth arrest, genetic stability and apoptosis [1]. p53 exhibits DNA binding activity and functions as a transcription factor. The protein induces the expression of genes (GADD45, WAF1/p21/CIP1, cyclinG, Bax and MDM2) [2–6] regulating cell cycle arrest and apoptosis. In normal cells, p53 is maintained at very low, often undetectable levels. The level of p53 increases, in response to various types of stress and the inhibition of its degradation is one of the mechanism of this increase. p53 degradation is regulated by the ubiquitin-proteolysis system [7]. In cells infected with human papillomavirus, HPV16 or HPV18, papillomavirus E6 and cellular E6-AP form a complex and function as ubiquitin ligase, E3 for p53 [8]. In uninfected cells, however, E6-AP functions as ubiquitin ligase, not for p53, but for HHR23A without E6 [9]. Since tight regulation of p53 seems critical not only for various forms of stress response but also for normal cell growth and genetic stability, the ubiquitin ligase for p53 is of intense interest.

GADD45, WAF1/p21/CIP1, cyclinG, Bax and MDM2 are identified as target genes of p53 [2–6]. Except for MDM2 [10], the expression of these genes is immediately induced after induction of p53. The expression of MDM2 increases at least one hour later than the induction of p53. The MDM2 protein has the ability to bind p53 and has been thought to suppress the activity of p53 as the transcription factor by interacting with p53 in its transcriptional activation domain in the N-terminal region [11]. Furthermore, recent reports show that MDM2 accelerates p53 degradation [12,13], although the mechanism of the degradation has not been clarified.

In the ubiquitin-proteolysis system, the ubiquitin-protein adduct is formed in three sequential steps [14] which contain the ubiquitin activating enzyme, E1, the ubiquitin conjugating enzyme, E2 and the ubiquitin ligase, E3. E1 is a common enzyme involved in all kinds of ubiquitination. The specificity of the targeted protein is dependent on the E2 and E3 used in the reaction. The resultant ubiquitinated protein is degraded by proteasome.

2. Materials and methods

2.1. Expression of proteins

Human MDM2 [15], E6-AP [16], UbcH7 [17], E2-C/UbcH10 [18], EFP [19], cdc34 [20] and UbcH5 [21] cDNAs were obtained by RT-PCR using RNA from HeLa S3 cells. E6 cDNA was amplified from the prototype HPV16 genome. Each cDNA of human p53, MDM2, E6-AP and E6 was inserted into a GST-fused or 6×His pFastBac vector (GIBCO) and the proteins expressed in Sf9 cells in accordance with the manufacturer's protocol. Each E2 (UbcH7, E2-C/UbcH10, EFP, cdc34 and UbcH5) was ligated into a pET3 vector and expressed in *Escherichia coli* BL [21]. Baculovirus expressed mouse E1 [22] protein was purified by ubiquitin affinity column. In some experiments, GST-p53 and GST-MDM2 were purified by use of glutathione sepharose 4B resin.

2.2. In vitro ubiquitination assay

The reaction mixture (50 μl) contained Sf9 cell extract expressing GST-p53 (500 ng), mouse E1 (500 ng), E2 (500 ng), biotinylated ubiquitin (15 μg) and MDM2 (500 ng), 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM ATP and 2 mM dithiothreitol (DTT). When the cell extract was used, it was prepared from HeLa S3 cells (1×10^5 cells) at the log phase or aphidicolin treated G1/S phase. After incubation at 25°C for 30 min, the GST-p53 was pulled down with glutathione sepharose 4B resin (Pharmacia) and resolved by SDS-7.5% PAGE. The proteins in the gel were transferred to a PVDF membrane (Millipore). The membrane was incubated with ExtrAvidin peroxidase (Sigma) at room temperature for 1 h and washed. Then the ubiquitinated proteins were visualized by ECL (Amersham).

2.3. Thioester formation assay

The reaction mixture (50 μl) containing Sf9 cell extract expressing GST-MDM2, UbcH5, E1 and biotinylated ubiquitin, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM ATP and 0.2 mM DTT was incubated at 25°C for 5 min. The GST-MDM2 was pulled down with glutathione sepharose 4B resin and then treated with SDS-PAGE/sample buffer with (+) or without (−) 100 mM DTT. The DTT(−) sample buffer contained 4 M urea. DTT(+) samples were boiled for 5 min and DTT(−) samples were incubated at 30°C for 15 min prior to SDS-7.5% PAGE. The following steps were performed as described in Section 2.2.

3. Results and discussion

When GST-p53 is incubated with MDM2 and various kinds of E2, multiple ubiquitination of p53 was detected using UbcH5 as E2 (Fig. 1A, B). When other kinds of E2 were used, the ubiquitination of p53 was not detected. The polyubiquitinated adducts of p53 were also detected using E6/E6-AP as described previously [8]. Though the ubiquitination of cyclin B or Cut2 required HeLa cell extract as the source of cyclosome/APC in this in vitro ubiquitination system [23] (Tanaka, H., Miyauchi, Y. and Yasuda, H., unpublished results), the

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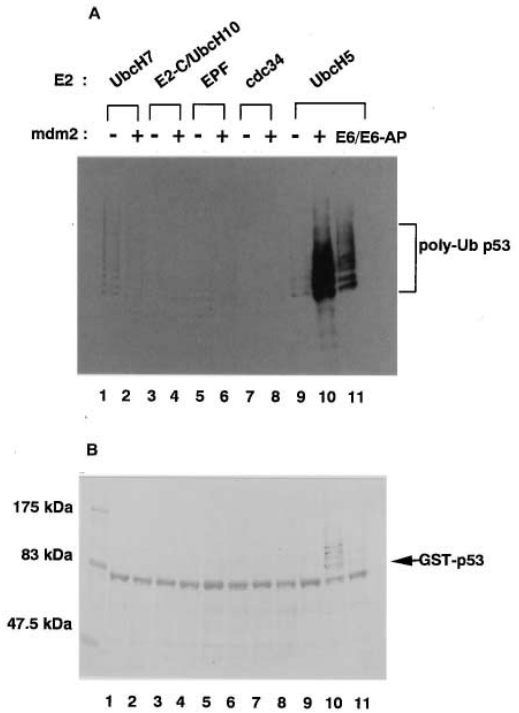

Fig. 1. Ubiquitination of p53 by MDM2 in vitro. A: MDM2 co-operates for the ubiquitination of p53 with UbcH5. GST human p53, 6×His-human MDM2 and 6×His-E6/E6-AP protein were expressed in Sf9 cells. Sf9 cell extracts expressing GST-p53 were incubated with E1, E2, biotinylated ubiquitin and HeLa cell extract in the presence (lane 2, 4, 6, 8 and 10) or absence (lane 1, 3, 5, 7 and 9) of MDM2. As a control, E6/E6-AP was used instead of MDM2 (lane 11). The ubiquitin onjugating enzyme E2s, UbcH7 (lane 1, 2), E2-C/UbcH10 (lane 3, 4), EFP (lane 5, 6), cdc34 (lane 7, 8) and UbcH5 (lane 9–11) were expressed in *E. coli*. B: The membrane used in A was stained by amido black, and the multiubiquitinated ladders of p53 were detected in lane 10 and 11.

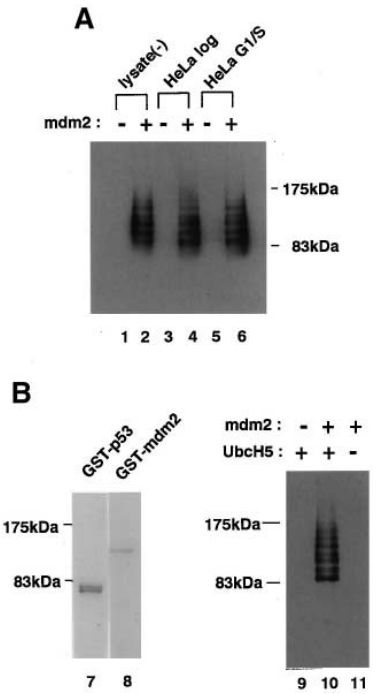

Fig. 2. MDM2-dependent ubiquitination of p53 in the absence of cell extract. The reaction was performed as described in A using UbcH5 as E2, in the absence (lane 1, 2) or presence of HeLa cell extract (log phase: lane 3, 4, G1/S phase: lane 5, 6). B: Ubiquitination assay using purified p53 and MDM2 [15]. SDS-12.5% PAGE of the purified GST-p53 and MDM2 (lane 7 and 8, respectively) was stained with coomasie brilliant blue R-250. Glutathione sepharose 4B resin conjugated with GST-p53 was incubated with (lane 10, 11) or without (lane 9) purified MDM2. In the absence of UbcH5 (lane 11), polyubiqutinated p53 was not detected.

multiple ubiquitination of p53 was observed without HeLa cell extract (Fig. 2A). Furthermore, when purified p53 and MDM2 were used to remove the effect of proteins originated from sf9 cell lysate, the polyubiquitination of p53 was still detected (Fig. 2B).

A ubiquitination of MDM2 was also detected in the presence of UbcH5 and was sensitive to dithiothreitol, indicating that the bond between MDM2 and ubiquitin is thioester (Fig. 3). This indicated that the ubiquitin molecule bound MDM2 through a sulfhydroxy bond which was characteristic of ubiquitin ligase (E3)-ubiquitin binding [24].

The E3 has been thought to contain the HECT (homologous to the E6-AP carboxyl terminus) domain in the carboxyl terminus. The carboxyl terminal peptide of MDM2 was compared to the HECT domain [25] (Fig. 4A). The identical amino acid residues are shown by asterisks in Fig. 4A, showing the homology of this region to HECT to be very weak. The cysteine, shown in bold, has been thought to be the essential residue for E3 having a HECT domain. A ubiquitin is thought to bind this cysteine through sulfhydroxy bond. Whether or not the correspondent cysteine residue of MDM2 was essential for its ligase activity was tested for. When the residue was mutated to alanine, the mutated MDM2 (C464A) did not have ubiquitin ligase activity (Fig. 4B).

As shown above, the multiple ubiquitination of p53 was detected using UbcH5 as E2 (Fig. 1). Although we did not use Ubc4 in this experiment, Ubc4 was thought to work as UbcH5 in this reaction, because the Ubc4 was highly homologous to UbcH5. Although the ubiquitination of p53 was detected in the presence of purified MDM2, it can not be denied that a small amount of the protein(s) or low molecular weight substance(s) originating from sf9 cell extract was necessary in this reaction. The data shown here, however, clearly show that the MDM2 had catalytic activity of ubiquitin ligase, E3, for p53.

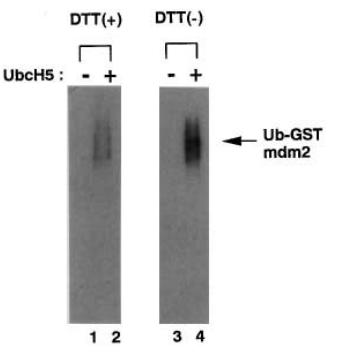

Fig. 3. Thioester formation of MDM2. Baculovirus expressed GST-MDM2 was incubated with UbcH5, E1 and biotinylated ubiquitin. After incubation, GST-MDM2 was pulled down with glutathione sepharose 4B resin (Pharmacia) and the reaction terminated by addition of SDS/PAGE sample buffer containing (lane 1, 2) or not containing (lane 3, 4) 100 mM DTT. The ubiquitin thioester formation on MDM2 was dependent upon the presence of UbcH5.

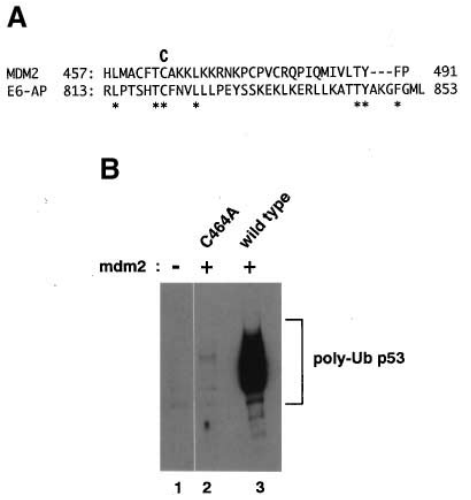

Fig. 4. Cysteine in the carboxyl terminal region of MDM2 is essential for the ubiquitin ligase activity. A: Comparison of amino acid sequence between the carboxyl terminal peptide of human MDM2 and the HECT domain of E6-AP. The identical amino acid residues are shown by asterisks. The cysteine shown in bold was the essential residue of the E3 with a HECT domain. B: The mutated MDM2 (C464A) did not have ubiquitin ligase activity. The ubiquitination assay was performed as described in Fig. 1 without MDM2 (lane 1), with mutated MDM2 (C464A) (lane 2) and with wild-type MDM2 (lane 3).

The MDM2 has ubiquitin ligase activity for p53 and only a very low homology to the HECT domain. MDM2 itself binds p53 and E6-AP cannot bind p53 without E6. MDM2 binds to p53 through its N-terminal domain and there are several phosphorylated residues in the domain. Phosphorylation was catalyzed by DNA-dependent protein kinase or casein kinase I, and the effects of the phosphorylation on MDM2 E3 activity are interesting subjects that need to be clarified.

MDM2 is expressed later than other induced genes by p53 after DNA damage. This suggests that p53 expression induced by DNA damage is regulated by auto-feedback via the ubiquitin-proteolysis system using MDM2 as E3 [26,27].

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References

[1] Gottlieb, T.M. and Oren, M. (1996) Biochim. Biophys. Acta 1287, 77–102.
[2] Kastan, M.B., Zhan, Q., el-Deiry, W.S., Carrier, F., Jacks, T., Walsn, W.V., Plunkett, B.S., Vogelstein, B. and Fornace Jr., A.J. (1992) Cell 71, 587–597.
[3] Barak, Y.M., Juven, T., Haffner, R. and Oren, M. (1993) EMBO J. 12, 461–468.
[4] el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B. (1993) Cell 75, 817–825.
[5] Miyashita, T. and Reed, J.C. (1995) Cell 80, 293–299.
[6] Okamoto, K. and Beach, D. (1994) EMBO J. 13, 4816–4822.
[7] Maki, C.G., Huibregtse, J.M. and Howley, P.M. (1996) Cancer Res. 56, 2649–2654.
[8] Scheffner, M., Huibregtse, J.M., Vierstra, R.D. and Howley, R.D. (1993) Cell 75, 495–505.
[9] Kumar, S., Kao, W.H. and Howley, P.M. (1997) J. Biol. Chem. 272, 13548–13554.
[10] Levine, A.J. (1997) Cell 88, 323–331.
[11] Oliner, J.D., Pietenpol, J.A., Thisagalingam, S., Gyuris, J., Kinzler, K.W. and Vogelstein, B. (1993) Nature 362, 857–860.
[12] Kubbutat, M.H.G., Jones, S.N. and Vousden, S.N. (1997) Nature 387, 299–303.
[13] Haupt, Y., Maya, R., Kazaz, A. and Oren, M. (1997) Nature 387, 296–299.
[14] Ciechanover, A. (1994) Cell 79, 13–22.
[15] Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L. and Vogelstein, B. (1992) Nature 358, 80–83.
[16] Huibregtse, J.M., Scheffner, M. and Howley, P.M. (1993) Mol. Cell. Biol. 13, 775–784.
[17] Nuber, U., Schwarz, S., Kaiser, P., Schneider, R. and Scheffner, M. (1996) J. Biol. Chem. 271, 2795–2800.
[18] Townsley, F.M., Aristarkhov, A., Beck, S., Hershko, A. and Ruderman, J.V. (1997) Proc. Natl. Acad. Sci. USA 94, 2362–2367.
[19] Liu, Z., Diaz, L.A., Hass, L.A. and Giudice, G.J. (1992) J. Biol. Chem. 267, 15829–15835.
[20] Plon, G.J., Leppig, K.A., Do, H.N. and Groudine, M. (1993) Proc. Natl. Acad. Sci. USA 90, 10484–10488.
[21] Scheffer, M., Huibregtse, J.M. and Howley, P. (1994) Proc. Natl. Acad. Sci. USA 91, 8797–8801.
[22] Imai, N., Kaneda, S., Nagai, Y., Seno, T., Ayusawa, D., Hanaoka, F. and Yamao, F. (1992) Gene 118, 279–282.
[23] Funabiki, H., Yamano, H., Nagao, K., Tanaka, H., Yasuda, H., Hunt, T. and Yanagida, M. (1997) EMBO J., in press.
[24] Scheffner, M., Nuber, U. and Huibregtse, J.M. (1995) Nature 373, 81–83.
[25] Huibregtse, J.M., Scheffner, M., Beaudenon, M. and Howley, P.M. (1995) Proc. Natl. Acad. Sci. USA 92, 2563–2567.
[26] Perry, M.E., Piette, J., Zawadzki, J.A., Harvey, D. and Levine, A.J. (1993) Proc. Natl. Acad. Sci. USA 90, 11623–11627.
[27] Wu, X., Bayle, J., Olson, H.D. and Levine, A.J. (1993) Genes Dev. 7, 1126–1132.