The ubiquitin–protein ligase activity of Hdm2 is inhibited by nucleic acids

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Abstract The proto-oncoprotein Hdm2 is a member of the RING finger-type family of ubiquitin–protein ligases E3. The RING finger domain is assumed to mediate the specific interaction of an E3 with its cognate ubiquitin-conjugating enzyme E2, which catalyzes the covalent attachment of ubiquitin to substrate proteins. In addition, the RING finger domain of Hdm2 is involved in Hdm2 homooligomer formation and has the capacity to bind to RNA in a sequence-specific manner. Here we report that interaction with nucleic acids interferes with both Hdm2/Hdm2 complex formation and auto-ubiquitination of Hdm2 in vitro. Furthermore, although binding of Hdm2 to the tumor suppressor p53 is not inhibited by nucleic acids, Hdm2-mediated ubiquitination of p53 is significantly decreased. Taken together, these results provide the first example of an E3 whose activity can be regulated by direct interaction with nucleic acids.

Key words: Hdm2; Ubiquitin–protein ligase; RNA; p53

1. Introduction

Amplification of the gene encoding the proto-oncoprotein Hdm2 (Mdm2 in mouse) has been implicated in the development of several human tumors including sarcomas and osteosarcomas [1,2]. Moreover, genetic analyses in mice demonstrated that Mdm2 deficient mice are only viable in a p53 null background, indicating that Mdm2 is a functional antagonist of the tumor suppressor protein p53 [3]. Structurally, Hdm2 consists of an N-terminal p53 binding domain, a central acidic region of yet unknown function, a zinc binding motif, and a C-terminal RING finger motif. RING finger motifs are often indicative of proteins with E3 ubiquitin ligase activity [4–6]. Indeed, Hdm2 facilitates ubiquitination and subsequent proteasome-mediated degradation of p53 [7–11], thereby inactivating the growth-suppressive properties of p53 [1,2,12]. In addition, Hdm2 itself is a target for ubiquitination ('auto-ubiquitination') and degradation [10,11].

Members of the RING finger family of E3s comprise at least two functional domains [4–6]. One domain determines the substrate specificity of the respective E3. The other domain, the RING finger motif, mediates the interaction of the E3 with its cognate E2 ubiquitin-conjugating enzyme, which covalently conjugates ubiquitin to a substrate protein. Thus, in a simplified mechanistic view, RING finger E3s can be considered as adaptor proteins bringing substrate proteins and E2s into close proximity for E2-catalyzed ubiquitination. Notably, the substrate interaction domain and the RING finger motif can be present on a single polypeptide chain, as in the case of Hdm2, or on several distinct proteins that form an E3 complex.

In vitro studies have shown that Hdm2 functionally interacts with the human E2 UbcH5 [10,11,13]. In addition to mediating the E2 interaction, the RING finger domain of Hdm2 is required for homooligomerization of Hdm2 and is involved in complex formation with the Hdm2-related protein HdmX [14,15]. Furthermore, the Hdm2 RING finger domain has the capacity to bind to RNA in a sequence-specific manner [16,17]. Here we show that the presence of nucleic acids inhibits the ability of Hdm2 to target itself and p53 for ubiquitination. Furthermore, nucleic acids interfere with the ability of Hdm2 to form homomeric complexes. Since nucleic acids do not interfere with, or even slightly stimulate, the interaction of Hdm2 with p53, these results suggest that homooligomerization is required for Hdm2 to be active as an E3.

2. Materials and methods

2.1. Plasmids and protein expression

The constructs for in vitro translation of p53, E6-AP, and RLIM, respectively, and the construct for bacterial expression of a glutathione *S*-transferase fusion protein of Hdm2 were described previously [13,18,19]. For in vitro translation of Hdm2, the cDNA for Hdm2 was cloned into the expression vector pcDNA3.1. The cDNA encoding a C-terminally truncated Hdm2 (Hdm2ΔRING, encompassing amino acid residues 1–423; deletion of RING finger domain) was generated by PCR-based methods and cloned into pcDNA3.1.

For in vitro ubiquitination experiments, Hdm2 was expressed as glutathione *S*-transferase fusion protein in *Escherichia coli* DH5α. The ubiquitin-activating enzyme E1 and E6-AP were expressed in the baculovirus system, and the ubiquitin-conjugating enzyme UbcH5 was expressed in *E. coli* BL21 as described [18,20].

2.2. In vitro ubiquitination assays

For in vitro ubiquitination, 1 μl of rabbit reticulocyte lysate-translated ^{35}S-labeled substrate (p53, Hdm2, E6-AP, or RLIM) was incubated in the presence of 50 ng E1, 50 ng UbcH5, and 10 μg ubiquitin in 50 μl volumes. For ubiquitination of p53 and Hdm2, the reaction additionally contained bacterially expressed Hdm2 (500 ng). Ubiquitination of E6-AP was performed in the additional presence of baculovirus-expressed E6-AP (50 ng). In addition, reactions contained various RNAs or DNAs as indicated (1 μg), 25 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 2 mM ATP, and 4 mM $MgCl_2$. After incubation at 30°C for 2 h, total reaction mixtures were electrophoresed in 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and ^{35}S-labeled substrates detected by fluorography.

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2.3. In vitro binding assays

Coprecipitation experiments using glutathione *S*-transferase fusion proteins were performed as previously described [18]. Briefly, 10 μl of rabbit reticulocyte lysate-translated ^{35}S-labeled Hdm2 or p53 was incubated with bacterially expressed glutathione *S*-transferase or the Hdm2 glutathione *S*-transferase fusion protein in 200 μl volumes containing 0.1 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, and 0.5% of the non-ionic detergent IGEPAL in the presence or absence of 5 μg of polyG or polydA at 4°C. After 3 h, bound proteins were purified by glutathione affinity chromatography, electrophoresed in 10% SDS–polyacrylamide gels, and ^{35}S-labeled proteins detected by fluorography. The ability of in vitro translated Hdm2, Hdm2ΔRING, E6-AP, and RLIM to bind to polyG was determined under similar conditions using polyG-agarose as an affinity matrix.

3. Results

3.1. Nucleic acids inhibit auto-ubiquitination of Hdm2

The RING finger domain of Hdm2 has been reported to have the ability to bind to RNA in a sequence-specific manner, suggesting that interaction with nucleic acids may affect the E3 activity of Hdm2 [16,17]. To test this hypothesis, the effect of different homopolymeric nucleic acids on Hdm2-induced ubiquitination of itself ('auto-ubiquitination') was determined [10,11]. In vitro translated radiolabeled Hdm2 was incubated in the presence of recombinant ubiquitin-activating enzyme E1, recombinant UbcH5, ubiquitin, and bacterially expressed Hdm2 under standard ubiquitination conditions (see Section 2). This resulted in the formation of high molecular weight forms of Hdm2 (Fig. 1A). Since the appearance of these high molecular weight forms is dependent on the presence of UbcH5 and ubiquitin [21], it can be concluded that these forms represent highly ubiquitinated species of Hdm2. Addition of various homopolyribonucleotides (polyA, polyC, polyG, polyA-U), tRNA (not shown), or homopolydesoxyribonucleotides (polydA, polydAT) completely abolished Hdm2 auto-ubiquitination.

Since Hdm2 was reported to bind specifically to polyG but not to other homopolyribonucleotides [16], titration experiments were performed with polyG and polydA (Fig. 1B). However, no significant difference between polyG and polydA was observed with respect to their ability to inhibit Hdm2 auto-ubiquitination, suggesting that Hdm2 binds to polyG and polydA with similar efficiency under the conditions used. Alternatively, the nucleic acids used may not interfere with Hdm2 auto-ubiquitination at the level of Hdm2 but rather inhibit the activity of E1 and/or UbcH5. To address this possibility, the effect of polyG on the auto-ubiquitination capacity of two Hdm2-unrelated E3s (E6-AP, RLIM) was determined [19,22]. As shown in Fig. 1C,D, auto-ubiquitination of these E3s was either not inhibited (E6-AP) or even slightly stimulated (RLIM) by polyG. Since auto-ubiquitination of both E6-AP and RLIM requires the activity of E1 and UbcH5 [19,22], this indicates that polyG and other homopoly(desoxy)ribonucleotides inhibit Hdm2 auto-ubiquitination by directly interfering with the E3 activity of Hdm2.

The ability of Hdm2, E6-AP, and RLIM, respectively, to bind to polyG was tested in coprecipitation experiments using polyG-agarose as an affinity matrix. As expected, Hdm2 bound to polyG (Fig. 2A) and binding of Hdm2 to polyG was dependent on the RING finger domain (Fig. 2B). Furthermore, RLIM bound to polyG (Fig. 2D), whereas an interaction of E6-AP with polyG was not observed under the conditions used (Fig. 2C). Since polyG interferes with auto-

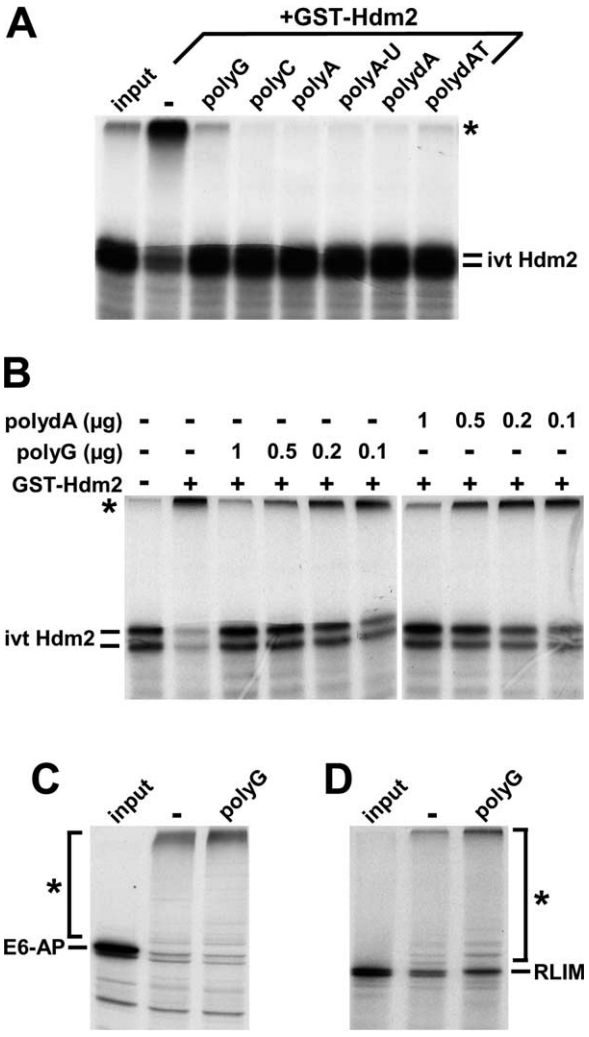

Fig. 1. RNA and DNA homopolymers interfere with Hdm2 auto-ubiquitination. One μl of the indicated in vitro translated radiolabeled protein (Hdm2, E6-AP, RLIM) was incubated under standard ubiquitination conditions (see Section 2) in the absence (−) or in the presence of different nucleic acids as indicated. After 2 h, the respective reaction mixtures were analyzed by SDS–polyacrylamide gel electrophoresis(PAGE) followed by fluorography. A: Reactions were performed in the presence of 1 μg of the nucleic acids indicated and in the additional presence of bacterially expressed glutathione *S*-transferase-Hdm2 fusion protein (GST-Hdm2). B: Reactions were performed in the presence of the indicated amounts of polyG or polydA and in the absence (−) or presence (+) of GST-Hdm2. The reaction in the absence of GST-Hdm2 was performed in the presence of GST. C: Reactions were performed in the additional presence of baculovirus-expressed E6-AP. D: Note that the use of in vitro translated RLIM is sufficient to observe auto-ubiquitination activity [19]. 'Input' represents 1 μl of the respective in vitro translated protein. The running positions of ubiquitinated forms of Hdm2, E6-AP, and RLIM are indicated by an asterisk.

ubiquitination of Hdm2 but not with RLIM auto-ubiquitination, these data (both Hdm2 and RLIM interact with polyG) indicate that the inhibitory effect of nucleic acids is specific for Hdm2 auto-ubiquitination.

3.2. Nucleic acids interfere with Hdm2/Hdm2 interaction

Addition of bacterially expressed Hdm2 is required to observe efficient ubiquitination of in vitro translated Hdm2, indicating that the concentration of Hdm2 has to reach a threshold level for auto-ubiquitination [21]. A likely explana-

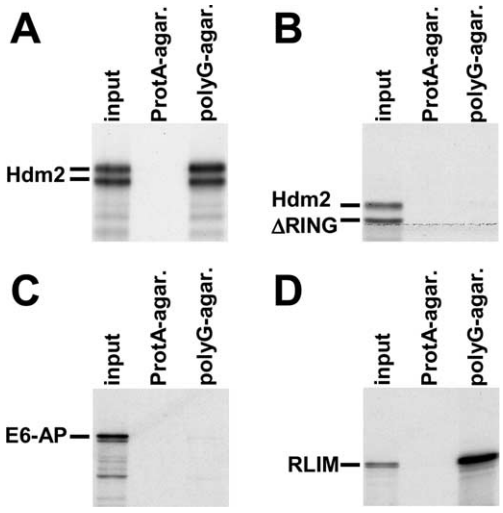

Fig. 2. Binding of Hdm2 to polyG depends on the RING finger domain. Ten μl of in vitro translated radiolabeled Hdm2 (A), Hdm2ΔRING (a C-terminally truncated Hdm2 mutant devoid of the RING finger domain) (B), E6-AP (C), or RLIM (D) were incubated with polyG-agarose (polyG-agar.) or protein A-agarose (ProtA-agar.) as control. The amount of proteins bound to protein A-agarose or polyG-agarose was determined by SDS–PAGE and fluorography. 'Input' represents 20% of the amount of the respective radiolabeled protein used for coprecipitation analysis.

tion for this observation is that auto-ubiquitination of Hdm2 occurs in trans (see Section 4) and proceeds via homooligomer formation. Moreover, since Hdm2 forms homooligomeric complexes via its RING finger domain [14,15] and RNA binds to the RING finger (Fig. 2A,B) [16,17], a possible explanation for the inhibitory effect of nucleic acids on Hdm2 auto-ubiquitination is that nucleic acid binding interferes with Hdm2/Hdm2 interaction. Indeed, addition of polyG or polydA to coprecipitation experiments inhibits the binding of in vitro translated radiolabeled Hdm2 to a glutathione *S*-transferase-Hdm2 fusion protein (Fig. 3A).

3.3. *Nucleic acids do not interfere with binding of Hdm2 to p53 but inhibit Hdm2-mediated ubiquitination of p53*

Hdm2 is best known for its role in ubiquitination and degradation of p53 [1,2,7–12]. To determine the effect of nucleic acids on Hdm2-mediated ubiquitination of p53, the effect of polyG on the ability of Hdm2 to bind to p53 was investigated next. This showed that polyG did not interfere with binding of Hdm2 to p53 (Fig. 3B). This observation is readily explained by the fact that binding of Hdm2 to p53 is mediated by the N-terminal region of Hdm2 and is not dependent on the presence of the C-terminal RING finger domain [1,2,12]. However, addition of nucleic acids significantly interfered with Hdm2-mediated ubiquitination of p53 in vitro (Fig. 4), suggesting that p53 ubiquitination requires Hdm2 oligomerization. It should be noted, however, that the C-terminal region of p53 has been implicated in the interaction with single-stranded nucleic acids (reviewed in [23]). Thus, an alternative but not mutually exclusive explanation for the inhibitory effect of nucleic acids is that nucleic acid-bound p53 is not as efficiently recognized as a substrate by Hdm2 as nucleic acid-free p53. However, this hypothesis cannot be directly tested, since the C-terminus of p53 is also required for efficient Hdm2-mediated ubiquitination of p53 in vitro [24,25].

4. Discussion

Several biochemical properties have been attributed to the RING finger domain of Hdm2 including the ability to form homooligomers, to interact with RNA, and to function as an E3 ubiquitin–protein ligase [9–11,14–17]. If these different properties of Hdm2 are mechanistically linked, and, thus, affect each other in a negative or positive manner has remained unclear. In this study, we show that binding to nucleic acids inhibits both the E3 activity of Hdm2 and the ability of Hdm2 to form homooligomeric complexes. This provides a novel mechanism, by which Hdm2-mediated ubiquitination processes can be regulated.

The observation that binding to nucleic acids inhibits Hdm2-mediated auto-ubiquitination can be explained by at least two possibilities. Firstly, since nucleic acid binding also interferes with Hdm2 homooligomerization (see Fig. 3A), homooligomerization may be a prerequisite for Hdm2 auto-ubiquitination. This hypothesis is supported by the fact that in order to observe auto-ubiquitination, Hdm2 levels need to reach a certain threshold [21]. This indicates that Hdm2 auto-ubiquitination occurs in trans (i.e. ubiquitination of a given Hdm2 molecule is mediated by another Hdm2 molecule) rather than in cis (a given Hdm2 molecule mediates its own ubiquitination). Secondly, in analogy to other RING finger E3s [4–6], the RING finger domain of Hdm2 is assumed to mediate the interaction with its cognate E2. Thus, an alternative but not mutually exclusive possibility is that nucleic acid binding interferes with the interaction of Hdm2 with its cognate E2. This possibility cannot be experimentally addressed at present. Although UbcH5 supports auto-ubiquitination of Hdm2 and Hdm2-mediated ubiquitination of p53 in vitro, a stable physical interaction between Hdm2 and UbcH5 has not been reported and has not been observed by us under the conditions of a coprecipitation experiment (unpublished observation). In any event, both mechanisms (RNA interferes with Hdm2 homooligomerization and/or Hdm2/E2 interaction) would also explain the observation that nucleic acids inhibit Hdm2-mediated ubiquitination of p53, although nu-

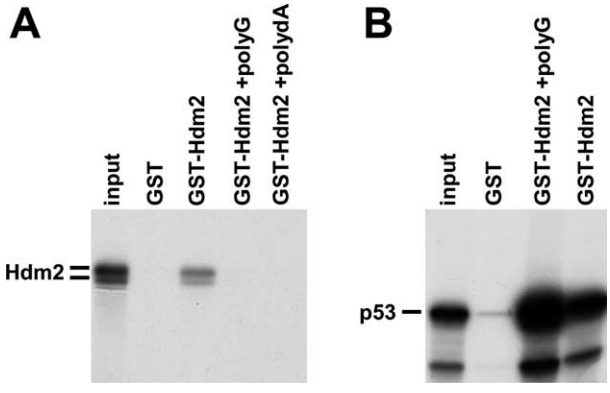

Fig. 3. PolyG interferes with Hdm2/Hdm2 interaction but not with the interaction of Hdm2 with p53. Ten μl of in vitro translated radiolabeled Hdm2 (A) or p53 (B) were incubated with bacterially expressed glutathione *S*-transferase (GST) or a glutathione *S*-transferase-Hdm2 fusion protein (GST-Hdm2) in the absence or presence of polyG or polydA as indicated (for conditions, see Section 2). The amount of proteins bound to GST or GST-Hdm2 was determined by SDS–PAGE and fluorography. 'Input' represents 20% of the amount of the respective radiolabeled protein used for coprecipitation analysis.

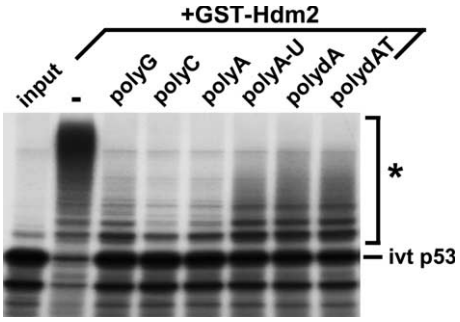

Fig. 4. RNA and DNA homopolymers inhibit Hdm2-mediated ubiquitination of p53. In vitro translated p53 was incubated in the presence of Hdm2 under standard ubiquitination conditions (Section 2) in the absence or in the presence of 1 μg of different nucleic acids as indicated. After 2 h, the respective reaction mixtures were analyzed by SDS–PAGE followed by fluorography. The running positions of ubiquitinated forms of p53 are indicated by an asterisk.

cleic acids do not interfere with Hdm2/p53 complex formation under the conditions used.

Although we did not observe significant differences between different RNA and DNA homopolymers in their ability to inhibit the E3 activity of Hdm2, there is experimental evidence that Hdm2 has the property to bind to certain RNAs in a sequence-specific manner [16,17]. This suggests that RNA-mediated inhibition of the E3 activity of Hdm2 is of physiological significance. To experimentally address this issue, an Hdm2 mutant that has lost the ability to bind to nucleic acids but is still active as an E3 should prove helpful. However, if such a mutant can be generated is presently unclear, since both properties (nucleic acid binding, E3 activity) depend on the RING finger domain of Hdm2. Finally, the observation that both Hdm2 and RLIM bind to polyG may indicate that RING finger E3s in general have the ability to interact with nucleic acids. Thus, it will be interesting to determine if the E3 activity of other RING finger E3s can be modulated by nucleic acids.

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