Human mdm2 Mediates Multiple Mono-ubiquitination of p53 by a Mechanism Requiring Enzyme Isomerization*

Received for publication, December 20, 2000, and in revised form, June 1, 2001
Published, JBC Papers in Press, June 7, 2001, DOI 10.1074/jbc.M011517200

Zhihong Lai‡, Katherine V. Ferry§, Melody A. Diamond§, Kevin E. Wee‡, Young B. Kim‡,
Jianhong Ma®, Tao Yang®, Pamela A. Benfield§, Robert A. Copeland‡§, and Kurt R. Auger$¶

From the §Cancer Research, DuPont Pharmaceuticals Company, Glenolden, Pennsylvania 19036 and ¶Chemical Enzymology, DuPont Pharmaceuticals Company, Wilmington, Delaware 19880

The mdm2 gene product is an important regulator of p53 function and stability. mdm2 is an E3 ubiquitin ligase for p53 and the RING finger domain of mdm2 is critical for ligase activity. Ubiquitin (Ub) conjugation is a general targeting modification and poly-ubiquitin chains specifically target proteins to the proteasome for degradation. In this report, we show that the multistep cascade of mdm2-mediated p53 ubiquitination can be reduced to three purified recombinant proteins: ubiquitin-conjugated E2, mdm2, and p53. This simplification allows enzymatic analysis of the isolated ligase reaction. The simplified reaction recapitulates the ubiquitination of p53 observed with individual components and the p53-Ub

levels of the protein include phosphorylation, dephosphorylation, acetylation, sumoylation, and ubiquitination. The stability and half-life of p53 are tightly regulated by mdm2 and the ubiquitin-proteasome pathway (5–7). Recent evidence suggests that mdm2 is an E3 ubiquitin ligase for p53 (8, 9).

A number of critical regulatory proteins in the cell are modified by ubiquitin (Ub) conjugation. Proteasomal degradation of key regulatory proteins control biological events involving the cell cycle, differentiation, immune responses, DNA repair, chromatin structure, and apoptosis (10). The initial step in the Ub cascade is the activation of Ub by the ubiquitin-activating enzyme (E1). E1 hydrolyzes ATP to AMP and pyrophosphate to generate a thioester bond between the active site Cys of E1 and the carboxyl-terminal Gly of Ub. The activated Ub is transferred to one of several different ubiquitin-conjugating enzymes (Ubc or E2) in an ATP-independent manner. The Ubc enzymes are catalytically similar to E1 in that a thioester bond is formed with Ub. The ubiquitin-conjugated E2 then functions in combination with an E3 ligase to transfer Ub to the target protein. The final Ub transfer results in an isopeptide bond between the carboxyl-terminal Gly of Ub and the ε amino group of a Lys residue on the target protein. The E3 ubiquitin ligase binds specifically to the target protein and participates in the transfer of Ub. Multiple Ub moieties are transferred through this cascade to generate a poly-ubiquitin chain. The ability of different E2 enzymes to function with a given E3 has led to a model that different E2-E3 complexes provide a level of substrate specificity. This model is attractive as combinatorial specificity can be achieved with a limited number of E2 and E3 enzymes (11).

Recently, it has become evident that ubiquitin E3 ligases can be divided into two major classes, the Hect domain E3 enzymes and the RING finger domain E3 enzymes. E6AP is the prototype for the Hect domain class, and members of this family are homologous to E6AP carboxyl terminus (12). This E3 class utilizes an active site Cys to form a thioester bond with Ub, analogous to E1 and E2. In contrast to the Hect domain family, members of the RING finger class of ligases are thought to interact with the Ub-E2 conjugate and “activate” the complex for the transfer of Ub to the acceptor protein (13, 14). Current models suggest an E3-ubiquitin intermediate is not formed with the RING finger domain class of E3s.

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¶ To whom correspondence may be addressed: Chemical Enzymology, DuPont Pharmaceuticals Co., Rt. 141 & Henry Clay Rd., Wilmington, DE 19880, Tel.: 302-695-7173; Fax: 302-695-8313; E-mail: Robert.A.Copeland@dupontpharma.com
† To whom correspondence may be addressed: Cancer Research, DuPont Pharmaceuticals Co., 500 S. Ridgeway Ave., Glenolden, PA 19036. Tel.: 610-237-7833; Fax: 610-237-7937; E-mail: Kurt.R.Auger@dupontpharma.com

This paper is available on line at http://www.jbc.org

31357
mmd2 is a member of the RING finger domain class of E3 ubiquitin ligases. The carboxyl terminus of mmd2 contains a variant of the RING finger domain (15). RING finger domain E3 ligases can be further classified into two general groups. The first group is the modular ligases where the RING finger domain and the substrate specificity domain reside in two different proteins within the E3 ligase complex, e.g. Rbx1 and the F-box protein in the SCF complex, respectively. In the second group, the ligase have the substrate recognition and the RING finger domain within the same polypeptide chain, such as mmd2 and cbl (16, 17).

In this report we characterize the mmd2-mediated ubiquitination of p53. Surprisingly, the p53-Ub, product has Ub at multiple sites on p53 and does not appear to form a poly-ubiquitin chain. Using pre-conjugated Ub-Ubc4, the kinetics of the mmd2-mediated enzymatic transfer of Ub to p53 has been characterized, and the results support a mechanism requiring enzyme isomerization.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Modification**—E1 and Ubc4 (Ubc4, Ubc4b and Ubc4c) and purified pre-conjugated Ub were generated as previously described (18). The human mmd2-mediated ubiquitination of p53 was expressed and purified as previously described (19). The transfer of ubiquitin in the mmd2/p53 ubiquitin ligase complex was measured as described (19).

**Materials**—Materials for the kinetic analyses were obtained from Sigma-Aldrich, and Sigma-Aldrich was chemically modified with Oregon Green (og) succinimidyl ester as described by the manufacturer (Molecular Probes) and purified as described previously (19). The Lys48 to Arg (K48R) and Lys63 to Arg (K63R) mutants and double mutant K48R/K63R of Ub were generated with the QuikChange site-directed mutagenesis kit from Stratagene. The wild-type Ub clone was a generous gift from the laboratory of Dr. Tracey Handel from the University of California at Berkeley. DNA sequencing as well as analysis of the purified protein by mass spectrometry confirmed the presence of the desired mutations.

A glutathione S-transferase fusion of the human mmd2 protein was expressed in baculovirus-infected Sf9 cells. Cell pellets were lysed by sonication and centrifuged prior to a 2-h incubation with GSH-Sepharose resin (Amersham Pharmacia Biotech). The resin was collected on a coarse sintered glass filter, washed, and loaded into a column for elution with reduced glutathione (pH 7.0–7.2). Fractions were analyzed by SDS-PAGE and HPLC using a C4 column. Peak fractions were pooled, the DTT content was adjusted to 50 mM, and bovine serum albumin was added to 100 μg/ml. The purified protein was aliquoted and stored at −80 °C.

A cell pellet from baculovirus-infected insect cells (Sf9), expressing human p53, was lysed by sonication in buffer containing Nonidet P 40 and centrifuged. The soluble fraction was diluted to 0.1 M NaCl and centrifuged, and the supernatant was incubated with Q-Sepharose (Amersham Pharmacia Biotech) for 30 min. The mixture was passed over a coarse sintered glass filter, and the resin was washed and loaded into a column for step elution with NaCl. The p53 protein peak eluted at ~0.1 M NaCl. The eluted material was diluted to 0.1 M NaCl, loaded onto an SP-Sepharose column, and eluted with a 0.1 to 1.0 M linear NaCl gradient. The eluted material was adjusted to 25 mM Tris-HCl (pH 7.5), 10 mM DTT, 10% glycerol, and 100 mM NaCl by the addition of the appropriate stock solutions. Analysis by SDS-PAGE, Western blotting, and reverse phase HPLC revealed that the final protein was greater than 95% pure.

**Conjugation of Ubc4 with Labelled Ubiquitin**—The conjugation of og-Ub and Ubc4 was performed at 37 °C in reaction buffer (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.1 mM DTT). The mixture contained E1 (0.5 μM), adenylate kinase (32 units/ml), og-Ub (100 μM), Ubc4 (50 μM). Pre-made ATP/MgCl2 (2 mM) was added to start the reaction and then incubated for 10 min. The reaction was transferred to ice and EDTA (final concentration 10 mM) and NaCl (final concentration 400 mM) were added to stop the reaction. The volume was reduced using an Amicon ultrafiltration cell equipped with a 3000-molecular weight cutoff filter. The concentrated reaction was loaded onto an Superdex 75 gel filtration column equilibrated with 50 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 0.1 mM DTT and eluted isocratically. og-Ub-Ubc4 was collected in the fractions representing the second peak of absorbance at 280 nm. Pooled fractions were concentrated as above, aliquoted, and stored at −80 °C. The active conjugate was defined as the fraction of og-Ub-Ubc4 sensitive to reduction by 100 mM DTT. The samples were analyzed by HPLC on a C4 column, and typically 80–90% was DTT-sensitive, suggesting an active thiolester linkage. The conjugate appeared stable over time as determined by activity and HPLC analysis.

**Analysis of mgd2-mediated Ubiquitination of p53**—Reactions were carried out in 15 mM HEPES (pH 7.5), 5 mM NaCl, and 10 mM octyl- d-glucoside in a 20-μl reaction volume. The reactions were performed at room temperature with varying incubation times, stopped with 4× SDS-reducing sample buffer and heating at 95 °C for 5 min. Reactions performed with the free components contained E1 (100 mM), og-Ub (5 μM), Ubc4 (1 μM), p53 (1 μM), mmd2 (3 μM), and ATP (1 mM). Reactions run in the absence of E1 included p53 (5 μM), og-Ub-Ubc4 (5 μM), and mmd2 (3 mM). Reactions were analyzed using SDS-PAGE gels from Novex (Carlsbad, CA), and the fluorescence intensity of the p53-Ub, bands was quantified on a FluorImager 595 (Molecular Dynamics) using an excitation at 488 nm and an emission filter of 530 nm, or on a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Fluorescence intensity was converted to molar units of og-Ub by reference to an og-Ub calibration curve and assuming that the quantum yield for og-Ub fluorescence was not significantly affected by formation of the og-Ub/p53 complex. Little or no change in the quantum yield for Oregon Green was detected after conjugating to Ub (19).

**Steady-state Analysis**—The majority of steady-state experiments were run at a single time point of 20 min, using p53 (1 μM), og-Ub-Ubc4 (5 μM), and mmd2 (3 mM). The velocity of product formation was fit to one of the following equations by global fitting of the entire data set using the software program Graft (Erthacus Software Ltd.). The equations below describe the following situations: Eq. 1, simple steady-state kinetics for one varied substrate at a fixed concentration of all others; Eq. 2, a Ping Pong Bi Bi steady-state mechanism; Eq. 3, a Rapid Equilibrium Random Bi Bi ternary complex steady-state mechanism; Eq. 4, competitive inhibition; Eq. 5, noncompetitive inhibition (mixed type); Eq. 6, substrate inhibition.

\[
\frac{v}{V_{\text{max}}} = \frac{[S]}{K_m + [S]}
\]  
\[
\frac{v}{V_{\text{max}}[A][B]} = \frac{K_B[A] + K_{Ax}[B] + [AX][B]}{[AX][B]}
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\frac{v}{V_{\text{max}}[A][B]} = \frac{K_B[A] + K_{Ax}[B] + [AX][B]}{[AX][B]}
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\frac{v}{V_{\text{max}}[S]} = \frac{[S] + K_B + [I]/K_C}{[S] + K_B + [I]/K_C}
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\frac{v}{V_{\text{max}}[S]} = \frac{[S] + K_B + [I]/K_C}{[S] + K_B + [I]/K_C}
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In these equations \( V_{\text{max}} \) is the maximal velocity obtained at infinite concentrations of all substrates \([AX] \) and \([B] \) are the concentrations of the group transfer donor and acceptor substrates (in our systems \([AX] = \text{[og-Ub-Ubc4]} \) and \([B] = \text{[p53]} \), respectively; \([S] \) is the concentration of the varied substrate when the other substrate is held constant; \( K_m \) is the Michaelis constant for the varied substrate; \( K_B \) and \( K_{Ax} \) are either Michaelis constants (in Eq. 2, Ping Pong mechanism) or equilibrium dissociation constants (in Eq. 3, Rapid Equilibrium Random Bi Bi mechanism) for substrates \([AX] \) and \([B] \), respectively; \( [I] \) is the substrate cooperativity factor (e.g. the Michaelis constant is equal to \( [I] \) times the equilibrium dissociation constants when all other substrates are saturating); \( K_C \) is the dissociation constant for the inhibitor-enzyme binary complex and \( \beta K_B \) is the dissociation constant for the inhibitor-enzyme-substrate ternary complex.

**Materials**—The methylated ubiquitin and ubiquitin aldehyde were obtained from Boston Biochemical. The lactacystin was from Calbiochem. The p53 (clone DO1) and 1B (SC-371) antibodies were from Santa Cruz Biotechnologies, and the 4B2 anti-mmd2 antibody was a generous gift from the laboratory of Dr. Arnold Levine.

**RESULTS**

mmd2 Mediates Ubiquitin Transfer to p53 Uncoupled from E1—The transfer of ubiquitin in the mmd2/p53 ubiquitin ligase assay was monitored by Oregon Green fluorescence and detected by reducing SDS-PAGE. Previous results have shown that the Oregon Green label is incorporated on Lys6 of Ub (19).
Reactions were monitored as a function of time with all the components of the ubiquitin cascade: the ubiquitin-activating enzyme (E1), Ub, ATP, Ubc4 (E2), mdm2, and p53. As shown in Fig. 1A, Ub was detected on p53 after 2.5 min and was incorporated for up to 60 min of reaction time. The p53-Ub (n) reaction products exhibited increased fluorescence intensity and "laddering" to slower mobility species in the gel. Western blots using an anti-p53 antibody (DO-1) demonstrated that the bands were indeed "laddered" p53 species (Fig. 3A). The reaction products from three independent experiments were quantified, and the results (Fig. 1C, solid circles) demonstrate the time-dependent incorporation of Ub onto p53 reaching a plateau after 60 min. The incorporation of Ub on p53 required the activation of Ub by the E1 enzyme prior to the mdm2-mediated transfer to p53 (data not shown). Product formation was also dependent on the addition of p53 to the reaction (Fig. 1A). Next we determined if the E1 activation and transfer of Ub to Ubc4 could be dissociated from Ub-Ubc4 and mdm2. To this end, og-Ub was conjugated to Ubc4 in the presence of E1, and the resulting product (og-Ub-Ubc4) was purified. The pre-conjugated E2 (og-Ub-Ubc4) was then used in the mdm2/p53 ubiquitination assay, and the results are shown in Fig. 1B. As with the free components, og-Ub was incorporated into p53 in a time-dependent manner. No incorporation was seen at time = 0 nor if p53 was left out of the reaction. Quantitation of the p53-Ub(n) from three independent experiments is shown with the open circles in Fig. 1C. These results demonstrate that pre-conjugated Ub-Ubc4 results in reaction kinetics and p53-Ub(n) products similar to what is produced with the free components. Both reactions were shown to be dependent on mdm2 (data not shown). The ability to use pre-conjugated E2 (og-Ub-Ubc4) eliminated the E1-coupled reaction and simplified kinetic characterization of mdm2-mediated Ub transfer to p53.

The Pre-conjugated Reaction Is Resistant to Thiol Modification—Initial studies of mdm2-mediated p53 ubiquitination suggested that mdm2 utilized a thiolester intermediate similar to the Hect domain E3 ligases (20). The ability to use the pre-conjugated Ub-Ubc4 eliminated the E1 thiolester intermediate (21) from the mdm2 ubiquitination of p53. A titration of the sulfhydryl-modifying reagent NEM was compared in the p53 ubiquitination reactions using the free components and the pre-conjugated Ub-Ubc4. As shown in Fig. 2, the pre-conjugated condition rendered the reaction resistant to NEM relative to the free components. The concentration of NEM required to achieve 50% inhibition was ~100-fold higher for the pre-con-
jugated system than for the free component assay. The NEM sensitivity in the free component assay may be attributed to E1, because the inhibition curve for formation of Ub-E1 can be superimposed with the inhibition of p53-Ub formation in the free component assay (Fig. 2B). The inhibition of the pre-conjugated reaction observed at higher NEM concentrations may reflect modification of the Cys residues in the RING finger domain that are required for ligase activity. Ligase activity is dependent on an intact RING finger domain as conversion of Cys461 to Ala eliminates ligase activity both in vitro and in vivo for both p53 and mdm2 (8, 9).

**mdm2 Mediates Multiple Mono-ubiquitination of p53**—Ubiquitination of proteins in the cell often results in poly-ubiquitin chain formation. A peptide bond is formed between the ε-amino group of Lys in the target protein and the carboxy terminus of Ub. Chain extension results from additional isopeptide bond formation with the carboxyl-terminal Gly of an additional Ub and usually Lys48 of the previously conjugated Ub molecule. Current data suggest that efficient recognition by the proteasome requires at least four Ub molecules in the chain linked in this Lys48 configuration (22, 23). Other Lys residues of Ub have been reported to support polymeric Ub-chain formation, including Lys63 (24, 25). To investigate the Lys residue(s) involved in mdm2-mediated ubiquitination of p53, we used a Lys48 to Arg (K48R), Lys63 to Arg (K63R), or a double mutant (K48R/K63R) of Ub to eliminate the ability to form poly-ubiquitin chains with those residues. Use of these Ub mutants resulted in p53-Ub(n) that appeared identical to the use of wild-type Ub (data not shown). This result suggested that neither Lys48 nor Lys63 were involved in the putative chain formation. Because there was no diminution of product formed using the mutated ubiquitins, Ub that had been subjected to reductive methylation to block all the Lys residues was used in the in vitro ligase reaction. Again, the reaction produced a p53 laddering pattern as seen with wild-type Ub (Fig. 3A). These results suggest that Ub was conjugated to p53 monomerically at multiple sites and not as a poly-ubiquitin chain. To ensure the fidelity of the methylated Ub, wild-type and methylated Ub were used in the poly-ubiquitination reaction of IκB by the SCF/TrCP complex from THP.1 cell lysates. The formation of high molecular weight poly-ubiquitinated IκB was detected when wild-type Ub was used but was inhibited in the presence of methylated Ub or without the addition of exogenous Ub (Fig. 3B). The partial shift in bands immunoreactive with anti-IκB probably represents the use of endogenous Ub from the cell extract. Previous data has shown IκB to be poly-ubiquitinated (26, 27). The methyl Ub was also tested in the “auto-ubiquitination” reaction of mdm2 (8, 9), and as seen in Fig. 3C, use of wild-type Ub led to formation of high molecular weight species whereas methylated Ub inhibited the formation of the ubiquitin-mdm2 spe-
Human mdm2 Mediates Multiple Mono-ubiquitination of p53

Steady-state Analysis of mdm2-mediated Ub Transfer to p53—Preconjugated og-UbUbc4 and p53 were used as substrates to characterize mdm2-mediated Ub transfer to p53. This reaction reduces the ubiquitination of p53 to a simple two substrate (Bi Bi) reaction. mdm2 (1–5 nM) was incubated with 5 μM og-UbUbc4 and 1 μM p53 and formation of the product p53−(og-Ub)α was linear with time over 20 min (Fig. 4 and inset of Fig. 1C). The initial velocity of product formation was also linearly dependent on mdm2 concentration (Fig. 4, inset).

The experimental data presented below were obtained at a fixed mdm2 concentration of 3 nM.

To define the mechanism of catalysis, we varied both og-UbUbc4 and p53 concentration simultaneously. Fig. 5A illustrates the untransformed data from experiments in which the velocity of p53−(og-Ub)α formation was determined by varying p53 concentration with several fixed concentrations of og-UbUbc4. A series of parallel lines were obtained when these data were presented in a double-reciprocal format. The reverse experiment was performed in which the velocity was measured as a function of og-UbUbc4 concentration at several fixed concentrations of p53. The data from this set of experiments also yielded a series of parallel lines when plotted as a double-reciprocal plot (Fig. 5B). Parallel lines are usually reflective of a Bi Bi reaction conforming to a double-displacement, or Ping Pong mechanism. The data in Fig. 5, A and B, were fit to Eq. 2 for such a Ping Pong mechanism. The kinetic constants obtained from either data set were essentially the same and were therefore averaged and are presented in Table I.

Although parallel line double-reciprocal plots are most often associated with a Ping Pong mechanism, such plots can also result from a random ordered ternary complex mechanism under conditions of large negative cooperativity between substrates (29). Hence, both data sets were also fit to the alternative model for a Rapid Equilibrium Random Bi Bi mechanism (Eq. 3). The best fit of the data to this model yielded the following values for the kinetic parameters: $k_{cat} = 4.8 \pm 2.2$ min$^{-1}$; $K_{m}$, og-UbUbc4 = 9.4 ± 4.2 nM; $K_{m}$, p53 = 3.1 ± 1.5 nM; $\alpha = 320 \pm 200$. Because the $K_{m}$ of each substrate, at saturating concentrations of the other, can be calculated as $aK_{m}$, we can obtain the following $K_{m}$ values for each substrate: $K_{m}$, og-UbUbc4 = 3.0 ± 1.7 μM; $K_{m}$, p53 = 1.0 ± 0.6 μM. The $k_{cat}$ and $K_{m}$ values from this fitting are virtually identical to those obtained from fitting to a Ping Pong mechanism (Table I). To obtain a reasonable fit of the experimental data to the Rapid Equilibrium Random Bi Bi mechanism, however, requires an unusually large value of $\alpha = 320 \pm 200$. The implications of this magnitude of negative cooperativity among substrates, and a more detailed discussion of the potential mechanisms consistent with all the kinetic data, are presented under “Discussion.”

Careful inspection of the data in Fig. 5 reveals that at lower concentrations of og-UbUbc4 and higher concentrations of p53, the data deviate from simple Michaelis-Menten behavior, demonstrating a lower velocity than expected. This behavior is more apparent in Fig. 6 where the p53 titration data are plotted at low (1 μM) and high (5 μM) og-UbUbc4 on a normalized velocity scale. The diminution of velocity at higher p53 concentrations and lower og-UbUbc4 concentrations is consistent with substrate inhibition by p53.

Kinetic Analysis with Alternative Substrates—There is compelling evidence that p53 is a substrate for mdm2 within cells. However, in vitro mdm2 is capable of catalyzing ubiquitination of other proteins as well. For example, we have found that mdm2 can catalyze the ubiquitination of the retinoblastoma protein (Rb) in vitro, although it is unclear if this reaction is physiologically meaningful. When Rb was substituted for p53 in our reaction mixture, we observed mdm2-dependent transfer of og-Ub from og-UbUbc4 to Rb. When the velocity of this reaction was studied as a function of og-UbUbc4 concentration, at several fixed Rb concentrations, the resulting data appeared as a series of parallel lines when plotted in double-reciprocal
Thus, we observe that the $K_m$ of the velocity of mdm2-Ub (n) formation as a function of og-concentrations and varying og-Ub ubiquitination. This reaction was also studied at fixed mdm2. When p53 was added to the assay, we could measure mdm2 auto-ubiquitination. As seen in Fig. 1, the ratio remains constant over the entire time course (i.e. in the initial velocity region) of the steady state.

In contrast to the patterns seen for p53 ubiquitination, the incorporation of Ub into mdm2 by auto-ubiquitination is quite different. As seen in Fig. 7C, the molecular weight distribution of mdm2-Ub$_{\text{ub}}$ products changes with time so that the intensity is shifted to higher molecular weight forms as the reaction time course proceeds. We cannot make any mechanistic inferences from these data without further studies. However, the data displayed in Fig. 7 suggest fundamental differences in the reaction pathways for p53 and mdm2 ubiquitination by mdm2.

**Kinetic Analysis of Product Inhibition**—To investigate further the mechanism of mdm2-catalyzed ubiquitination of p53 we studied the effects of the reaction product, Ubc4 on catalysis. Fig. 8, A and B, illustrates, in double-reciprocal form, the effects of varying Ubc4 concentration on the velocity of the mdm2 reaction. In panel A, the p53 concentration was used at its apparent $K_m$ (vide supra) and the initial velocity was determined at varying concentrations of og-Ub-Ubc4 and several fixed concentrations of Ubc4. The untransformed data from this experiment were fit to the Michaelis-Menten equation (Eq. 1) at each inhibitor (Ubc4) concentration, and the kinetic parameters from this fitting were then used to generate double-reciprocal plots. This results in a series of lines that converge at the y axis of the double-reciprocal plot, as expected for a competitive inhibitor (Fig. 8A). The untransformed data from this experiment were therefore fit to Eq. 4 for competitive inhibition yielding a $K_i$ (i.e. $K_{in}$) of $3.4 \pm 0.5$ $\mu$M for Ubc4. In Fig. 8B, the reaction was studied at varying concentrations of p53 at several concentrations of Ubc4, with the og-Ub-Ubc4 concentration held constant at its apparent $K_m$ (vide supra). These data were analyzed as described above and yielded a series of lines in the
A double-reciprocal plot that converged on the x axis at a negative value of reciprocal substrate concentration. These data are most consistent with Ubc4 as a noncompetitive inhibitor with respect to p53 (mixed type, with $\beta = 1$, Eq. 5).

**DISCUSSION**

The results presented in this manuscript demonstrate that mdm2 ubiquitinates p53 in a catalytic fashion, and the product of this reaction is a p53 molecule that has Ub at a number of different residues. The ubiquitinated p53 generated *in vitro* is similar to p53-Ub(n) obtained from lactacystin-treated cells. We have also demonstrated that mdm2-mediated transfer of Ub from Ub-Ubc4 to p53 can occur independent of E1 if Ub is pre-conjugated to Ubc4 and used as the Ub-donating substrate. This observation suggests that Ub-E2 binds to the E3 with the subsequent transfer of Ub followed by dissociation of E2 from mdm2, which then allows another Ub-E2 to bind. Although it does not rule out the possibility that the E2 stays bound and is recharged by E1 in the cell for subsequent Ub transfer. We also note that the physiological E2 for mdm2 is currently not defined. Regardless of these issues, the isolated reaction has allowed us to characterize in kinetic detail the enzymatic transfer of Ub by mdm2.

**TABLE I**

Average values ($n = 3$) of the kinetic constants for mdm2-mediated ubiquitin transfer to p53

All values were obtained through global fitting using Grafit. See text for further details.

| Kinetic constant | Ping Pong mechanism | RERBB$^a$ mechanism |
|------------------|---------------------|---------------------|
| og-Ub Ubc4, $K_m$ | $3.0 \pm 1.0 \mu M$ | $3.2 \pm 1.7 \mu M$ |
| p53, $K_m$      | $1.1 \pm 0.4 \mu M$ | $1.0 \pm 0.6 \mu M$ |
| $k_{cat}$        | $5.0 \pm 2.0 \text{ min}^{-1}$ | $4.8 \pm 2.2 \text{ min}^{-1}$ |
| $\alpha$         | NA$^b$              | $320 \pm 200$       |

$^a$ RERBB, Rapid Equilibrium Random Bi Bi.

$^b$ NA, not applicable to this mechanism.
The finding that mdm2-mediated ubiquitination of p53 results in multiple mono-ubiquitin moieties was surprising, because mdm2 expression and p53 stability are integrally linked (5, 6). The expectation was that a poly-ubiquitin chain would be formed as this configuration is most efficiently recognized by the proteasome (22, 23). The ability of the methylated Ub to inhibit the poly-ubiquitination reaction of IκB and the auto-ubiquitination of mdm2 but not the mdm2-mediated ubiquitination of p53 confirms the integrity of the methylated Ub substrate. These data also represent the first direct evidence that the auto-ubiquitination of mdm2 generates a chain of poly-ubiquitin and is consistent with previous observations that mutation of Lys466 is sufficient to eliminate the ubiquitination of mdm2 (30). Our data are also consistent with published observations for multiple ubiquitination sites on p53 (31, 32). Mutagenesis of specific Lys residues, or sets of residues, does not eliminate the ubiquitination and degradation of p53 in cells. However, truncation of the carboxyl terminus of p53 or mutation of multiple Lys residues at once confers resistance to mdm2-mediated ubiquitination and degradation (31–33). These data suggest a model wherein the primary Ub moieties on p53 could serve an alternative function from direct recognition by the proteasome (see below) or could represent the precursor for recognition by the recently described E4 activity to generate the poly-ubiquitin chain (34), or both. Indeed, two recent papers suggest that ubiquitination of p53 is required for nuclear export and that ubiquitination of p53 could be required to expose the nuclear-export signal within the tetramerization domain of p53 (36, 37). Multiple mono-ubiquitination is also consistent with the requirement for nuclear export for proteosomal degradation in the cytoplasm (36–39). As previously mentioned, the multiple mono-ubiquitin configuration would lack the efficient recognition motif for proteasomes in the nucleus, although a less efficient nuclear degradation cannot be completely ruled out (35). Regardless, it is clear that Ub-modified proteins have specific functions and that Ub modification is not simply a tag for directing proteasomal degradation (24, 25, 40). Three recent reports of Ub-modified proteins where the Ub modification does not lead directly to degradation involve proteins, like p53, that are involved transcriptional regulation; these include histone H2B (41), Met4 (42), and TAFII250 ubiquitination of histone H1 (43).

Using pre-conjugated Ub-Ubc4, we were able to characterize the mdm2-mediated Ub transfer to p53 reaction with two substrates (Ub-Ubc4 and p53) and two products (p53-Ub)n and Ub-Ubc4. Our findings are summarized as follows: 1) Parallel double-reciprocal plots were obtained when one substrate was titrated at several fixed concentrations of the other substrate. 2) The reaction product Ubc4 was competitive with respect to Ub-Ubc4 and noncompetitive with respect to p53. 3) Substrate inhibition by p53 was observed at low Ub-Ubc4 concentrations whereas higher Ub-Ubc4 concentrations eliminate the p53 substrate inhibition. 4) The Km for Og-Ub-Ubc4 was unaffected by the identity of the Ub-accepting substrate, similar Km values (within a factor of 2) were obtained for Og-Ub-Ubc4 when p53, Rb, or mdm2 was used as the Ub acceptor.

Parallel double-reciprocal lines are usually indicative of a Ping Pong mechanism. A classical Ping Pong mechanism predicts that a reaction product will be noncompetitive with respect to its cognate substrate (at nonsaturating levels of the second substrate) and competitive with respect to the second substrate of the enzyme. However, we observed a very different Ubc4 inhibition pattern (competitive with Ub-Ubc4 and noncompetitive with p53), indicating that our kinetic data are not consistent with a simple Ping Pong mechanism. Parallel, or apparently parallel, double-reciprocal plots can arise from several kinetic mechanisms other than a classical Ping Pong mechanism. Segel (29) lists six potential mechanisms that can yield such results. The inhibition pattern observed for Ubc4 allows us to eliminate all but three potential mechanisms for mdm2 catalysis.

The first two mechanisms that are consistent with all of the kinetic data both involve the transient formation of a covalent Ub-mdm2 intermediate (i.e. a Ping Pong mechanism). The first of
these is the Hybrid Di Iso Ping Pong-Di Theorell Chance (HDIPPDTC) mechanism and is illustrated in Fig. 9A. As with a classical Ping Pong mechanism, the HDIPPDTC mechanism does not involve formation of a ternary Ub,Ubc4,mdm2,p53 complex. In this mechanism, productive p53 binding can only occur after transfer of Ub from Ubc4 to mdm2. The feature that distinguishes this mechanism from a classical Ping Pong mechanism is two compulsory enzyme isomerization steps: 1) after mdm2 accepts Ub from Ubc4, and 2) after Ub-mdm2 transfers the Ub to p53. The HDIPPDTC mechanism can involve distinct binding sites on mdm2 for each substrate that are in conformational communication with one another. The mdm2 conformational state that initially accepts Ub from Ub-E2 would be designated as E3, and the mdm2 conformational state that is capable of transferring Ub to p53 would be designated as E3'. The conformational change from E3 to E3' occurs after Ub transfer to mdm2 and prior to Ub transfer to p53. After the product p53-Ub has dissociated, the free enzyme is in state E3' and must undergo a second conformational change to return it to the original E3 state to complete the catalytic cycle. With this mechanism, the free mdm2 in state E3 can also bind directly to p53, consistent with experimental evidence. Within the context of this mechanism, however, formation of the binary mdm2:p53 complex does not lead to productive catalysis. This is reflected in the kinetic meas-

**Fig. 9.** Product inhibition of the mdm2/p53 Ub reaction by Ubc4. A, double-reciprocal plot of the initial velocity of p53-Ub, formation as a function of og-Ub-Ubc4 concentration. Reactions were run at 1 μM p53 and different Ubc4 concentrations (C, 0 μM; A, 1 μM; □, 5 μM; ●, 20 μM). Inset, the untransformed data with lines depicting global fitting of the entire data set to Eq. 4 (competitive inhibition). B, double-reciprocal plot of the initial velocity of p53-Ub, formation as a function of p53 concentration. Reactions were run at 5 μM og-Ub-Ubc4 and different Ubc4 concentrations (C, 0 μM; ●, 1 μM; □, 5 μM; ■, 20 μM). Inset, the untransformed data with lines depicting global fitting of the entire data set to Eq. 5 (non-competitive inhibition).
site residue on mdm2; 2) A conformational rearrangement of mdm2 brings the bound Ub moiety from the Ub-Ubc4 binding pocket into proximity with the p53 binding pocket; 3) The Ub is transferred from mdm2 to p53; and 4) relaxation of mdm2 to bring the active site residue, which is transiently modified by Ub, back into register with the Ub-Ubc4 binding pocket. Requisite characteristics of this mechanism are formation of a transient, covalent complex between mdm2 and Ub and conformational changes in mdm2 that facilitate Ub acceptance from Ub-Ubc4 and subsequent Ub donation to p53. The observed p53 substrate inhibition could result from decreased binding affinity of Ub-Ubc4 to a binary mdm2-p53 complex relative to free mdm2 and increasing Ub-Ubc4 concentration would diminish the p53 substrate inhibition.

As stated above, the mechanisms depicted in Fig. 9, A and B, share some common features. Both mechanisms involve formation of a transient Ub-mdm2 intermediate and isomerization of mdm2 to complete the catalytic cycle. A distinction between the two mechanisms is that the HDIPPDTC depicted in Fig. 9A does not permit formation of a catalytically productive ternary Ub-Ubc4-mdm2-p53 complex, whereas such a complex is permitted by the HPPRERBB mechanism in Fig. 9B. Recently, a crystal structure has been reported for the RING finger E3 ligase c-cbl in complex with E2 (UbcH7) and a peptide derived from ZAP-70, the c-cbl ubiquitination target (44). The crystallization of this E2-enzyme-peptide ternary complex raises the possibility that a similar ternary complex between Ub-E2-enzyme-target protein could occur. If this is true for c-cbl, and by extension to mdm2 and other RING finger E3 ligases, then the mechanism outlined in Fig. 9A is less likely.

The third mechanism that is consistent with our kinetic data is the Rapid Equilibrium Random Bi Bi mechanism (RERBB) with a substrate cooperativity factor, \( \alpha \), of \( \gg 1 \) (Fig. 9C). This mechanism involves a random order of substrate addition, resulting in formation of a ternary Ub-Ubc4-mdm2-p53 complex. The \( K_{AX} \) and \( K_B \) in Fig. 9C are equilibrium dissociation constants (\( K_d \)) rather than Michaelis constants (\( K_m, K_m = \alpha K_d \)). No covalent intermediate on mdm2 is involved in the transfer. Therefore, the role of mdm2 in this mechanism is to bring Ub-Ubc4 and p53 into close proximity and appropriate orientation for direct transfer. The magnitude of the \( \alpha \) factor reflects strong negative cooperativity between the two substrates, such that the binding of one substrate diminishes the affinity of the enzyme for the other substrate through some form of enzyme allostery. The anti-cooperativity between substrates would have to be mediated through a form of enzyme isomerization to communicate, in structural terms, substrate binding at one site to the other substrate binding pocket. Seemingly parallel-lined double-reciprocal plots in the context of this mechanism require a minimum \( \alpha \) value of 100 over the substrate concentration range studied here. Global fitting of the data in Fig. 5, A and B, to this mechanism provides a best fit estimate of \( \alpha \) of 320. This is an unusually large degree of anti-cooperativity, but may reflect the need for very tight control of cellular p53 ubiquitination. The commonality of og-Ub-Ubc4 \( K_m \) for three different Ub-accepting substrates (p53, Rb, and mdm2) is easily explained by a Ping Pong mechanism. However, consistency with a Rapid Equilibrium Random Bi Bi mechanism would further require that the value of \( \alpha \) be invariant with all the Ub-accepting substrates. This seems unlikely, particularly given the fact that the \( K_f \) for Rb under this mechanism would have to be subnanomolar (data not shown). Also, it should be noted that a simple Rapid Equilibrium Random Bi Bi mechanism does not account for the substrate inhibition observed for p53. Substrate inhibition within this mechanism would require a second p53 binding site on mdm2 and its occupation would lead to a nonproductive p53-mdm2-p53 complex. The elimination of substrate in-

![Fig. 9. Schematic representations of potential mechanisms, consistent with the kinetic data, for mdm2-mediated Ub transfer to p53. A, Hybrid Di Iso Ping Pong-Di Theorell Chance (HDIPP-DTC) mechanism. B, Hybrid Ping Pong Rapid Equilibrium Random (Two Site) Bi Bi (HPPRERBB) mechanism. C, Rapid Equilibrium Random Bi Bi mechanism (RERBB). In all of these schemes substrate AX represents Ub-Ubc4, substrate B represents p53, X represents Ub, and AX represents p53-Ubc4.](image-url)
hilitation at high Ub-Ubc4 concentrations would require that both p53 binding sites be in strong anti-cooperative communication with the Ub-Ubc4 binding site of the enzyme. The mechanisms illustrated in Fig. 9, B and C, predict that the two substrates bind to mdm2 in rapid equilibrium. A cat-

calytically productive ternary Ub-Ubc4-mdm2-p53 complex is present in both reaction schemes. However, the major distinc-
tion between these mechanisms is that a covalent Ub-mdm2 intermediate is required in the HPPRERBB mechanism, whereas in the RERBB mechanism, transfer of Ub occurs di-
rectly from Ub-Ubc4 to p53.

A Ub-E3 covalent intermediate, as required by the first two Ping Pong mechanisms, has been isolated for enzymes of the Hect family of ubiquitin ligases. In this case the covalent ac-
duct results from formation of a thiolester bond between an active site Cys of the E3 and the carboxyl-terminal Gly of Ub. The isolation of a covalent Ub-mdm2 intermediate has been previously reported (20); however, this result must be viewed with caution due to the auto-ubiquitination activity of mdm2. Our attempts to identify a Ub-mdm2 intermediate have been con-
firmed by the coconatin formation of mdm2-Ub product catalyzed by mdm2 auto-ubiquitination. Our NEM sensitivity study (Fig. 2) along with published data from other groups (8, 45) suggest that a free sulfydryl group is not available on mdm2 for thiolester bond formation with Ub. Hence, we have no direct evidence for the proposed Ub-mdm2 intermediate.

The mechanisms illustrated in Fig. 9, B and C, predict that the two substrates bind to mdm2 in rapid equilibrium. A cat-

calytically productive ternary Ub-Ubc4-mdm2-p53 complex is present in both reaction schemes. However, the major distinc-
tion between these mechanisms is that a covalent Ub-mdm2 intermediate is required in the HPPRERBB mechanism, whereas in the RERBB mechanism, transfer of Ub occurs di-
rectly from Ub-Ubc4 to p53.

Although auto-ubiquitination involves formation of a pol-
y ubiquitin chain on mdm2, mdm2-catalyzed ubiquitination of p53 results in multiple mono-ubiquitination events. The mech-


Acknowledgments—We thank Robert Brukner for purifying Ubc4, Denise McCabe for purifying mdm2 and p53, Jodie Duke for help with the preparation of Ub-Ubc4, and Robert Graffstrom for many helpful discussions and critical reading of the manuscript. Early aspects of this work benefited from a collaboration with Peter Strack, Maureen Caliguri, and Mark Rolfe at Mitotix, Inc.