Topors Functions as an E3 Ubiquitin Ligase with Specific E2 Enzymes and Ubiquititates p53*

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The human topoisomerase I- and p53-binding protein topors contains a highly conserved, N-terminal C3HC4-type RING domain that is homologous to the RING domains of known E3 ubiquitin ligases. We demonstrate that topors functions in vitro as a RING-dependent E3 ubiquitin ligase with the E2 enzymes UbcH5a, UbcH5c, and UbcH6 but not with UbcH7, CDC34, or UbcH2b. Additional studies indicate that a conserved tryptophan within the topors RING domain is required for ubiquitination activity. Furthermore, both in vitro and cellular studies implicate p53 as a ubiquitination substrate for topors. Similar to MDM2, overexpression of topors results in a proteasome-dependent decrease in p53 protein expression in a human osteosarcoma cell line. These results are similar to the recent finding that a Drosophila topors orthologue ubiquititates the Hairy transcriptional repressor and suggest that topors functions as a ubiquitin ligase for multiple transcription factors.

Topors was originally discovered in a screen for proteins that bind to the N terminus of topoisomerase I (1) and was also identified in a screen for proteins that interact with p53 (denoted p53BP3) (2). Furthermore, topors was identified in an assay for RING domain proteins that are expressed in normal lung tissue (denoted LUN) (3). While topors is widely expressed in normal human tissues, topors mRNA and protein levels are commonly decreased or undetectable in colon adenocarcinomas and cell lines (4). The topors protein contains an N-terminal C3HC4-type RING domain that is conserved in orthologues from various species (5) and is closely related in sequence to the RING domains of known E3 ubiquitin ligases such as the herpesvirus protein ICP0 and Chl (6, 7). Recently, a Drosophila topors orthologue was shown to interact physically and genetically with the Hairy transcriptional repressor (8). Furthermore, the Drosophila topors protein was shown to ubiquitinate Hairy in vitro and to decrease expression of an epitope-tagged Hairy protein in co-transfection studies (8).

Topors is also known to associate with promyelocytic leukemia (PML) nuclear bodies in the nuclei of exponentially growing cells (5, 9). Treatment with transcriptional inhibitors or with the topoisomerase I-targeting drug camptothecin results in rapid dispersion of topors to the nucleoplasm, suggesting that topors is involved in the cellular response to transcriptional perturbation (5).

To gain insight into the role of the topors and the conserved RING domain, we determined whether topors functions as a ubiquitin ligase. Our results indicate that topors acts as a RING domain-dependent, E3 ubiquitin ligase with specific E2 enzymes. Similar to the E3 ubiquitin ligase Cbl, a conserved tryptophan within the topors RING domain is required for ubiquitin ligase activity. Furthermore, additional in vitro and cellular studies implicate p53 as a ubiquitination substrate for topors.

EXPERIMENTAL PROCEDURES

Expression Plasmids—A plasmid expressing a GST-N-terminal topors fusion protein (pGEX-topors) was constructed using PCR with pEGFP-topors (1), the pGEX-4T3 vector (Amersham Biosciences), and primers designed to amplify the entire topors coding region. pGEX-topors-1–195 was created by digestion of pGEX-topors with EcoRI and DraIII sites were utilized. A plasmid expressing an N-terminal polyhistidine-topors fusion protein (pET-topors) was constructed by digestion of pKG-topors (5) with SmaI and HindIII, followed by ligation into the pET-28a(+) vector (Novagen). Mutagenesis of tryptophan 131 in the topors RING domain to alanine was achieved using PCR (oligonucleotide sequences available upon request). EcoRI and DraIII sites were used to place the mutant fragment in the pGEX-topors vector. All plasmids were sequenced to confirm that the correct recombinant had been obtained.

A plasmid expressing GST-tagged human MDM2 (pGEX-MDM2) was a gift from Jiandong Chen (10). Mammalian expression plasmids for human p653 (pRCCMV-p53) and MDM2 (pCHDM1B) were kindly provided by Arnold Levine (11). A mammalian expression plasmid for polyhistidine-tagged ubiquitin (pMT.107) was obtained from Dirk Bohmann (12).

In Vitro Ubiquitination Assays—Purification of GST fusion proteins from bacterial (BL21(DE3); Invitrogen) lysates was performed as described (13). His fusion proteins were purified using a cobalt-based affinity resin (Talon resin, Clontech). Briefly, bacterial pellets were lysed in 500 mM NaCl, 50 mM Tris, pH 8.0 (buffer A), with 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM pepstatin, followed by incubation with Talon resin for 2 h at 4 °C. The beads were washed three times with buffer A containing 5 mM imidazole, then eluted with an equal volume of buffer B containing 500 mM imidazole.

Eluted GST and His fusion proteins were dialyzed against a buffer containing 50 mM Tris·Cl, pH 8.0, 20% glycerol, 0.2 mM EDTA, 300 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT.

In vitro ubiquitination reactions were performed in a buffer contain-
proteins that are likely contaminating bacterial proteins, since they are not recognized by a topors antibody. B.

0.1% phenol red) containing 1 mM DTT. Reaction products were analyzed by SDS-PAGE and immunoblotting using anti-p53 antibodies. Brackets indicate the migration of polymeric ubiquitin conjugates, with the migration of free ubiquitin indicated by arrows. Topors stimulates formation of low mobility ubiquitin conjugates by UbcH5a, with the RING domain necessary and sufficient for this activity. Reactions were performed with full-length topos, a fragment containing the RING domain (1–195), or a fragment lacking this domain (196–1045). C. His-topors induces formation of low mobility ubiquitin conjugates by UbcH5a. D. His-topors stimulates formation of polymeric ubiquitin chains by UbcH5a. Reactions contained either ubiquitin or methylated ubiquitin, as indicated. E, a panel of E2 enzymes was assayed for ubiquitin-conjugating activity in the absence (left panel) and presence (right panel) of GST or GST-topors. The line with an asterisk indicates ubiquitin conjugates formed by E2 enzymes in the absence of topos. F, tryptophan 131 in the topors RING domain is required for ubiquitin ligase activity. In vitro ubiquitin ligase reactions were performed using the indicated E2 and either no E3, wild-type GST-topors, or a GST-topors W131A mutant. A representative silver stain of the purified W131A protein is included on the left.

FIG. 1. Topors functions as a RING-dependent ubiquitin ligase with specific E2 enzymes. A, silver stain of purified recombinant topos proteins. 1–195 and 196–1045 refer to GST fusion proteins containing the indicated topos amino acids. In the His-topors lane, brackets indicate proteins that are likely contaminating bacterial proteins, since they are not recognized by a topors antibody. B–E, results of in vitro ubiquitin assays. Reactions were analyzed by SDS-PAGE and immunoblotting with a ubiquitin antibody. Brackets indicate the migration of polymeric ubiquitin conjugates, with the migration of free ubiquitin indicated by arrows. B. GST-topors stimulates formation of low mobility ubiquitin conjugates by UbcH5a, with the RING domain necessary and sufficient for this activity. Reactions were performed with full-length topos, a fragment containing the RING domain (1–195), or a fragment lacking this domain (196–1045). C. His-topors induces formation of low mobility ubiquitin conjugates by UbcH5a. D. His-topors stimulates formation of polymeric ubiquitin chains by UbcH5a. Reactions contained either ubiquitin or methylated ubiquitin, as indicated. E, a panel of E2 enzymes was assayed for ubiquitin-conjugating activity in the absence (left panel) and presence (right panel) of GST or GST-topors. The line with an asterisk indicates ubiquitin conjugates formed by E2 enzymes in the absence of topos. F, tryptophan 131 in the topos RING domain is required for ubiquitin ligase activity. In vitro ubiquitin ligase reactions were performed using the indicated E2 and either no E3, wild-type GST-topors, or a GST-topors W131A mutant. A representative silver stain of the purified W131A protein is included on the left.

In Vivo Ubiquitination Assays—H1299 (p53 null) and 2KO (p53−/−, mdm2−/− murine embryonic fibroblast) cells were obtained from Arnold Levine (University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, NJ) and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Equal numbers of exponentially growing cells were seeded into 60-mm dishes and were transfected using LipopectAMINE 2000 (Invitrogen) in the presence of serum- and antibiotic-free media. Cells were co-transfected with 200 ng of pRC-CMV-p53, 500 ng of pMT.107 (expressing His-tagged ubiquitin), or empty CMV vector, and 2.5 µg of either pEGFP, pCHDM1B (expressing human MDM2), pEGFP-topors, or pEGFP-topors-231–1045 expression vectors (5). In each case, the total amount of DNA transfected was 2.2 µg/60-mm dish. Twenty-four hours after transfection, MG132 was added to 4 µM, and the cells were incubated for an additional 12 h. Cells were harvested in 1 ml of ice-cold phosphate-buffered saline, and the cell suspension was divided into two parts; 100 µl were lysed using 1× SDS-PAGE sample loading buffer containing 10% DTT, and 900 µl were lysed in guanidine HCl buffer B (6 M guanidine HCl, 0.1% Nonidet P-40, 5% glycerol, and 20 mM imidazole). 500 µl of a 1:3 mixture of buffers B and C, and finally with 500 µl of buffer C. Bound proteins were analyzed by boiling the beads in SDS sample buffer with 1 mM DTT, followed by SDS-PAGE and immunoblotting using anti-p53 antibodies.
Topors Functions as an E3 Ubiquitin Ligase

**RESULTS**

Topors Induces Formation of Polyubiquitin Chains with Specific E2 Enzymes in Vitro—To test whether human topors functions as a ubiquitin ligase, we employed an *in vitro* assay using purified recombinant proteins expressed in bacteria (Fig. 1A). Previous work using similar assays demonstrated that many RING proteins stimulate formation of polymeric ubiquitin chains in the absence of a specific substrate (14, 15). In the presence of E1 and the E2 UbcH5a, GST-topors, but not GST, stimulated formation of ubiquitin conjugates with low mobility on SDS-polyacrylamide gels (Fig. 1B). Furthermore, formation of these conjugates required the topors RING domain, since a 196–1045 fragment was inactive (Fig. 1B). Moreover, similar to other RING proteins (6), a small topors fragment containing the RING domain (1–195) was sufficient for this activity (Fig. 1B). Since previous results suggest that dimerization conferred by GST could contribute to E2- or E3-type ubiquitin ligase activity (16, 17), we also evaluated a bacterially expressed His-topors fusion protein in this assay. His-topors also stimulated the formation of low mobility ubiquitin conjugates in reactions with UbcH5a (Fig. 1C).

To determine whether the low mobility ubiquitin conjugates represented polymeric ubiquitin chains, we repeated the assay using methylated ubiquitin, which due to lysine modification cannot form chains. Low mobility ubiquitin conjugates were not detected when methylated ubiquitin was used in the assay, indicating that topors induces formation of polymeric ubiquitin chains (Fig. 1D). Immunoblotting with antibodies directed against either GST or topors demonstrated that the majority of the ubiquitin conjugates did not involve topors (data not shown), suggesting that they represent conjugates to either the E1, E2, or alternatively, as has been suggested for ICP0, unanchored ubiquitin chains (14). We further analyzed the low mobility conjugates using MALDI-MS and LC-MS/MS methods. Analysis of the resulting mass spectra yielded five highly significant matches (Table I). Four of the masses matched unmodified ubiquitin peptides, whereas one of the masses matched the predicted mass of a tryptic peptide of ubiquitin in which lysine 48 is ubiquitinated, which commonly occurs in polymeric ubiquitin chains (18) (Table I). There were no matched masses suggesting that the low mobility bands contained unmodified or ubiquitinated topors, E1, or E2 peptides. Taken together with the immunoblotting data, these results indicate that the low mobility bands are predominantly polymeric free ubiquitin chains. Thus, similar to other RING proteins, the topors RING domain confers an E3-type ubiquitin ligase activity in the absence of a specific substrate.

Next, we analyzed the ability of topors to interact with specific E2 enzymes. In the absence of topors, ubiquitin conjugates migrating from about *M*<sub>r</sub> 35 to 100 that were stable under reducing conditions were produced commonly by certain E2 enzymes, most notably UbcH6 (Fig. 1E). Similar observations were reported previously and presumably reflect E2 auto-ubiquitination (19, 20). Inclusion of topors resulted in the formation of very low mobility ubiquitin conjugates in reactions with human UbcH5a, UbcH5c, and UbcH6 but not UbcH7, CDC34, or UbcH2b (Fig. 1E). Thus, topors stimulates polymeric ubiquitin conjugates with some, but not all, E2 enzymes.

A Conserved RING Tryptophan Is Required for the Ubiquitin Ligase Activity of Topors—Previous studies identified a tryptophan residue within the Cbl RING domain as required for ubiquitin ligase activity (6) and as important in the physical interaction between Cbl and the E2 UbcH7 (21). By contrast, mutation of the corresponding tryptophan in the ICP0 RING domain did not affect ubiquitination activity *in vitro* (14). These conflicting results prompted investigation of the role of a corresponding and conserved tryptophan residue in the topors RING domain. Reactions with a topors mutant consisting of replacement of the RING domain did not affect ubiquitination activity in the topors RING domain. Reactions with a topors mutant consisting of replacement of the RING domain with alanine did not yield polymeric ubiquitin chains with any of the E2 enzymes.

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**Table I**

| Peptide      | Ubiquitin region | Calculated mass of the indicated peptide with a diglycine linked to lysine 48 (noted by an asterisk) as a result of ubiquitination (18). |
|--------------|------------------|--------------------------------------------------------------------------------------------------|
| EGIPPDQQR    | 128             | 34–42                                                                                           |
| ESTLHVLVR    | 139             | 64–72                                                                                           |
| TLDYNIQK     | 148             | 55–63                                                                                           |
| LIFAKG*QLEDGR| 157             | 43–54                                                                                           |
| TITLVEPSDTIENVK | 177          | 12–27                                                                                           |

*Calculated mass of the indicated peptide with a diglycine linked to lysine 48 (noted by an asterisk) as a result of ubiquitination (18).
presence of UbcH5a, topors induced monoubiquitination of p53, with this activity similar to results obtained with MDM2 using our assay conditions (Fig. 2A). Topors also ubiquitinated p53 in the presence of UbcH5c and UbcH6 (data not shown). Mutant proteins either lacking the entire RING domain (196–1045; Fig. 2A) or containing an alanine substitution for the conserved RING tryptophan (W131A; Fig. 2B) were inactive, indicating that the RING domain is required for p53 ubiquitination by topors. Furthermore, despite induction of polymeric ubiquitin chains by the RING-containing 1–195 topors fragment in reaction mixtures containing p53, this fragment did not induce p53 ubiquitination (Fig. 2A). This finding indicates that the 196–1045 region of topors is required for p53 ubiquitination and is consistent with previous results indicating that a 456–882 region of topors binds p53 (2).

**Topors Induces Ubiquitination and Proteasome-dependent Down-regulation of p53 in Cells**—To investigate whether topors ubiquitinates p53 in cells, we performed transient co-transfection studies using topors, p53, and His-ubiquitin plasmids. To enhance detection of ubiquitin conjugates, transfectants were treated with the proteasome inhibitor MG132 prior to lysis. In co-transfected H1299 cells, lower mobility p53 bands, consistent with mono- and polyubiquitination of p53, were evident in SDS-based whole cell lysates of cells transfected with the MDM2 vector, and to a lesser extent, the full-length topors construct (Fig. 3A). When guanidine-based lysis and affinity chromatography were used to isolate His-ubiquitin conjugates, polyubiquitination of p53 was strikingly apparent in cells transfected with MDM2, and to a lesser degree, the full-length topors plasmid (Fig. 3A). Furthermore, p53 ubiquitination was not observed in cells transfected with a RING-less topors plasmid (Fig. 3A).

To exclude the possibility that ubiquitination of p53 by topors in H1299 cells was occurring indirectly via an interaction with MDM2, similar experiments were performed in 2KO cells, which lack both p53 and MDM2. The results indicate that MDM2 is not required for ubiquitination of p53 by topors in vivo (Fig. 3B).

Next, we evaluated the effects of topors overexpression on endogenous p53 protein levels. U2OS cells were transfected with vectors expressing either MDM2, topors, or the RING-less topors 231–1045 fragment. Immunoblotting analyses performed 48 h after transfection indicated that relative to mock transfectants, p53 protein levels decreased markedly in cells

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**FIG. 3. Topors ubiquitinates p53 in cells.** A, H1299 cells were co-transfected with plasmids expressing the indicated proteins, followed by incubation with MG132 for 12 h prior to lysis and analysis by SDS-PAGE and immunoblotting with a p53 antibody. As indicated under “Experimental Procedures,” 10% of the cells were lysed using SDS (upper panel), while the remainder were lysed with 6 M guanidine HCl, followed by affinity chromatography to isolate His-ubiquitin conjugates (lower panel). B, 2KO cells (p53; Mdm2−/−) were co-transfected with the indicated plasmids and incubated with MG132 for 12 h prior to lysis in 6 M guanidine HCl. His-ubiquitin conjugates were purified by affinity chromatography and analyzed by SDS-PAGE and immunoblotting with a p53 antibody. C, overexpression of topors induces a proteasome-dependent decrease in p53 protein expression. U2OS cells were transfected with either no DNA (mock) or 5 µg of plasmid expressing either GFP-topors (Topors FL), MDM2, or GFP-topors-231–1045. After 48 h, cells were lysed and analyzed for p53 and actin protein content by immunoblotting. As indicated, 1 µM MG132 was added in some cases for the final 24 h of the incubation. D, quantitative analysis of topors-induced decreases in p53 protein expression. p53 and actin bands were analyzed by densitometry using NIH ImageJ. p53 levels were normalized for actin and are expressed as percent of the p53 level in mock transfectants. The graph includes means and S.E. values of four replicate experiments.

**FIG. 4. Alignment of Cbl, herpes simplex ICP0, and topors RING domains.** The alignment was performed using a ClustalW algorithm. Residues in the Cbl RING domain implicated in direct interactions with UbcH7 (21) are indicated by a plus sign.
transfected with MDM2 or full-length topors plasmids but not the RING-less topors plasmid (Fig. 3, C and D). The greater effect of MDM2 relative to topors on p53 protein expression is consistent with results obtained in the cellular ubiquitination assays (Fig. 3). Moreover, in MDM2 and topors transfecteds treated with MG132 for 24 h prior to lysis, p53 protein levels were similar to those observed in mock transfecteds, indicating that the decrease in p53 protein expression induced by topors and MDM2 is dependent on intact proteasome function (Fig. 3C).

**DISCUSSION**

Our results indicate that topors functions as a RING domain-dependent, E3-type ubiquitin ligase with specific E2 enzymes. Among the E2 enzymes that were analyzed, the specificity exhibited by topors is identical to that of ICP0, which contains a RING domain that is very similar to that of topors (Fig. 4). The RING domains of topors and ICP0 are also highly similar to that of Cbl (Fig. 4). Structural analyses of a complex between Cbl and the E2 Ubch7 implicate nine residues within the Cbl RING domain as determinants of E2 binding specificity (21). The corresponding residues in the topors and ICP0 RING domains are identical to those of Cbl, with the exception of four amino acids (Fig. 4). These differences may explain the observation that Cbl functions with Ubch7 but not Ubch5a (6, 22), whereas topors and ICP0 exhibit the converse specificity. Since residues implicated in E2 binding are almost completely conserved in the ICP0 and topors RING domains (Fig. 4), it is not surprising that these proteins exhibit similar E2 specificity.

The finding that tryptophan 131 is required for topors function as a ubiquitin ligase is similar to results obtained with Cbl (6). However, this finding contrasts with results obtained with ICP0, where mutation of the corresponding RING tryptophan did not affect ubiquitin ligase activity in vitro (14). Therefore it is possible that despite their sequence similarities, the ICP0 and topors RING domains bind E2 enzymes using distinct mechanisms. Notably, recent NMR studies of a CNOT4/UbcH5b complex highlight the potential for differing mechanisms of interaction between RING domains and E2 enzymes by demonstrating that the RING domain of CNOT4 binds UbcH5b in a manner that is significantly different than that observed for the Cbl RING-Ubch7 interaction (23).

With regard to potential substrates, human topors was reported to interact physically with topoisomerase I, p53, and certain adeno-associated virus replication proteins (1, 2, 24). However, this finding contrasts with results obtained with ICP0, where mutation of the corresponding RING tryptophan did not affect ubiquitin ligase activity in vitro (14). Therefore it is possible that despite their sequence similarities, the ICP0 and topors RING domains bind E2 enzymes using distinct mechanisms. Notably, recent NMR studies of a CNOT4/UbcH5b complex highlight the potential for differing mechanisms of interaction between RING domains and E2 enzymes by demonstrating that the RING domain of CNOT4 binds UbcH5b in a manner that is significantly different than that observed for the Cbl RING-Ubch7 interaction (23).

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