Cancer Research CAN-11-2410R1

Supplementary materials and methods, discussion, and references

Dmp1 physically interacts with p53 and positively regulates p53's stability, nuclear localization, and function

Donna P. Frazier, Robert D. Kendig, Fumitake Kai, Dejan Maglic, Takayuki Sugiyama, Rachel L. Morgan, Elizabeth A. Fry, Sarah J. Lagedrost, Guangchao Sui, and Kazushi Inoue

Supplementary Materials and Methods

Cell culture, retrovirus preparation, and infection

NIH 3T3, H1299, A549, and U2OS cells were cultured and transfected as described previously (16, 20). Passage 6 *Arf*; *p53*-double knockout MEFs were received from Drs. C. Sherr and M. Roussel. For preparation of retroviruses, human kidney 293T cells were transfected with a helper ecotropic retrovirus plasmid defective in psi-2 packaging sequences, together with pMSCV-IRES-puro vectors containing *Dmp1*, *Dmp1* mutant, or *p53* cDNA (16).

Immunoprecipitation of co-transfected NIH 3T3 cells

To identify novel Dmp1 interaction partners, NIH 3T3 cells were co-transfected with 5 μg of pFLEX1-Dmp1 and 5 μg of pCMV-p53, c-Myb, Arf, Mdm2, c-Myc, E2F2 or E2F3. Cells were lysed in EBC buffer (20), sonicated, and cleared by centrifugation at 20,000g for 10 minutes. To immunoprecipitate FLAG-Dmp1, anti-FLAG M2 affinity gel (Sigma) was used. 200 μg of cell lysates were incubated with 20 μL of the M2 affinity gel for 2 hrs at 4°C with rotation. The samples were then centrifuged at 10,000g for 2 minutes at 4°C and the supernatant (unbound) lysate was removed and saved. After extensive washing, the M2 affinity gel was incubated with 0.6 μg of FLAG peptide (Sigma) in 100 μL EBC buffer for two hrs at 4°C. The samples were then centrifuged at 10,000g for 2 minutes at 4°C and the supernatant (immunoprecipitated protein) was analyzed by Western blotting. A reciprocal immunoprecipitation was performed for p53 with αHA affinity gel (Sigma). Immunoprecipitation-Western blottings were conducted using Protein G-Sepharose and mouse/rabbit Trueblot antibodies (eBioscience).

Dmp1-p53 binding assay *in vitro*

Recombinant p53 protein (4 μg) was incubated with p53 antibody (sc-6243G) for 30 minutes at 4°C with rotation, respectively. Additionally, recombinant Dmp1 protein (4 μg) was incubated with Dmp1 antibody RAX (26). As a control, these proteins were incubated with normal rabbit or goat IgG (Santa Cruz). Each mixture was then incubated with 20 μL of pre-washed protein G sepharose for 30 minutes at 4°C with rotation. The samples were then centrifuged at 10,000 g for 2 minutes at 4°C and washed four times with 800 μL EBC. Recombinant Dmp1 protein (4 μg) prepared under the presence of DNase (3 units per culture) was then incubated with the p53 conjugated sepharose while recombinant p53 (4 μg) was incubated with the Dmp1 conjugated sepharose for one hour at 4°C with rotation. The samples were then centrifuged at 10,000 g for 2 minutes at 4°C and washed twice with stringent wash buffer, twice with RIPA buffer, and then eluted in 1x SDS-PAGE sample buffer.

***In vitro* ubiquitination assay**

The assay was performed as described by Grossman *et al* (33). Hdm2 (500 ng) and p53 (500 ng) were pre-incubated in ubiquitin conjugation reaction buffer for 30 minutes on ice. Dmp1 or Dmp1 deletion mutants (1 μg) were then added to the Hdm2/p53 complex and incubated for 1 hr on ice. Other reaction components were added next: 3 mM Mg-ATP, 200 ng E1; 150 ng E2; and 10 μg Ub or 10 μg Me-Ub (all from Boston Biochem). The reaction was carried out for 30 minutes at 37°C and p53 ubiquitination was analyzed by Western blotting.

Immunofluorescence and confocal microscopy

Cells were plated on Lab-Tek 4-well chamber slides (Nunc). Cells were washed in PBS and fixed in 2% formaldehyde diluted in PBS for 15 minutes at room temperature (500 µL total volume per well). Following fixation, cells were washed 3 times with 500 µl PBS for 10 minutes with gentle rocking at room temperature. Cells were permeabilized with 0.2% Triton X-100 in 1% BSA/0.05% Saponin/PBS for 5 minutes with gentle rocking on ice. Following permeabilization, cells were washed 3 times with 500 µL 0.5% BSA/0.05% Saponin/PBS for 10 minutes with gentle rocking at room temperature. Cells were incubated in primary antibodies diluted 1:50 and To-Pro-3 diluted 1:1000 in 500 µL, 1% BSA/0.05% Saponin/PBS in a humidified box at 4ºC with gentle rocking. Cells were washed 3 times with 500 µL 0.5% BSA/0.05% Saponin/PBS for 10 minutes with gentle rocking at room temperature. Cells were incubated in secondary fluorescently labeled antibodies for at least 1 hr at 4ºC with gentle shaking in the dark. After washing with PBS, the chambers were removed from the slides according to the manufacturers' instructions, mounted using ProLong Gold anti-fade reagent (Invitrogen), and coverslipped. The mounted slides were allowed to cure for 24 hrs in the dark at room temperature and then visualized using a laser scanning confocal microscope (Zeiss, LSM510).

Immunohistochemistry of mouse paraffin sections

Mouse thymic tissues isolated from doxorubicin-injected mice were fixed in formalin and were stained with specific antibodies to cleaved caspase 3 (#9661, Cell Signaling), $p21^{Cip1/Waf1}$ (sc-6246), and bbc3 (ab9645, Abcam) as previously described (26).

Luciferase assays

Reporter assays were conducted as described previously with a luciferase construct containing 2 kb mouse *p21*$^{Cip1/Waf1}$ promoter with *Actin* promoter SEAP as an internal control (16, 20).

Supplementary Discussion

Our data show that Ser15/20 phosphorylation of p53 by etoposide was significantly decreased in *Dmp1*$^{-/-}$ cells than in wild-type cells, (Fig. 4*D*). Since Ser15 phosphorylation is mediated by ATM/ATR in cellular DNA damage response, it is possible that Dmp1 modulates the activity of ATM/ATR kinases in response to genotoxic stress. Interestingly, it was reported that forced expression of ARF enhances phosphorylation of p53 Ser15 mediated by ATM/ATR (34), which is a critical mechanism in ARF-mediated tumor suppression. Interestingly, it was reported that ARF interacts with ATR and auguments its activity (35). Although it is currently unknown how Dmp1 modulates the phosphorylation of p53 in response to DNA damage response, it is possible that the response is also mediated by ATM/ATR. Further study will be required to elucidate the role of Dmp1 as a mediator of DNA damage response.

Supplementary References

33. Grossman SR, Deato ME, Brignone C, Chan HM, Kung AL, Tagami H, et al. Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science 2003;300:342-4.

34. Li Y, Wu D, Chen B, Ingram A, He L, Liu L, et al. ATM activity contributes to the tumor-suppressing functions of p14ARF. Oncogene 2004;23:7355-65.

35. Rocha S, Garrett MD, Campbell KJ, Schumm K, Perkins ND. Regulation of NF-kappaB and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. EMBO J. 2005;24:1157-69.