Human PTIP Facilitates ATM-mediated Activation of p53 and Promotes Cellular Resistance to Ionizing Radiation*

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† The abbreviations used are: ATM, ataxia telangiectasia-mutated; ATR, ataxia telangiectasia-related; PTIP, Pax2 transactivation domain-interacting protein; hPTIP, human PTIP; Gy, gray; BRCA1, breast and ovarian cancer susceptibility gene 1; 53BP1, p53-binding protein 1; siRNA, small interfering RNA; IR, ionizing radiation; RACE, rapid amplification of cDNA ends; PBS, phosphate-buffered saline; HA, hemagglutinin; SCR, scrambled version of SI20.

Mus musculus Pax2 transactivation domain-interacting protein (Ptip) is an essential gene required for the maintenance of genome stability, although its precise molecular role is unclear. Human PTIP (hPTIP) was recently isolated in a screen for proteins, translated from cDNA pools, capable of interacting with peptides phosphorylated by the ATM (ataxia telangiectasia-mutated)/ATR (ataxia telangiectasia-related) protein kinases. hPTIP was described as a 757-amino acid protein bearing four BRCT domains. Here we report that instead full-length endogenous hPTIP contains 1069 amino acids and six BRCT domains. hPTIP shows increased association with 53BP1 in response to ionizing radiation (IR) but not in response to other DNA-damaging agents. Whereas translocation of both 53BP1 and hPTIP to sites of IR-induced DNA damage occurs independently of ATM, IR-induced association of PTIP and 53BP1 requires ATM. Deletion analysis identified the domains of 53BP1 and hPTIP required for protein-protein interaction and focus formation. Data characterizing the cellular roles of hPTIP are also presented. Small interfering RNA was used to show that hPTIP is required for ATM-mediated phosphorylation of p53 at Ser15 and for IR-induced up-regulation of the cyclin-dependent kinase inhibitor p21. Lowering hPTIP levels also increased cellular sensitivity to IR, suggesting that this protein plays a critical role in maintaining genome stability.
nous hPTIP binds 53BP1 specifically in response to IR and demonstrate that, whereas this association of hPTIP with 53BP1 requires the ATM protein kinase, translocation of these proteins to sites of DNA damage occurs independently of ATM. We identify the regions of both hPTIP and 53BP1 that are required for partner interaction and for translocation to sites of damage and show that hPTIP is required for the efficient ATM-mediated activation of p53 and for cellular resistance to ionizing radiation.

MATERIALS AND METHODS

Cell Lines—HEK 293 and HeLa cells were grown in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% fetal bovine serum (HyClone). HCT-116 cells were grown in McCoy's 5A medium (Invitrogen) and 10% fetal bovine serum. PT-EB57 (A-T cells) and YZ5 (A-T cells stably transfected with ATM) were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM l-glutamine (Invitrogen), and 100 μg/ml hygromycin (Roche Applied Science). All of the cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

Antibodies and Plasmids—Sheep polyclonal antibodies raised against the N terminus of hPTIP (amino acids 198–307) were passed over a glutathione-Sepharose column before affinity purification on glutathione S-transferase-hPTIP by the antibody production team in the Division of Signal Transduction Therapy (University of Dundee). Other primary antibodies used were raised against the FLAG epitope (M2, Sigma), HA epitope (Covance Research Products), 53BP1 (Abcam), p53 (DO-7, Novoceastra), CHK1, CHK2 (14), p53 phospho-Ser⁶⁵ (16G8), p53 phospho-Ser³⁷⁵, CHK1 phospho-Ser⁷⁹⁵, CHK2 phospho-Thr²⁰⁵, phospho-5T-Q (Cell Signalling), p21 (187, Santa Cruz Biotechnology), SMC1 phospho-Glu⁶⁸, p213, and SMC1 (Bethyl Laboratories). Plasmids expressing HA-tagged 53BP1 were described previously (15). hPTIP was amplified from human testis cDNA (Clontech) and cloned into the EcoRI/HindIII sites of pCMV5 with a Kozak consensus sequence and an N-terminal FLAG tag. The full sequence of hPTIP was cloned with the nuclear localization signal from SV40 large T-antigen at their C termini.

Immunoprecipitation and Western Blotting—For Western blotting, cells were lysed directly into SDS sample buffer. For immunoprecipitation, cells were lysed in ice-cold buffer containing 50 mM Tris, pH 7.4, 0.27 M sucrose, 1% Triton X-100, and 105 in 35-mm tissue culture plates) using 1 μl of LipofectAMINE 2000 (Invitrogen). After 24 h, cells were split 1:4 and retransfected with siRNA. Maximal depletion was achieved after an additional 48 h.

Colonies Formation Assay—Cells were then trypsinized and seeded out at 2000 cells/10-cm plate. Cells were allowed to adhere for 18 h before exposure to the indicated dose of IR using a Cs137 source at a delivery rate of 3 Gy/min. Cells were allowed to grow for 10–14 days before fixing in 100% ethanol and staining with Giemsa (Sigma), and the number of colonies with >50 cells were counted.

RESULTS

hPTIP Is a Widely Expressed Protein of 1069 Amino Acids with Six BRCT Domains That Associates with 53BP1 Specifically in Response to Double-stranded Breaks—To investigate the nature of endogenous hPTIP, an antibody (anti-PTIPN) was raised against part of the N-terminal hPTIP sequence identified here (amino acids 198–307) and affinity-purified before use. Western blot analysis with anti-PTIPN revealed a single cross-reactive band in extracts of HeLa, HEK 293, U2OS, HCT-116, and KB cells with an apparent molecular mass of 118 kDa (Fig. 1C, first panel), in agreement with a size of 1069 amino acids. The relative abundance of hPTIP did not change in response to DNA damage (data not shown). The full hPTIP open reading frame was amplified from a human testis cDNA library, and a single PCR product of the expected size (3.2 kb) for a 1069-amino acid protein was obtained. Extensive RACE analysis failed to isolate any extra coding 5′-sequence and indicated that all of the hPTIP coding sequence had probably been identified (data not shown; see “Discussion”). When the full hPTIP sequence was transiently expressed in HEK 293 cells bearing a FLAG epitope tag, the anti-PTIPN antibody recognized a single band with apparent molecular mass similar to endogenous hPTIP (Fig. 1C, second panel). To further investigate the specificity of the anti-PTIPN antibody, HeLa cells were transfected with two different small interfering (si)RNA duplexes. One (S120) was aimed at a specific target sequence toward the N terminus of hPTIP, and the other (SCR) corresponded to a scrambled version of S120. As shown in Fig. 1C (third panel), transfection of cells with S120 but not with SCR led to a marked and reproducible decrease in the 118-kDa band recognized by the anti-PTIPN antibody without affecting the levels of GAPDH (Fig. 1C, fourth panel), 53BP1, or BRCA1 (data not shown). These results demonstrated that hPTIP is a widely expressed protein of 1069 amino acids with six BRCT domains.

It was recently reported that the C-terminal BRCT domains of hPTIP expressed in bacteria and immobilized on an insoluble support could deplete 53BP1 from extracts of cells that had been exposed to IR (6). As shown in Fig. 1D, the exposure of cells to IR led to increased association of endogenous 53BP1 and hPTIP (Fig. 1D, upper panel). This increased binding was associated with 53BP1 phosphorylation on ATM/ATR consensus sites S/T-Q motifs; Fig. 1D, middle panel). Increased binding of 53BP1 to hPTIP was not observed after exposure to UV light, camptothecin, hydroxyurea, methylmethanesulfonate, suggesting that association of hPTIP and 53BP1 is induced specifically by DNA double-strand breaks.

ATM is Required for IR-stimulated Association of hPTIP with 53BP1 but Not for Translocation of either Protein to Sites of DNA Damage—ATM is activated specifically by DSBs, whereas most types of DNA damage activate ATR (2). The observation that the association of 53BP1 and hPTIP increases after IR but not after other DNA-damaging agents (Fig. 1D) suggested a role for ATM. To test this possibility, A-T cells (lacking ATM) complemented with ATM cDNA or not were co-transfected with HA-tagged 53BP1 and FLAG-tagged hPTIP since the levels of endogenous 53BP1 and hPTIP were very low in these cells (data not shown). As shown in Fig. 2A, no...
FIG. 1. hPTIP is a widely expressed protein of 1069 amino acids that is induced to associate with 53BP1 specifically in response to DNA double strand breaks. A, sequence alignment of PTIP from Homo sapiens, Mus musculus, and Xenopus laevis. The N-terminal sequence identified in this study is underlined, and the start ATG of the previously described hPTIP fragment is underscored with an asterisk. B, schematic diagram of full-length hPTIP (top), mouse Ptip (middle), and the previously described hPTIP fragment (bottom). The region of hPTIP used to raise antibodies, and the glutamine-rich region, is indicated. C, Western blot analysis of endogenous hPTIP in different cell lines. Extracts of HeLa, HEK...
increase in the association of 53BP1 with PTIP was observed after IR treatment in cells lacking ATM, in contrast with A-T cells complemented with ATM. To further investigate the role of phosphorylation in the binding of hPTIP to 53BP1, hPTIP immunoprecipitates were treated with λ-phosphatase. This resulted in loss of 70% 53BP1 from hPTIP (Fig. 2B, upper panel) (see “Discussion”) at the highest possible concentration of protein phosphatase (data not shown) judged by densitometric analysis of the ratio of 53BP1 to hPTIP. This was accompanied by the loss of the anti-phospho S/T-Q signal in anti-53BP1 immunoprecipitates (Fig. 2B, middle panel).

Both 53BP1 and hPTIP translocate to sites of DNA damage where they form nuclear “foci” (6, 16). We next investigated whether the ATM-stimulated interaction of hPTIP with 53BP1 mediates the association of hPTIP with DNA damage. In A-T cells, hPTIP formed foci that were indistinguishable from those formed in cells complemented with ATM (Fig. 2C) as reported previously for 53BP1 (16). These results demonstrated that ATM is required for IR-induced association of 53BP1 and hPTIP but not for association of either protein with sites of DNA damage.

Mapping the Domains on hPTIP and 53BP1 Required for Protein-Protein Interaction and for Focus Formation—We next investigated the regions of each protein responsible for focus formation and for protein-protein interaction. HeLa cells were co-transfected with various fragments of HA-tagged 53BP1 (Fig. 3A) (15) and full-length FLAG-hPTIP. Cells were then treated or not with IR, and anti-FLAG immunoprecipitates were probed for HA-53BP1. As shown in Fig. 3B, the removal of both BRCT domains of 53BP1 did not prevent its association with hPTIP but did diminish the basal association of the two proteins. Deletion of an additional 658 amino acids from the C-terminal end of 53BP1 abrogated its interaction with hPTIP, suggesting that amino acids 1052–1710 of 53BP1 are important for interacting with hPTIP. However, a fragment correspond-
Characterization of hPTIP

A.

B.

C.

D.

E.

Fig. 2. ATM is required for IR-stimulated association of hPTIP with 53BP1 but not for translocation of either protein to sites of DNA damage. A. Association of hPTIP and 53BP1 requires ATM. YZ5 cells (ATM−) and FT-EBS7 (ATM−) cotransfected with pCMV5-FLAG-hPTIP and pCMV5-FLAG-53BP1 were exposed to IR. After cell lysis, anti-FLAG immunoprecipitates (IP) were subjected to SDS-PAGE followed by Western blot analysis with the indicated antibodies (top panels). The two lower panels show Western blot analysis of cell extracts before immunoprecipitation. B. Phosphatase treatment of hPTIP immunoprecipitates. hPTIP immunoprecipitates were incubated with λ-phosphatase (400 units) (New England Biolabs) in the presence or absence of EDTA (50 mM) for 30 min at 30 °C. Immunoprecipitates were washed with tris-buffered saline containing 0.2% Tween 20 and subjected to Western blot analysis using the indicated antibodies. Densitometry was performed using NIH Image software to determine the fold increase in the ratio of 53BP1 signal to hPTIP signal in each immunoprecipitate. C. Focus formation by hPTIP and 53BP1 does not require ATM. FT-EBS7 cells grown on 13-mm-diameter glass coverslips were treated with 0 or 10 Gy IR and left to recover at 37 °C for 60 min before fixation and extraction. Cells were washed and blocked before incubation with anti-PTIPN (1 μg/ml) or anti-53BP1 (1 μg/ml) for 60 min at room temperature. After washing in PBS-T, coverslips were incubated with secondary antibodies (2 μg/ml) conjugated to fluorescein isothiocyanate or Texas Red for 45 min. After thorough washing in PBS-T, coverslips were mounted on glass slides and images were acquired using an Olympus IX70 microscope.

HeLa cells were cotransfected next with various fragments of FLAG-hPTIP (Fig. 3B) and neither could a fragment with amino acids 1052–1972 (fragment 6) could not interact with hPTIP (Fig. 3B) and neither could a fragment with amino acids 1052–1710 (fragment 4). These data suggested that sequences that lie between amino acids 1052 and 1710 are necessary but not sufficient to interact with hPTIP and that sequences upstream of 1052 are also required. The ability of these 53BP1 fragments to form foci was reported previously, and the results were confirmed in this study (Fig. 3A). Interestingly, fragment 4 (amino acids 1052–1972) cannot interact with PTIP but can still form foci (Fig. 3B) (15), supporting the notion that focus formation by hPTIP and 53BP1 occurs independently of their physical association (Fig. 2).

HeLa cells were cotransfected next with various fragments of FLAG-hPTIP (Fig. 3C) and full-length HA-53BP1. Cells were then treated or not with IR, and anti-FLAG immunoprecipitates were probed for HA-53BP1. As shown in Fig. 3D, whereas IR induced the association of full-length hPTIP with 53BP1, deletion of the four C-terminal BRCT domains (fragment 3) of hPTIP prevented its association with 53BP1. Deletion of the third and fourth BRCT domains (fragment 4) also abrogated hPTIP interaction with 53BP1. Although Manke et al. (6) demonstrated that these two extreme C-terminal BRCT domains (fragment 5), immobilized on beads, could pull down 53BP1 from irradiated cell extracts, this fragment could not interact with 53BP1 in cells after IR (Fig. 3D, fragment 5). Instead, all four C-terminal BRCT domains (fragment 6) are required for the IR-induced interaction with 53BP1. The two N-terminal BRCT domains of hPTIP did not interact with 53BP1 (Fig. 3D, fragment 2).

We next examined the ability of the hPTIP fragments to form foci. All but fragment 3, which was both cytoplasmic and nuclear despite the presence of a nuclear localization signal, showed diffuse nuclear staining in the absence of DNA damage. As shown in Fig. 3E, only hPTIP fragments 1, 6, and 7 formed foci after IR, suggesting that all four C-terminal BRCT domains of hPTIP are required for association with sites of DNA damage. The N-terminal BRCT domains (Fig. 3E, fragment 2) and the two extreme C-terminal BRCT domains alone (fragment 5) were unable to form foci. Overexpression of the hPTIP fragments did not affect focus formation by endogenous 53BP1 (Fig. 3E, right panels). Taken together, these data suggested that the four C-terminal domains of hPTIP are required for both focus formation and interaction with 53BP1.

hPTIP Facilitates ATM-mediated p53 Activation and Promotes Cellular Resistance to Ionizing Radiation—To investigate the cellular role of hPTIP, human colorectal HCT-116 cells were transfected with the SI20 and SCR siRNAs described in Fig. 1. Although depletion of hPTIP with SI20 caused cells to grow more slowly, cell viability was not affected up to 144 h after hPTIP had been maximally depleted (data not shown). We next examined the effect of hPTIP depletion on IR-induced
activation of DNA damage signaling pathways. Although deple- 
tion of hPTIP had little effect on IR-induced stabilization 
of p53, there was a significant decrease in phosphorylation of 
P53 on Ser15, the residue phosphorylated by ATM (~2-fold 
increase in phosphorylation 6 h post-IR with SI20 compared 
with 4-fold with SCR; Fig. 4, A and B) (2). This was associ- 
ated with a marked decrease in the induction of the p53 target 
gene, p21. Control cells (SCR) showed a 2.5- and 4-fold in- 
crease in p21 at 3 and 6 h post-IR, respectively, whereas in 
cells with low levels of hPTIP, no induction of p21 was ob- 
served at 3 h and only a 1.5-fold increase was seen 6 h post-IR 
(Fig. 4, A and B). ATM-mediated phosphorylation of CHK2 on 
Thr68 (17) was slightly reduced when hPTIP levels were low, 
whereas IR-induced phosphorylation of CHK1 on Ser345 (18) 
was more pronounced, most notably 6 h post-irradiation (Fig. 
4, A and B). Phosphorylation of p53 at Ser20 mediated by
CHK2 after IR was slightly reduced in cells depleted of hPTIP (a 3.7-fold increase with SCR compared with a 2.7-fold increase with SI20, 6 h post-IR). Phosphorylation of the cohesin subunit SMC1 at Ser966 (19), an ATM target site, was not significantly altered (Fig. 4, A and B).

The effect of reducing hPTIP expression on the ability of cells to survive after DNA damage was assessed using the colony formation assay. As shown in Fig. 4C, cells with reduced levels of hPTIP showed no significant difference from wild-type cells in their ability to proliferate after exposure to a low dose (1 Gy) of IR. However, at higher doses, cells with low levels of hPTIP showed greater sensitivity to IR than wild-type cells with an ~50% decrease in cell survival after 3 Gy IR (Fig. 4C).

DISCUSSION

In this study, we presented the full amino acid sequence of hPTIP that consists of 1069 residues with four BRCT domains at the C terminus and two BRCT domains at the N terminus,
confirmed by Western blot analysis of endogenous hPTIP in cell extracts (Fig. 1). It is unlikely that the hPTIP fragment isolated from *in vitro* translated cDNA pools by Manke et al. (6) (Fig. 1B) corresponds to a hPTIP splice variant, because the ATG encoding Met<sup>13</sup> of full hPTIP does not lie at an intron/exon boundary (data not shown). We showed that double-strand breaks preferentially stimulate the association of endogenous hPTIP with 53BP1 and that this association requires ATM, suggesting a phospho-dependent interaction. Furthermore, phosphatase treatment of hPTIP immunoprecipitates reduced the association of 53BP1 and hPTIP, although 30% 53BP1 remained bound at the highest possible phosphatase concentration. This is probably because of inaccessibility of some of the phosphorylated residues to the phosphatase. The phospho-dependent interaction of hPTIP and 53BP1 after IR is mediated by ATM (Fig. 2A).

Interestingly, the translocation of hPTIP to sites of DNA damage was not affected in cells lacking ATM, also shown previously for 53BP1 (16). These data suggested that the mechanisms regulating the association of 53BP1 and hPTIP are distinct from those regulating their association with sites of DNA damage. This is further supported by the notion that certain domains of 53BP1 that form foci are unable to interact with hPTIP (Fig. 3, A and B) (15). The four C-terminal BRCT domains of hPTIP are required for both focus formation and association with 53BP1. However, it is possible that these domains bind to phosphorylated 53BP1 and also to another distinct protein that promotes the association of hPTIP with sites of DNA damage. It is interesting to note that the three C-terminal domains of *Xenopus* Swift were also shown to mediate its interaction with Smad2 (13). This interaction is important for transforming growth factor-β-induced gene transcription that also depends on the CBP/p300 histone acetyltransferase, and the three C-terminal BRCT domains of Swift possess intrinsic transactivation activity (13). At present, the relevance of transcriptional regulation/chromatin regulation by hPTIP to DNA damage signaling is not clear.

The cellular role of hPTIP was investigated using siRNA conditions that decreased hPTIP levels by ~90%. Although cell viability was not affected under these conditions (even though cells grew more slowly), it is possible that residual hPTIP in these cells might be sufficient to prevent lethality given that mouse Ptp is an essential protein (12). Alternatively, it might be that PTIP is only essential during development and not in somatic cells, as previously described for CHK1 (20). Radiation sensitivity of cells with low levels of hPTIP is accompanied by defects in up-regulation of the p53 target gene, p21, and p53 phosphorylation on Ser<sup>15</sup> (Fig. 4). Other studies have shown that NBS1 (Nijmegen breakage syndrome 1) and BRCA1 are also required for the efficient phosphorylation of both p53 Ser<sup>15</sup> and Chk2 Thr<sup>68</sup> after IR (21, 22). The data presented here suggest that hPTIP is required for phosphorylation of some ATM substrates but not others, although this might be due to the redundancy of hPTIP with other factors (4). It will be interesting to investigate the contribution of the physical association of 53BP1 and hPTIP to the cellular functions of each protein, and this is currently under investigation. It is clear that there are differences in the roles of these proteins in responding to DNA damage because Ptp null mice are not viable (12), whereas 53BP1 null mice live (23). Because defects in p53 regulation are found in the majority of cancers, it will be important to look for hPTIP mutations that affect hPTIP expression and function in human tumors.

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