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Phospho-epitope binding by the BRCT domains of hPTIP controls multiple aspects of the cellular response to DNA damage

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ABSTRACT

Human (h)PTIP plays important but poorly understood roles in cellular responses to DNA damage. hPTIP interacts with 53BP1 tumour suppressor but only when 53BP1 is phosphorylated by ATM after DNA damage although the mechanism(s) and significance of the interaction of these two proteins are unclear. Here, we pinpoint a single ATM-phosphorylated residue in 53BP1—Ser25—that is required for binding of 53BP1 to hPTIP. Binding of phospho-Ser25 to hPTIP *in vitro* and *in vivo* requires two closely apposed pairs of BRCT domains at the C-terminus of hPTIP and neither pair alone can bind to phospho-Ser25, even though one of these BRCT pairs in isolation can bind to other ATM-phosphorylated epitopes. Mutations in 53BP1 and in hPTIP that prevent the interaction of the two proteins, render cells hypersensitive to DNA damage and weaken ATM signalling. The C-terminal BRCT domains of hPTIP are also required for stable retention of hPTIP at sites of DNA damage but this appears to be independent of binding to 53BP1. Thus, the BRCT domains of hPTIP play important roles in the cellular response to DNA damage.

INTRODUCTION

PTIP (Pax2 transactivation domain-interacting protein) is a key regulator of cellular responses to DNA damage that is vitally important for cell and organism function. It was originally identified in mice in a two-hybrid screen with the Pax2 transcription factor that regulates embryonic development (1). PTIP null embryos do not recapitulate the phenotype of mice lacking Pax2, but instead show very high levels of DNA damage and embryos die at day E8.5 because the DNA damage sustained in S-phase causes a mitotic block (2). These observations suggested that PTIP plays an important role in regulating genome stability.

Human (h)PTIP is required for survival of cells exposed to ionizing radiation (IR) (2,3). hPTIP binds to sites of DNA damage (3,4) and appears to function as an 'adaptor' protein in that it is required for IR-induced phosphorylation of a subset of targets of the ATM (ataxia telangiectasia-mutated) protein kinase (3). ATM is a key regulator of cellular responses to double-stand breaks (DSBs). Binding of ATM to sites of DNA damage appears to stimulate ATM kinase activity (5), leading to phosphorylation of target proteins at Ser–Gln or Thr–Gln (S/T–Q) motifs (5–8). For example, ATM, and the related kinase ATR, phosphorylate Ser129 of the core histone variant H2AX at sites of DNA damage (9–11), and phospho-H2AX in turn acts as a platform for the recruitment of proteins that are needed to signal and repair DNA damage. The MDC1 protein, for example, has a single pair of BRCT domains that bind to phospho-Ser129 of H2AX, thereby recruiting MDC1 and associated proteins to sites of DNA damage (12). BRCT domains, mostly found in pairs, are small modules of ~100 amino acids (13) that mediate protein–protein interactions. In some cases these domains recognize phosphorylated epitopes on target proteins (4,14).

hPTIP has a pair of BRCT domains at the N-terminus, and another two pairs at the C-terminus (Figure 2A). The most extreme C-terminal pair alone was shown *in vitro* to bind to a library of phospho-peptides based on the S/T-Q consensus sequence for phosphorylation by ATM. The optimal sequence bound by this pair of BRCT domains *in vitro* was found to be pS/T-Q-V-F (4). hPTIP was shown to interact with 53BP1 after exposure of cells to IR (3,4). 53BP1 is also an 'adaptor' protein for ATM (15,16) and mice lacking 53BP1 are tumour prone and are hypersensitive to IR (17–19). It was recently shown that 53BP1 is down-regulated in transition from pre-cancerous stage to carcinomas (20), and loss of a single 53BP1 allele in mice causes genome instability

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and lymphoma (18). Cells lacking 53BP1 show mild cell cycle checkpoint defects (16,17,21) and a pronounced defect in the repair of a subset of DNA breaks by non-homologous end joining (NHEJ) (22–24).

Intriguingly, the interaction of hPTIP and 53BP1 after DNA damage requires ATM-dependent phosphorylation of 53BP1 (3,4). Translocation of these proteins to sites of DNA damage does not require ATM, however, and is therefore independent of their physical association (3). Although it was implied that binding of a phosphorylated residue in 53BP1 that lies in the pS/T-Q-V-F motif, to the most C-terminal BRCT domain pair in hPTIP explained how these proteins interacted (4), this was not tested. Thus, the mechanism(s) of interaction of hPTIP and 53BP1 or the significance of their interaction for the DNA damage response is not yet known. In this study, we address these questions.

MATERIALS AND METHODS

Cell lines and treatments

HEK 293 and U2OS cells were grown in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum (FBS, HyClone) and penicillin/streptomycin. Cells were kept at 37°C in a 5% CO_2 atmosphere. Co-immunoprecipitation of FLAG-hPTIP and HA-53BP1 proteins, SDS–PAGE and western blot analysis were carried out as described previously (3).

Antibodies and peptides

The primary antibodies used in this study were: anti-HA (12CA5; Roche), anti-FLAG (M2; Sigma), anti-53BP1, anti-phospho Ser1524 BRCA1 (Bethyl Laboratories), anti-phospho Thr68 Chk2, anti-Chk2 (Cell Signaling Technologies) and anti-BRCA1 (Oncogene Research Products). Antibodies against hPTIP were described previously (3). All peptides were synthesized by Dr Graham Bloomberg, University of Bristol.

Plasmids, transfections, small interfering (si)RNA and mutagenesis

Full-length 53BP1 was amplified from plasmid pCMH6K-53BP1 (24) with an N-terminal HA tag, sub-cloned into pCR2.1 and cloned into the KpnI and SalI sites of pCMV5. To create the 53BP1 phospho-site mutants shown in Figure 1, up to five mutations in 53BP1 at a time were introduced into 53BP1 using the QuikChange Multi-Site mutagenesis kit (Stratagene) and PCR reactions were spiked with *Pfu* Ultra DNA polymerase (Stratagene). All plasmids were checked carefully by sequencing the entire insert forwards and backwards.

Plasmids expressing hPTIP were described previously (3). pCMV5-based plasmids were transfected into HEK293 cells using calcium phosphate. In the siRNA/rescue experiments, 53BP1 siRNA duplexes that recognize nucleotides 84–104 in human 53BP1 (AAGCCA GTTCTAGAGGATGA), or scrambled (SCR) duplexes (100 nM) were co-transfected into cells with 53BP1 siMUT plasmids (0.5 μg) in which 53BP1 bore a mutation at nucleotide T93 that did not affect the coding sequence but made 53BP1 refractory to silencing by siRNA. The calcium phosphate method of transfection of HEK293 cells was used for RNA interference and rescue because this allowed transfection efficiencies of >90% (data not shown). hPTIP depletion by siRNA duplexes was described previously (3).

Protein expression and peptide pulldowns

The hPTIP BRCT domains were amplified from pCMV5-hPTIP (3) and cloned into the BamHI site of pGEX6P3. Proteins expression in *Escherichia coli* BL21 (DE3)-RIL cells (Stratagene) was induced by addition of IPTG at 10 μM, when cells were at OD_{600} 0.6, at 16°C for 16 h. Cells were lysed in 50 mM Tris/HCl, pH 7.4 containing 1% (w/v) Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine and 0.2 mM phenyl methyl sulphonate. After sonication of cells and centrifugation to remove cell debris, proteins were purified on glutathione-sepharose (AP Biotech) and eluted with a gradient of reduced glutathione (Sigma). Proteins were dialysed free of glutathione and stored frozen at −80°C.

In each peptide pulldown, 3 μg of each peptide [Non(P), biotin-DTPCLIIEDSQPEQVLEDD; Ser25(P), biotin-DTPCLIIEDpSQPESQVLEDD; Ser29(P), biotin-DTPCLIIEDSQPEpSQVLEDD; Ser25(P)/Ser29(P), biotin-DTPCLIIEDpSQPEpSQVLEDD; Rad53(P), biotin-MENIpTQPpTQQSTQAT; pS—phosphoserine, pT—phosphothreonine] immobilized on 10 μl of streptavidin-Dynabeads (Invitrogen) was incubated with 10 μg of recombinant protein in 50 mM Tris/HCl, pH 7.4, 0.15 M NaCl and 0.1% 2-mercaptoethanol for 60 min at room temperature. Beads were washed three times in incubation buffer before bound proteins were eluted with boiling LDS sample buffer and subjected to SDS–PAGE on 4–12% Bis–Tris gels and Coomassie staining.

BiaCore analysis

Binding was analysed in a BiaCore 3000 system. The relevant biotinylated peptides were bound to an SA sensor chip (GE Healthcare). The indicated concentrations of bacterially expressed wild-type and mutant forms of GST–hPTIP (590–1069) (BRCT pairs C1 + C2) in HBS-EP [HEPES-buffered saline with EDTA and polysorbate 20; 10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% (v/v) polysorbate 20], were injected over the immobilized peptides at a flow rate of 90 μl/min. Interactions between each peptide and GST–hPTIP pair C1 + C2 (amino acids 590–1069) were analysed and steady-state binding was determined at each concentration. Dissociation of GST–hPTIP pair C1 + C2 forms from each peptide was monitored over 90 s. Regeneration of the sensor chip surface between each injection was performed with three consecutive 5 μl injections of a solution containing 50 mM NaOH and 1 M NaCl.

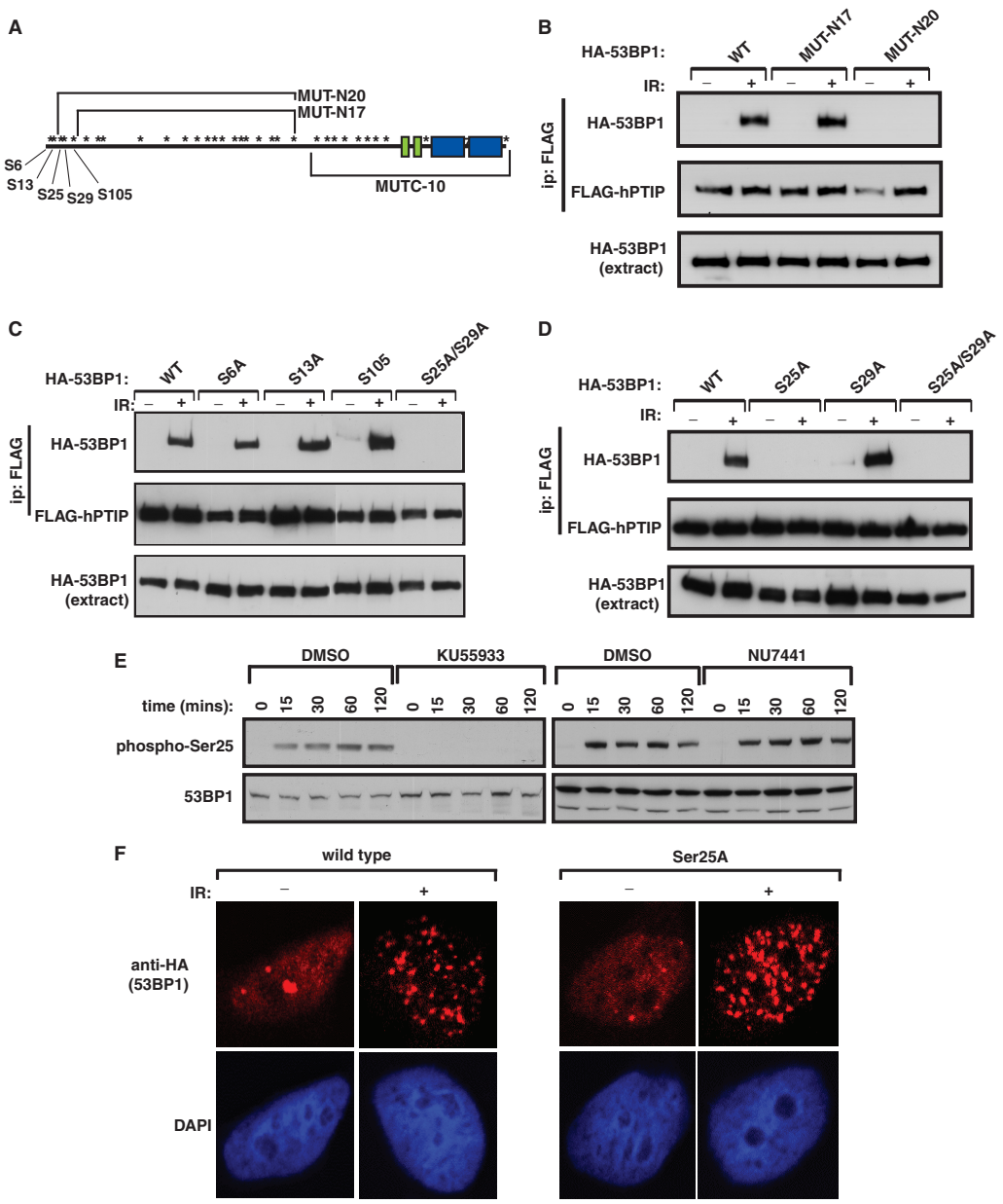

Figure 1. Identification of Ser25 of 53BP1 as the ATM-phosphorylated residue required for interaction with hPTIP after IR. (**A**) Schematic diagram of full-length 53BP1. Asterisks represent S/T-Q motifs. The TUDOR domains and BRCT domains of 53BP1 are shown in green and blue, respectively. In MUT-C10, the serine or threonine residue in each of the 10 most C-terminal S/T-Q motifs were mutated to alanine. In MUT-N17, the 17 S/T-Q motifs immediately upstream of those in MUT-C10 were mutated. In MUT-N20, a further three S/T-Q motifs were mutated in addition to those in MUT-N17. (**B**–**D**) HEK293 cells were co-transfected with FLAG-hPTIP and full-length HA-53BP1 bearing the indicated mutations. Cells were exposed to IR (0 Gy or 20 Gy) and after cell lysis anti-FLAG immunoprecipitates were subjected to SDS–PAGE and western blotting with the indicated antibodies. The lowest panel in each case shows HA-53BP1 levels in cell extracts. (**E**) HEK293 cells were pre-incubated with DMSO vehicle or with 10 μM KU55933 or 10 μM NU7441 for 1 h before exposure to IR (10 Gy) and then allowed to recover for the times indicated. Cells were lysed in LDS sample and extracts subjected to SDS–PAGE followed by western blot analysis with the indicated antibody. (**F**) HEK293 cells grown on 13-mm-diameter glass coverslips were transfected with full-length HA-53BP1: wild-type (WT) or the Ser25Ala mutant and then treated with 0 or 10 Gy IR and left to recover at 37°C for 45 min before fixation and permeabilization as described previously (3). Cells were washed and blocked before incubation with anti-HA (to detect transfected 53BP1).

RESULTS

Phosphorylation of 53BP1 at Ser25 is required for its interaction with hPTIP

We previously reported that hPTIP interacts with 53BP1 only after DNA damage and that this requires phosphorylation of 53BP1 by ATM. 53BP1 has a total of 32 ATM consensus motifs (S/T-Q) (Figure 1A). The Ser/Thr residues each of these motifs was mutated to alanine and the resulting mutants were transfected into HEK293 cells and tested for interaction with co-transfected with FLAG-hPTIP. Mutation of the

10 most C-terminal S/T-Q motifs in 53BP1 (termed MUT-C10; Figure 1A) or mutation of a combination of 17 S/T-Q motifs upstream of the MUT-C10 mutations (termed MUT-N17; Figure 1A) did not affect its interaction with hPTIP after exposure of cells to IR (Figure 1B). However, mutation of an additional three residues Ser25, Ser29 and Ser105 (termed MUT-N20), abolished the IR-inducible interaction of 53BP1 with hPTIP (Figure 1B). Whereas mutation of Ser29, Ser105, Ser6 or Ser13, singly had no effect, mutation of Ser25 of 53BP1 abolished the IR-induced interaction of 53BP1 and hPTIP (Figure 1C and D). Thus, Ser25 in 53BP1 is essential for the interaction of 53BP1 with hPTIP. It was reported previously that 53BP1 is phosphorylated at Ser25 by ATM (21). Consistent with this, we observed that Ser25 is phosphorylated after exposure of HEK 293 cells to IR, but not in cells pre-incubated with the ATM-specific inhibitor KU55933 (25) (Figure 1E). Pre-incubation of cells with NU7441 (26), a specific inhibitor of DNA-dependent protein kinase (Figure 1E), or ablation of ATR expression (27) (data not shown), did not affect IR-induced phosphorylation of 53BP1 Ser25. The 53BP1 Ser25Ala mutant was retained at sites of DNA damage in a manner indistinguishable from the wild-type protein (21) (Figure 1F; data not shown). Therefore, the involvement of Ser25 phosphorylation in promoting the interaction of 53BP1 and hPTIP is independent of translocation of 53BP1 to sites of DNA damage. This is consistent with previous reports that ATM is not required for formation of IR-induced nuclear foci by 53BP1 or hPTIP (3,28). The data in this section demonstrate that Ser25 that is phosphorylated by ATM is essential for the interaction of 53BP1 with hPTIP after DNA damage and that this is independent of 53BP1 translocation to IR-induced foci.

Two pairs of hPTIP BRCT domains are required for binding to 53BP1 phospho-Ser25 *in vitro* and *in vivo*

We tested if phosphorylated Ser25 of 53BP1 could bind directly to hPTIP. Two peptides corresponding to the sequence around Ser25, in which Ser25 is phosphorylated or not, were synthesized with a biotin moiety at the N-terminal end. As a test for specificity, a peptide in which nearby Ser29 is phosphorylated, and the Ser25/Ser29 diphospho-peptide, were also synthesized. Peptides were immobilized on streptavidin-coated magnetic beads and incubated with cell extracts. As shown in Figure 2B, endogenous hPTIP was retrieved by the phospho-Ser25 peptide and phospho-Ser25/phospho-Ser29 peptide but not by the unphosphorylated peptide or the phospho-Ser29 peptide or by an unrelated diphospho-peptide from yeast Rad53. Similar results were obtained with full-length FLAG-hPTIP transfected into cells, and with a transfected fragment of hPTIP (amino acids 590–1069) (Figure 2B), corresponding to the two C-terminal pairs of BRCT domains (pair C1 + pair C2; Figure 2A), that we previously showed were necessary and sufficient for hPTIP to interact with 53BP1 after DNA damage in cells (3). These data demonstrate that hPTIP interacts with phosphorylated Ser25 of 53BP1 and that this is required for the inducible interaction of the two proteins.

We assumed that just one of the two C-terminal pairs of hPTIP BRCT domains is responsible for binding phospho-Ser25. To test which of the two pairs was responsible, the two isolated pairs or both pairs together were expressed in bacteria as GST fusion proteins. Neither the first (pair C1) nor the second (pair C2) pair of BRCT domains in isolation bound to the Ser25 phospho-peptide whereas both domains together (pair C1 + pair C2) bound efficiently (Figure 2C). BiaCore analysis demonstrated that the dissociation constant (K_d) for binding of BRCT pair C1 + pair C2 to the phospho-Ser25 peptide was ~526 nM, whereas the K_d for binding to the non-phospho-peptide was >1000 μM, demonstrating a high degree of phospho-specificity (Figure 3A; data not shown). The K_d of ~526 nM is in good agreement with other physiological BRCT–phosphoepitope interactions; the K_d for Ser129 of H2AX and the BRCA1 BRCT domain pair was reported to be 2.2 μM (12). BiaCore measurements showed no detectable binding of hPTIP BRCT pairs C1 or C2 in isolation to the phospho-Ser25 peptide (Figure 3B).

The requirement for two BRCT domain pairs for recognition of phospho-Ser25 was surprising because in all reported cases of phospho-epitope recognition by BRCT domains, a single pair is sufficient and also because it was reported that the BRCT domain pair C2 of hPTIP alone bound to a library of degenerate pS/T-Q phosphopeptides. The optimal peptide used in these experiments by Yaffe and colleagues (4) was biotin-GAAYDI-pSQ-VFPFAKKK. In agreement with these data, even though hPTIP BRCT domain pair C2 did not bind to 53BP1 phospho-Ser25, it bound to this phospho-peptide (termed 'S-Q-V-F peptide') with a K_d of ~273 nM (data not shown), and not to the unphosphorylated version of this peptide (Figure 2D). These data taken together suggest that BRCT pair C1 + C2 of hPTIP are capable two modes of phospho-epitope recognition: one specific for pS-Q-V-F peptides mediated solely by pair C2, and another mode that is responsible for binding phospho-Ser25 that needs both pair C1 + pair C2.

To confirm the requirement for two separate pairs of BRCT domains in hPTIP to bind phospho-53BP1, the effect of mutation of conserved residues in these domains was determined. Inspection of the amino acid sequence of hPTIP revealed that BRCT domain pair C2 contains a small conserved motif that in other proteins recognizes phospho-peptides. MDC1 and BRCA1 each have a single pair of BRCT domains that interact with phospho-peptides (12,14). Structural analyses showed that Arg1933 of MDC1 and Arg1699 of BRCA1 coordinate phospho-Ser and are essential for phospho-peptide binding (12,29,30). Alignment of each of the two C-terminal pairs of BRCT domains from hPTIP, individually, with the BRCT domain pairs from MDC1 and BRCA1 revealed clear conservation of residues involved in phospho-specific binding in the second pair, but not in the first pair, of hPTIP C-terminal BRCT domains (Figure S1). Arg910 in BRCT pair C2 of hPTIP appears to be equivalent to Arg1699 of BRCA1 and Arg1933 of MDC1 (Figure S1). BiaCore measurements showed that whereas pair C1 + C2 bound to the 53BP1 phospho-Ser25

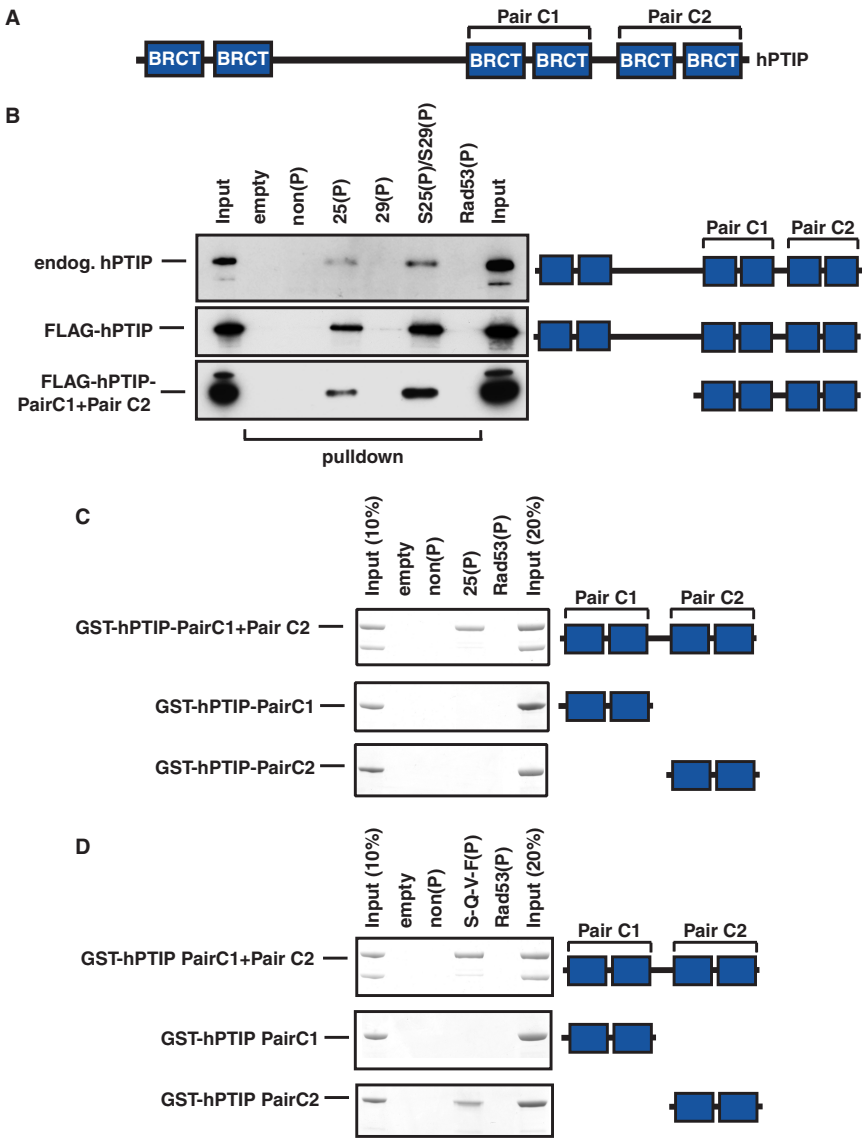

Figure 2. Both C-terminal BRCT domains of hPTIP mediate binding to phospho-Ser25 of 53BP1. (**A**) Schematic diagram of hPTIP. BRCT domains are shown as blue boxes. (**B**) Extracts from untransfected HEK 293 cells (top panel; 4 mg of extract protein), or cells transfected with full-length FLAG-hPTIP (middle panel; 100 µg of extract protein) or with FLAG-hPTIP (590–1069; 100 µg of extract protein) corresponding to the two C-terminal BRCT domains of hPTIP ('Pair C1 + Pair C2'; bottom panel) were incubated with Dynabeads conjugated to the indicated peptides, corresponding to the sequence around Ser25 and Ser29 of 53BP1. The peptides used were: Empty, no peptide; Non(P), biotin-DTPCLIIEDSQPEQVLEDD; Ser25(P), biotin-DTPCLIIEDpSQPESQVLEDD; Ser29(P), biotin-DTPCLIIEDSQPEpSQVLEDD; Ser25(P)/Ser29(P), biotin-DTPCLIIEDpSQPEpSQVLEDD; Rad53(P), biotin-MENIpTQPpTQQSTQAT. (pS, phosphoserine, pT, phosphothreonine). After extensive washing, beads were subjected to SDS–PAGE and western blot analysis with the indicated antibodies. (**C**) Bacterially expressed GST-hPTIP (590–1069) (BRCT pairs C1 + C2), GST-hPTIP (590–800) (BRCT pair C1) and GST-hPTIP (862–1069) (BRCT pair C2) were incubated with Dynabeads conjugated to the indicated peptides (Figure 2B). After extensive washing, beads were subjected to SDS–PAGE and gels were stained with Coomassie brilliant blue. (**D**) Same as C. except that Non(P) refers to the peptide biotin-GAAYDI-SQ-VFPFAKKK while S-Q-V-F (P) refers to the peptide biotin-GAAYDI-pSQ-VFPFAKKK (4). Rad53(P) is described in (B).

peptide with a K_d of ~526 nM, mutation of Arg910 (R910Q) severely reduced binding of BRCT pair C1 + pair C2 of hPTIP to phospho-Ser25 of 53BP1 so that the K_d was so high that it was difficult to determine (Figure 4A). Almost all BRCT domains have a conserved Trp on the α3 helix (13) that corresponds to Trp676 in hPTIP BRCT pair C1 and Trp929 in pair C2 (Figure S1). Mutation of either Trp676 (W676A) or Trp929 (W929A) to alanine severely reduced binding to Ser25 phospho-peptide and in each case the K_d was >1000 µM (Figure 4A). As shown in Figure 4B, mutation of Trp676, Arg910 or Trp929 abolished binding of hPTIP to 53BP1 in IR-treated cells *in vivo*. These data are consistent with the requirement for two pairs of BRCT domains for hPTIP to recognize phospho-Ser25 of 53BP1.

A previous report showed that hPTIP BRCT pair C2 alone fused to GST could retrieve phosphorylated 53BP1 from extracts of irradiated cells (4). However, the data

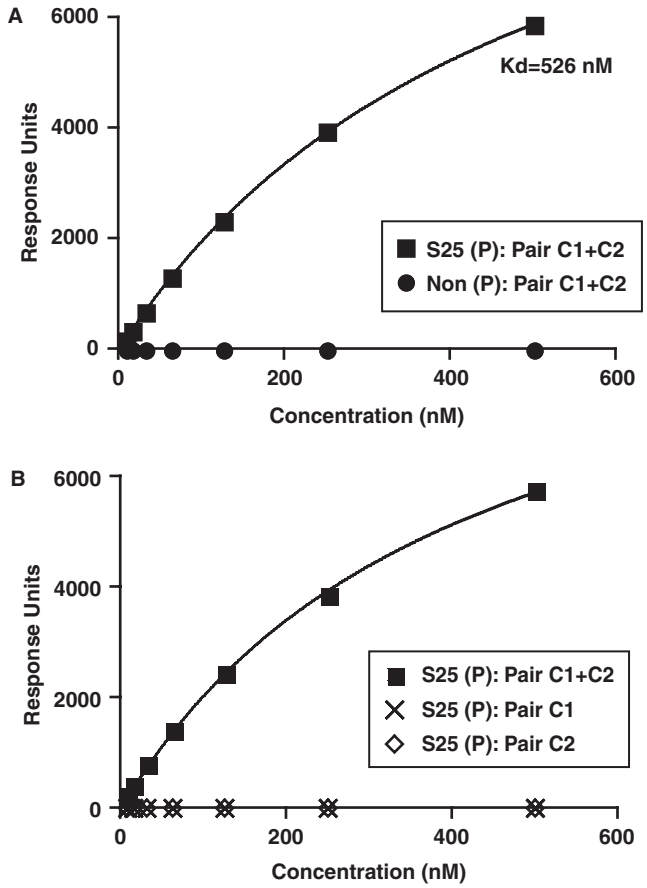

Figure 3. BiaCore analysis of binding of hPTIP BRCT domain pairs C1 + C2 to 53BP1 phospho-Ser25. (**A**) The binding of bacterially expressed hPTIP (590–1069) (BRCT pairs C1 + C2) to biotinylated 53BP1 peptides (Non(P), biotin-DTPCLIIEDSQPEQVLEDD; Ser25(P), biotin-DTPCLIIEDpSQPESQVLEDD) was analysed using BiaCore as described in the Materials and Methods section. The analysis was performed over a range of protein concentrations (7.8–500 nM) and the response level at steady state was plotted against protein concentration. The K_d values were calculated by fitting the data to the equation $y = B_{max} \cdot x/(K_d + x)$ using GraphPad 4 software, which describes the binding of a ligand to a receptor that follows the law of mass action. B_{max} is the maximal binding and K_d is the concentration of ligand required to reach half-maximal binding, whereas x and y correspond to the protein concentration and the response units, respectively. (**B**) Same as A., except that binding of hPTIP BRCT domain pair C1, C2 and pair C1 + pair C2 to the 53BP1 phospho-Ser25 peptide was compared.

presented in this study indicate that two hPTIP BRCT pairs are required to bind to phospho-53BP1, and this discrepancy was investigated. Protein fragments corresponding to the hPTIP BRCT domains were expressed as GST fusions in bacteria, immobilized and incubated with extracts of cells exposed, or not, to IR. As shown in Figure 4C, BRCT Pairs C1 + C2 of hPTIP retrieved 53BP1 from extracts of irradiated cells much more efficiently than from un-irradiated cells. The isolated BRCT pair C2 of BRCT domains also retrieved 53BP1 from extracts of irradiated cells but with much lower efficiency than hPTIP BRCT pairs C1 + C2 (Figure 4C), showing that pair C1 is important. Retrieval of 53BP1 from extracts by GST-hPTIP (590–1069) was prevented by mutation of the W676A mutation in pair C1 (Figure 4C). Therefore, BRCT pair C2 of hPTIP only binds very weakly to phospho-53BP1 in cell extracts compared with a combination of both C-terminal pairs of hPTIP BRCT domains. These data are consistent with our previous finding that both C-terminal pairs of hPTIP BRCT domains are necessary and sufficient to interact with 53BP1 in cells after DNA damage *in vivo* (3).

The interaction of 53BP1 and hPTIP is not required for recruitment of hPTIP to sites of DNA damage

We previously showed that both C-terminal pairs of BRCT domains in hPTIP are necessary and sufficient for the formation of nuclear foci by hPTIP after DNA damage (3). Since both these domains in hPTIP are also required for binding to phospho-Ser25 of 53BP1, we investigated if binding of the C-terminal domains of hPTIP is required for stable retention of hPTIP at sites of DNA damage. U2OS cells were transiently transfected with plasmids expressing wild-type FLAG-hPTIP and hPTIP bearing the following mutations: Trp676Ala (W676A), Arg910Gln (R910Q) or Trp929Ala (W929A). As shown in Figure 5A, formation of foci by endogenous 53BP1 was normal in all irradiated cells: this was used as an internal control for focus formation. Wild-type hPTIP formed nuclear foci after exposure of cells to IR (Figure 5A): almost all cells had >50 foci under these conditions, whereas most cells had <10 foci in untreated cells (Figure 5B). In contrast, mutation of Trp676 or Trp929 prevented focus formation by hPTIP (Figure 5A); very few cells had >50 foci after IR, and almost all cells had <10 (Figure 5B). Mutation of Arg910 had little effect on the ability of hPTIP to form foci after IR and this mutant was difficult to distinguish from wild-type hPTIP in this regard (Figure 5A and B). However, all three of these mutations—Trp676Ala, Trp929Ala and Arg910Gln—prevent the association of hPTIP with phospho-Ser25 of 53BP1 *in vitro* and *in vivo* (Figure 4B). Therefore, the two C-terminal BRCT domains of hPTIP are essential for binding to sites of DNA damage but it appears that this is independent of binding to phosphorylated Ser25 of 53BP1. This is consistent with our previous finding that ATM,which catalyses Ser25 phosphorylation, is not required for hPTIP to form nuclear foci after DNA damage (3).

The interaction of 53BP1 with hPTIP is required for an intact DNA damage response

We next wished to test if the interaction of 53BP1 and hPTIP has important responses to DNA damage and we first tested the effects of mutation of Ser25 of 53BP1. Transfection of HEK293 cells with a siRNA duplex to silence 53BP1 (16), but not with a scrambled (SCR) version of this duplex, caused a dramatic reduction in 53BP1 protein levels (Figure 6A). An assay to allow expression of ectopic HA-tagged 53BP1was then established to allow complementation analysis. This involved making a mutation in (HA-tagged) 53BP1 cDNA on a plasmid at a point (T93) in the target sequence recognized

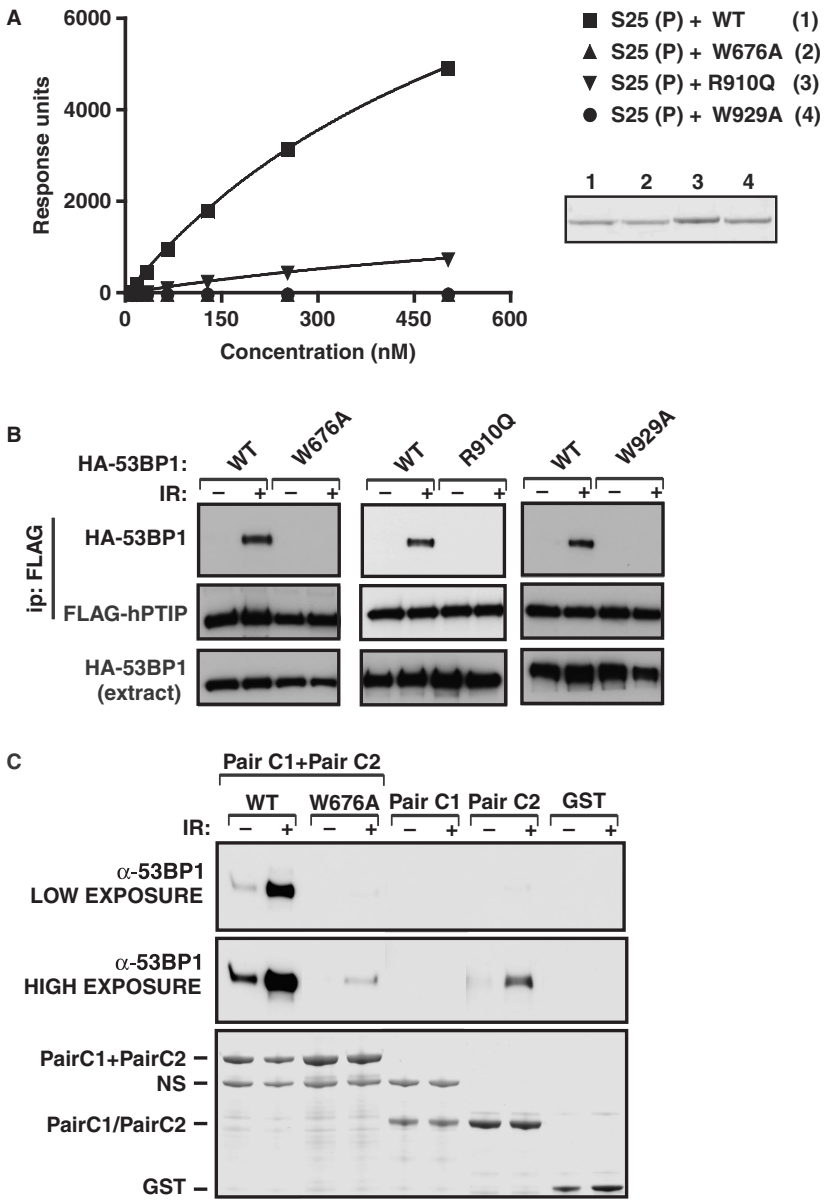

Figure 4. Effect of mutation of conserved residues in the hPTIP BRCT domains on binding to phospho-53BP1 (**A**) The binding of bacterially expressed hPTIP-BRCT pair C1 + C2 proteins: wild type, W676A, R910Q and W929A to the 53BP1 phospho-Ser25 peptide (biotin-DTPCLIIEDpSQPESQVLEDD) was analysed using BiaCore as described in the Materials and Methods section and in the legend to Figure 3A. The inset shows a Coomassie-stained gel of the GST fusion proteins used in this analysis. (**B**) HEK293 cells were co-transfected with full-length HA-53BP1 and FLAG-hPTIP bearing the indicated mutations. Cells were exposed to IR (0 Gy or 20 Gy) and after cell lysis anti-FLAG immunoprecipitates were subjected to SDS–PAGE and western blotting with the indicated antibodies. The lowest panel in each case shows HA-53BP1 levels in cell extracts. (**C**) Bacterially expressed versions of GST-hPTIP-BRCT pair C1 + C2: wild-type (WT) and Trp676Ala (W676A), GST-hPTIP-BRCT pair C1 and GST-hPTIP-BRCT pair C2, or GST alone were immobilized on glutathione-sepharose and incubated with extracts of HEK 293 cells that had been exposed, or not, to 10 Gy IR. After extensive washing, beads were subjected to SDS–PAGE and western blot analysis with antibodies against 53BP1. Low and high exposures of the blot are shown in the top and middle panels, respectively and the bottom panel shows a Coomassie-stained gel of the GST-fusions used in this experiment. NS, non-specific.

by the siRNA duplex, to render HA-53BP1 refractory to siRNA-mediated silencing. Conditions were established so that HA-53BP1 or HA-53BP1 in which Ser25 was mutated to Ala were expressed at close to endogenous levels, judged by immunoblotting with anti-53BP1 antibodies (Figure 6A). Immunoprecipitation with anti-HA antibody fully depleted 53BP1 from extracts of cells co-transfected with 53BP1 siRNA and HA-53BP1 (data not shown). This showed that all of the 53BP1 expressed under these conditions corresponded to exogenous 53BP1.

53BP1 is required for ATM-dependent phosphorylation of BRCA1 and CHK2 at low doses of radiation (16,17,21). Consistent with these reports, depletion of 53BP1 caused a severe reduction in phosphorylation of Chk2 at Thr68 and phosphorylation of BRCA1 at Ser1524 (Figure 6A). Whereas wild-type 53BP1 could rescue IR-induced

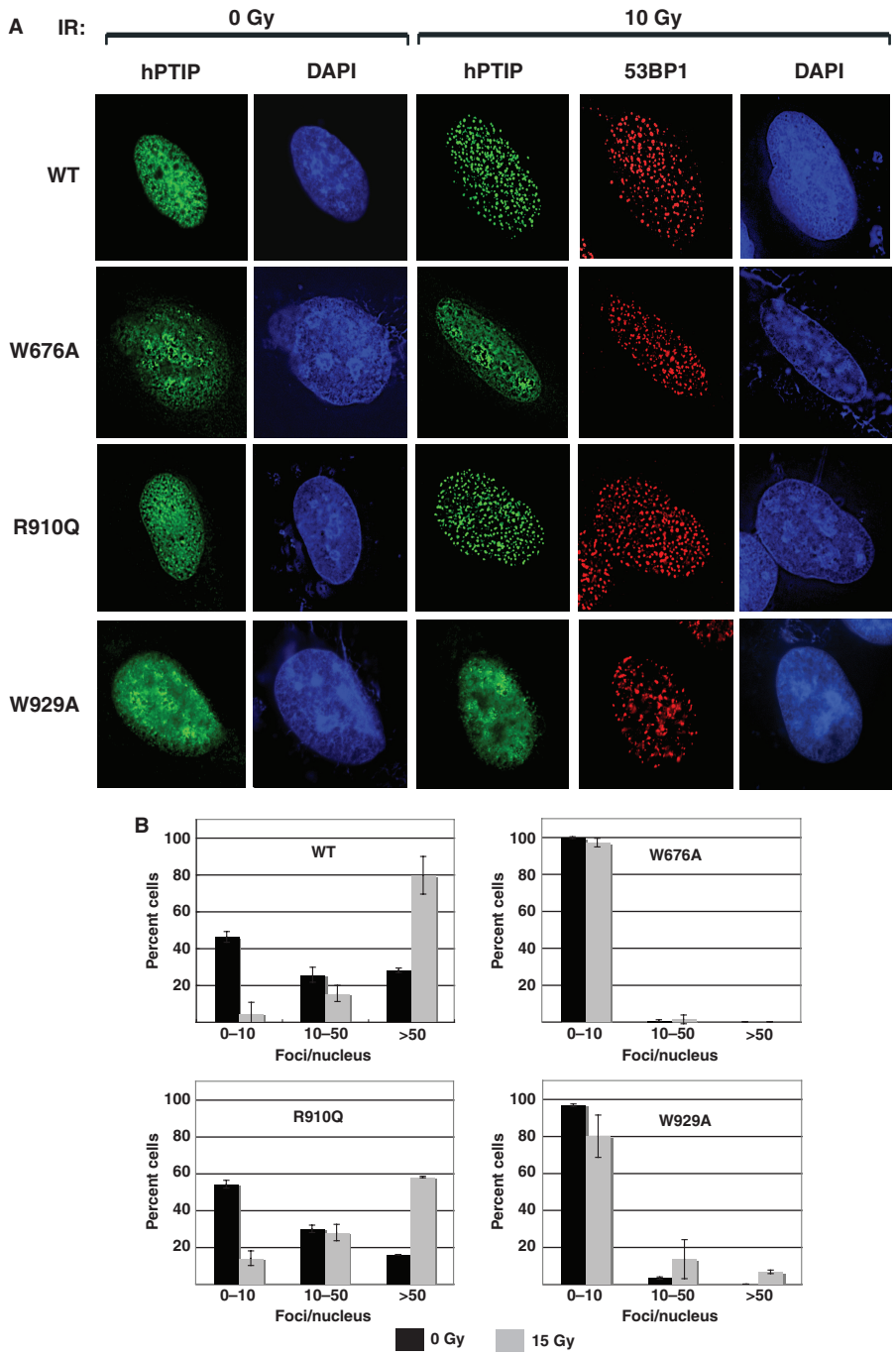

Figure 5. Interaction of 53BP1 with hPTIP is not required for stable retention of hPTIP at sites of DNA damage. (**A**) U2OS cells grown on 13-mm-diameter glass coverslips were transfected with full-length FLAG-hPTIP: wild-type (WT) or the W676A, R910Q or W929A mutants and then treated with 0 or 15 Gy IR and left to recover at 37°C for 45 min before fixation and permeabilization as described previously (3). Cells were washed and blocked before incubation with anti-FLAG (to detect transfected hPTIP) or with 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 Alexa488 or Alexa 594 (Jackson Laboratories) for 60 min. After thorough washing in PBS-T, coverslips were mounted on glass slides and images were acquired using an Olympus IX70 microscope. (**B**) The number of PTIP foci formed before and after exposure of cells to IR, in (A), was quantitated by counting the number of foci observed with anti-FLAG antibody in ~200 transfected cells. The mean number of foci ± SD are presented.

phosphorylation of Chk2 and 53BP1, mutation of Ser25 to Ala prevented this. We next looked at the effect of mutation of Ser25 on cellular resistance to IR. Depletion of 53BP1 rendered cells hypersensitive to IR (Figure 6B), consistent with previous reports (17,22). Treatment of 53BP1-depleted cells with 3 Gy of IR caused an ~90% decrease in cell viability, in contrast with an almost 40% decrease in viability in cells treated with a scrambled (SCR) RNA duplex (Figure 6B). 53BP1 in which Ser25 was mutated to alanine was unable to rescue this defect

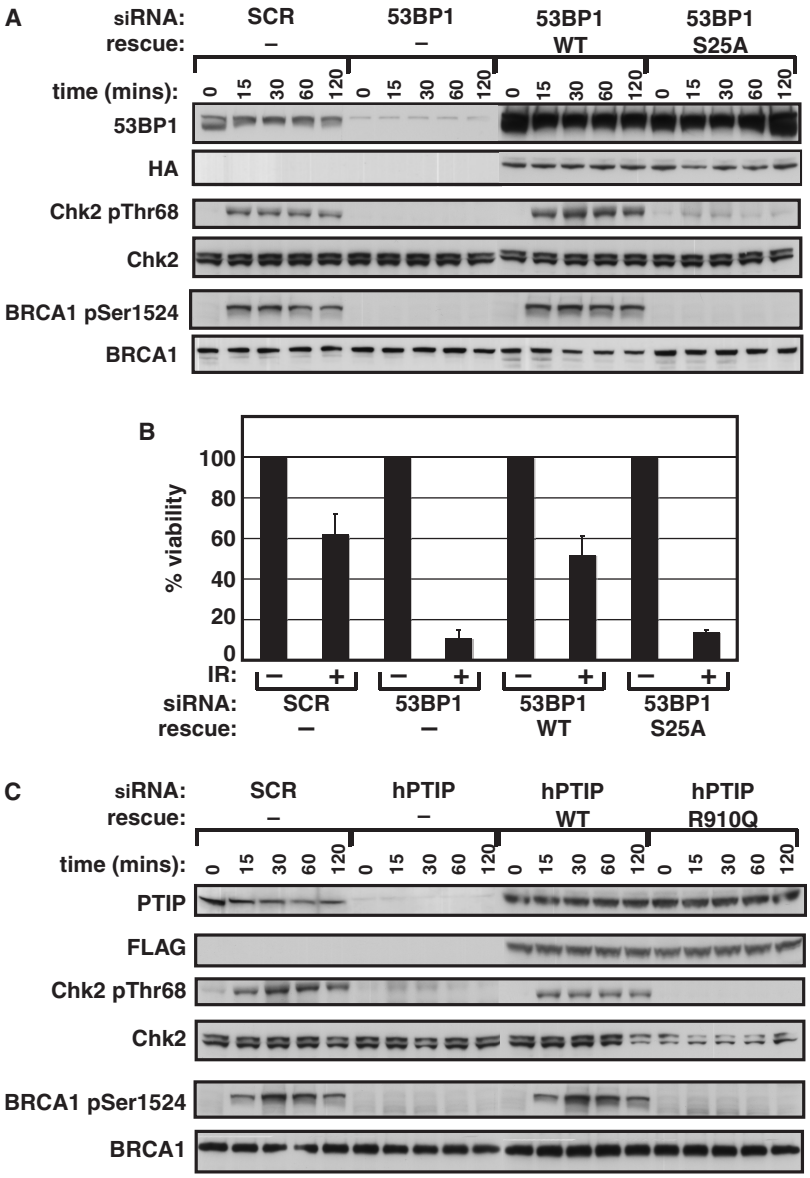

Figure 6. The interaction of 53BP1 with hPTIP is required for an intact cellular response to DNA damage. (**A**) HEK293 cells were co-transfected with siRNA to silence 53BP1 or a scrambled RNA duplex (SCR), and with various plasmids: pCMV5 (–), pCMV5-HA-53BP1 carrying a silent mutation (see Materials and Methods section) that renders HA-53BP1 refractory to silencing by siRNA (WT), or pCMV5-HA-53BP1 with the same mutation but with Ser25 mutated to alanine (S25A). Cells were exposed to IR (3 Gy) and allowed to recover for the indicated times before lysis. Extracts were subjected to SDS–PAGE and western blotting with the indicated antibodies. (**B**) Same as (A) except that cells were seeded at different dilutions, in triplicate in 10-cm culture dishes and irradiated with 0 or 3 Gy IR. Before plating, the viability of the cells was assessed during counting by a dye exclusion test with trypan blue. After 14 days, colonies were fixed with methanol, stained with crystal violet and were counted if they consisted of more than 50 cells. The fraction of cells surviving irradiation was normalized to the surviving fraction of the corresponding control. (**C**) Same as (A) except that cells were co-transfected with siRNA to silence hPTIP (3) or a scrambled RNA duplex (SCR), and with various plasmids: pCMV5 (–), pCMV5-FLAG-hPTIP carrying a silent mutation that renders hPTIP refractory to silencing by siRNA (WT), or pCMV5-FLAG-hPTIP with Arg910 mutated to glutamine (R910Q).

whereas wild-type 53BP1 restored cellular resistance to IR (Figure 6B). These data show that Ser25 of 53BP1, that mediates the inducible interaction of 53BP1 with hPTIP, is required for 53BP1 to act as an adaptor protein for ATM and for cellular resistance to DSBs.

Mutation of Arg910 in hPTIP BRCT pair C2 prevents binding of hPTIP to phospho-Ser25 of 53BP1 *in vitro* and prevents binding of hPTIP to 53BP1after DNA damage in cells, but does not affect hPTIP focus formation. This mutation is therefore useful for probing the consequences of the interaction of hPTIP with 53BP1 phospho-Ser25. hPTIP-specific RNAi was carried out in HEK293 cells and this, like depletion of 53BP1, resulted in a dramatic reduction in the IR-induced phosphorylation of Chk2 at Thr68 and of BRCA1 at Ser1524 (Figure 6C). Whereas wild-type FLAG-hPTIP could fully rescue these defects, the Arg910Gln (R910Q) mutant could not. Therefore, Arg910 of hPTIP BRCT pair C2 is required

for an intact response to DNA damage. These data strongly suggest that recognition of 53BP1 phospho-Ser25 by the C-terminal BRCT domains of hPTIP is important for maintenance of genome stability.

DISCUSSION

The data presented above demonstrate that the two pairs of C-terminal BRCT domains of hPTIP are capable of at least two modes of phospho-epitope recognition and that they control at least two important, but independent, facets of hPTIP function: interaction with phosphorylated 53BP1 and translocation to sites of DNA damage.

At the outset of this study, it was known that hPTIP interacts with 53BP1 after DNA damage in an ATM-dependent manner (3,4) but the mechanisms or significance of this interaction were unclear. In this study, we showed that a single ATM-phosphorylated residue in 53BP1—Ser25—is required for interaction with hPTIP in cells. Mutation of Ser25 did not grossly perturb 53BP1 function since the 53BP1 Ser25Ala mutant protein formed foci after DNA damage in a manner indistinguishable from the wild-type protein (31) (data not shown). Phosphorylated Ser25 was shown to interact with the two C-terminal pairs (Pair C1 + C2) of hPTIP (Figure 2). We found that, surprisingly, both of these BRCT pairs are required to bind to the phospho-Ser25 peptide and neither domain alone could bind to this peptide (Figure 2B). Consistent with these data, mutations of conserved residues in Pair C1 (Trp676) or in Pair C2 (Arg910 or Trp929) severely reduced binding of hPTIP to phospho-Ser25 *in vitro*. Furthermore, mutation of any of these residues abolished the binding of hPTIP to 53BP1 after DNA damage *in vivo*. This is the first reported example of a requirement of two pairs of BRCT domains for binding to a single phospho-epitope. It is not yet clear why two BRCT pairs are required to recognize 53BP1 phospho-Ser25, especially when in BRCA1 and MDC1 (and in other BRCT-proteins), a single BRCT pair in each case is sufficient for interacting with phospho-H2AX and phospho-BACH1, respectively (12,14). We predict that the small conserved basic patch containing Arg910 in hPTIP pair C2 contacts the phospho-Ser25 of 53BP1 and that pair C1 makes contact with residues nearby, possibly helping to determine the specificity of the interaction. Solving the crystal structure of hPTIP BRCT domain pairs C1 + C2 in complex with the 53BP1 phospho-Ser25 peptide should provide valuable insight into the detailed mechanism of this interaction.

The requirement for two pairs of BRCT domains for hPTIP to recognize a single phospsho-epitope—Ser25 of 53BP1—is somewhat surprising since isolated BRCT domain pair C2 of hPTIP was shown previously, in peptide selection experiments, to interact with synthetic phospho-peptides that lie in an pS/T-Q-V-F motif. Ser25 does not lie in this type of motif but there are two serine residues in 53BP1—Ser29 and Ser105—that do conform to the pS/T-Q-V-F motif, are not required for 53BP1to bind hPTIP after DNA damage (Figure 1C). The observation that hPTIP BRCT pairs C1 + C2 recognize a phospho-peptide different from the pS/T-Q-V-F peptides bound by pair C2 in isolation suggests that there are two modes of phospho-epitope recognition resident in pairs C1 + C2. Again, it would be interesting to compare the crystal structure of hPTIP BRCT pairs C1 + C2 in complex with the two types of phospho-peptide to ascertain whether different modes of interaction are at play. It would be interesting to know if two types of phosphopeptide bind in a mutually exclusive manner or if they can bind to hPTIP simultaneously.

We showed previously that both BRCT pairs C1 and C2 are required for translocation of hPTIP to sites of DNA damage. In this study, we showed that mutation of Trp676 in hPTIP BRCT pair C1 abolished formation of IR-induced nuclear foci by hPTIP, as did mutation of Trp929 in pair C2 (Figure 5). However, the R910Q mutation that also abolished binding of hPTIP to phospho-Ser25 and to 53BP1 in cells, did not affect formation of nuclear foci by hPTIP after IR. Therefore, both C-terminal pairs of BRCT domains in hPTIP appear to be essential for binding of sites of DNA damage but this appears to be independent of their ability to bind phospho-Ser25 of 53BP1. This is consistent with previous reports that ATM, the Ser25 kinase, is not required for binding of hPTIP or 53BP1 to sites of DNA damage (3,28). At present, the molecular mechanisms governing translocation of hPTIP are not clear and this will be interesting to investigate. Whatever the case, it is clear both C-terminal BRCT domains of hPTIP are required.

Mutation of 53BP1 Ser25, that abolished interaction with hPTIP, and mutation of Arg910 of hPTIP that prevents interaction with phospho-Ser25, prevented phosphorylation of BRCA1 and Chk2 by ATM. Thus, 53BP1 must interact with hPTIP to exercise its role as adaptor and to protect cells against DSBs. At present, it is not clear how 53BP1 or hPTIP functions to assist ATM phosphorylation but may involve recruitment of ATM substrates to sites of DNA damage. Mutation of Ser25 also caused cells to become hypersensitive to IR, probably indicative of a DNA repair defect, since 53BP1 are defective in NHEJ of a subset of DSBs in cells (22). It would be interesting to know if mutation of Ser25 in mice recapitulates the same spectrum of tumours seen in 53BP1 null mice, and if mutation of Ser25 affects immunoglobulin class switching. Neither 53BP1 nor hPTIP appear to have catalytic activity and probably act as 'scaffolding' proteins to recruit and direct 'effector' polypeptides to sites of DNA damage. Ultimately it will be vital to identify the effector molecules that hPTIP and 53BP1 bring to sites of DNA damage that facilitate DNA repair and that enable phosphorylation of ATM targets.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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