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Negative Cell Cycle Regulation and DNA Damage-inducible Phosphorylation of the BRCT Protein 53BP1*

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In a screen designed to discover suppressors of mitotic catastrophe, we identified the *Xenopus* ortholog of 53BP1 (X53BP1), a BRCT protein previously identified in humans through its ability to bind the p53 tumor suppressor. X53BP1 transcripts are highly expressed in ovaries, and the protein interacts with Xp53 throughout the cell cycle in embryonic extracts. However, no interaction between X53BP1 and Xp53 can be detected in somatic cells, suggesting that the association between the two proteins may be developmentally regulated. X53BP1 is modified via phosphorylation in a DNA damage-dependent manner that correlates with the dispersal of X53BP1 into multiple foci throughout the nucleus in somatic cells. Thus, X53BP1 can be classified as a novel participant in the DNA damage response pathway. We demonstrate that X53BP1 and its human ortholog can serve as good substrates *in vitro* as well as *in vivo* for the ATM kinase. Collectively, our results reveal that 53BP1 plays an important role in the checkpoint response to DNA damage, possibly in collaboration with ATM.

The eukaryotic cell cycle is a series of molecular events resulting in the accurate replication and segregation of the genome. To achieve this, it is essential that the successive phases of the cell cycle are coordinated in a precise and punctual manner, since failure to do so may result in abnormal growth and development, probably as a consequence of genomic instability. For this purpose, cells have evolved an elaborate network of "checkpoint controls" that monitor the proper completion of various events in the cycle including DNA replication in S phase and chromosome segregation during mitosis (reviewed in Ref. 1). Upon genetic damage by any of a number of mechanisms (UV light, ionizing radiation (γ-IR), chemicals, etc.), checkpoint response pathways become activated, leading to attenuation in cell cycle progression and a mobilization of the DNA repair machinery.

A growing body of information on the mechanisms of checkpoint control in organisms ranging from yeast to humans have shown the importance of these pathways with respect to maintaining genomic stability (1, 2). For example, patients with the recessive disorder ataxia telangiectasia (AT)[1] fail to delay DNA synthesis and mitosis in response to γ-IR, suggesting that the gene responsible for this pleiotropic disease, *ATM* (mutated in ataxia telangiectasia; Ref. 3), plays a major role in coordinating the checkpoint response (4). Indeed, one of the hallmarks of AT is a predisposition to cancer, probably as a result of the failure to repair damaged DNA (5). In addition to *ATM*, many tumors and cancer predisposition syndromes have defects in checkpoint-related gene products that are phosphorylated and controlled, at least in part, by the ATM kinase. This includes BRCA1 (breast cancer gene 1), the effector kinase Cds1/Chk2, p95/Nbs1, and p53 (6–14). BRCA1 has been reported to function in the transcriptional-coupled repair of damaged DNA, G_2/M checkpoint control, and the repair of double-stranded DNA breaks (DSBs; Refs. 15–18). In addition, BRCA1 resides in a large complex of proteins (BASC) dedicated to repairing various forms of damaged DNA (19). This includes DNA mismatch repair proteins, the BLM helicase, and the RAD50-MRE11-NBS1 complex that is believed to function in the repair of DSBs (21, 22). Recent work by Elledge and colleagues has suggested that mutations in the ATM/BRCA1 pathway may account for 10% of all cases of breast cancer (11).

The biological response to DNA damage is best understood in both the budding and fission yeast systems (1, 24). In many respects, the general framework of the pathways responsible for checkpoint signaling appears to be conserved from yeasts to humans. Here, DNA damage-sensing proteins (*e.g.* various Rad gene products) transduce signals to phosphoinositide (PI)-kinase like proteins (Mec1/Tel1 in budding yeast, ATM and ATR in higher eukaryotes) that regulate effector kinases and/or transcription factors (Rad53/Dun1 in budding yeast, Cds1/Chk2 in higher eukaryotes; Refs. 9, 23, and 25–30). In *Saccharomyces cerevisiae*, DNA damage slows S phase progression and can arrest cells at either the G_1/S or the G_2/M transitions (31, 32). In particular, Rad9 has been shown to be a critical factor for various checkpoint responses (31–34). In addition to Rad9, several other budding yeast gene products have been identified to play important roles in sensing DNA damage or blocks in DNA replication. These include Rad53, Mec3, Rad1, Rad24, Pol2, Dpb11, and Rfc5 and their orthologous counterparts in other species (1, 25, 35, 36). Although the precise biochemical functions for many of these proteins remain unknown, some DNA damage-sensing factors from various systems appear to possess similarities to DNA polymerase acces-

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[1] The abbreviations used are: AT, ataxia telangiectasia; DSB, double-stranded DNA break; aa, amino acid(s); kb, kilobase pair(s); RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; CSF, cytostatic factor; Gy, gray; PBS, phosphate-buffered saline; XTC, *Xenopus* tissue culture; IF, immunofluorescence; WT, wild type.

sory factors, repair proteins, and exonucleases (25, 37–40). Such properties might be expected for proteins whose functions are to sense DNA damage.

One major checkpoint response in higher eukaryotes is activated upon exposure to γ-IR or other agents that create DSBs. The PI-kinase like protein ATM responds to this type of genotoxic insult (41) in a manner that is most likely dependent upon DNA damage-sensing proteins. ATM-dependent phosphorylation of the p53 tumor suppressor is known to mediate cell cycle arrest in response to DNA damage (42–44). The gene encoding p53 is the most frequently mutated gene in all cases of human cancer (45). p53 protein levels are activated and stabilized in cells exposed to γ-IR. Here, the protein can act as a tetrameric transcription factor by inducing the expression of genes involved with the DNA damage response (42, 44). The precise mechanisms by which p53 is activated are not well understood and are likely to be cell type-specific (44). In other instances, p53 directs the apoptotic response (45). p53 protein levels are tightly regulated by the Mdm2 protein, an E3 ubiquitin ligase that targets p53 for degradation by the proteosome. It is believed that multiple modifications at the N and C termini contribute to the activation of latent p53 (44, 45). Such modifications include phosphorylation by ATM on serine residue 15 (7, 8) and phosphorylation of serine 20 by Cds1/Chk2 (46, 47). Both of these modifications are believed to contribute to the overall stability of p53, perhaps by interfering with its ability to be degraded by Mdm2.

One protein that interacts with p53 is 53BP1 (48). 53BP1 was initially identified in a two-hybrid screen with p53 as bait (48). Using murine p53 amino acids (aa) 73–390 fused to the Gal4 DNA-binding domain, two human proteins were identified which bound to p53. These proteins, designated as 53BP1 and 53BP2, bound to the transcriptional activation domain of p53 but not to a version of p53 with a point mutation (R175H) in this domain (48). R175H generates a transcriptionally incompetent p53 protein and has been found in patients with cancer (45), suggesting that the binding of 53BP1 and 53BP2 is sensitive to the conformation of p53. Additional studies have shown that 53BP1 can enhance p53-mediated transcription of reporter genes (58). 53BP1 possesses strong homology to the C-terminal repeats of BRCA1 (BRCT motifs). BRCT domains are autonomously folding modules consisting of ~100 aa and are believed to mediate protein-protein interactions, particularly with respect to proteins associated with the cell cycle and the various aspects of DNA metabolism: replication, recombination, and repair (49–51). Other proteins possessing BRCT motifs include budding yeast Rad9 and Dpb11, fission yeast Crb2 and Cut5, and mammalian XRCC1 and DNA ligase IV (1, 51, 52, 53). The C-terminal 271 aa of human 53BP1, the region encompassing the BRCT folds, was found to interact with p53 through its C-terminal BRCT repeats (48). Murine 53BP1 has also been isolated in the two-hybrid assay when the inteferon-inducible p202 protein is used as bait (54).

In this report, we present the isolation and characterization of the *Xenopus* ortholog of human 53BP1 (X53BP1). We discovered X53BP1 in a screen designed to identify suppressors of mitotic catastrophe (negative regulators of the cell cycle) of the fission yeast *Schizosaccharomyces pombe* strain SP984 (55). We have previously exploited the mitotic catastrophe phenotype of SP984 to isolate Xorc2, a component of the origin recognition complex (56). This strain contains a temperature-sensitive mutation in *wee1* and a null mutation in the partially redundant *mik1* gene. Both genes encode proteins that modify and negatively regulate the Cdc2 kinase (Cdk1), a Cdk that functions in the progression into mitosis (57). At the nonpermissive temperature of 35.5 °C, SP984 prematurely enters mitosis due to its inability to control the precocious activation of Cdc2. Thus, cDNA molecules capable of rescuing SP984 will express proteins that either directly or indirectly inhibit Cdc2 activity. The complete cDNA encoding X53BP1 presented here, in conjunction with the recently described human sequence (58), reveals that 53BP1 proteins from both frogs and humans are well conserved and contain numerous putative ATM family member phosphorylation sites (59) in addition to their BRCT motifs. Northern blotting reveals that X53BP1 expression appears largely restricted to ovaries. Consequently, we find that the protein is abundant in eggs but much less so in somatic cells. We present evidence that X53BP1 and Xp53 associate throughout the cell cycle in a cell-free system, confirming the original two-hybrid isolation of 53BP1 (48) in a specialized cellular setting. In contrast, no association between X53BP1 and Xp53 can be detected in somatic cells, suggesting that the interaction between these proteins is under developmental control or is restricted to germline tissue. Additionally, we find that X53BP1 is phosphorylated *in vivo* in response to DNA damage. Consistent with this, we demonstrate that both the frog and human 53BP1 proteins serve as substrates for the ATM kinase *in vitro*, particularly in an N-terminal region containing numerous ATM phosphorylation motifs. In addition, ATM phosphorylates human 53BP1 in an N-terminal region *in vivo* in response to γ-IR. Taken together, our data indicate that 53BP1 plays a significant role in the cellular response to DNA damage, classifying it as an important checkpoint response protein.

EXPERIMENTAL PROCEDURES

Isolation of the Gene Encoding X53BP1 as a Suppressor of Mitotic Catastrophe and Cloning of the Full-length Gene—The fission yeast strain SP984 (55) was transformed with a *Xenopus laevis* cDNA library prepared in the *S. pombe* expression vector pAX-NMT (56, 60) by the spheroplast method using Lipofectin (56). pAX-NMT is a fission yeast/*Escherichia coli* shuttle vector and contains the *S. cerevisiae LEU2* gene for selection. From ~200,000 transformants, about 60 colonies grew at the nonpermissive temperature of 35.5 °C on minimal media lacking leucine and thiamine. One rescuant, pC535, was chosen for further study. pC535 plasmid DNA was isolated and used to re-rescue SP984 from mitotic catastrophe at the nonpermissive temperature. Once the rescuing event was confirmed, the 1.9-kb *Apa*I/*Xho*I cDNA insert of pC535 was used to probe a *Xenopus* oocyte library as described previously (56, 60). This screen yielded S48-1, which contains a 5.6-kb cDNA insert corresponding to X53BP1. To isolate the 5′-end of X53BP1, sequence information at the 5′-end of S48-1 was used to perform RACE PCR as described below. DNA sequencing of the cDNA insert of S48-1 was done by primer walking with custom designed oligonucleotides. The complete cDNA sequence for X53BP1 was assembled with information generated from S48-1 and pZX-1 (see below).

Total RNA Extraction, 5′-RACE PCR, and Northern Hybridization—Various tissues as described under "Results" were collected from adult female *X. laevis* frogs and immediately frozen in liquid nitrogen. Total RNA was extracted from the various tissues or from somatic *Xenopus* tissue culture (XTC) cells using Trizol (Life Technologies, Inc.) in conjunction with DNase I. 5′-RACE PCR was performed with a RACE system (Life Technologies, Inc.) according to the manufacturer's instructions. First strand cDNA synthesis was synthesized from 1.0 μg of total XTC RNA using the GSP1 primer: 5′-AAAGAAGAATCCTTAG-3′. After dC tailing, a PCR was carried out using the cDNA as a template along with an abridged anchor primer and the nested GSP2 primer: 5′-CAGCATCTTGGGAAGGAGAAACCAGAT-3′. A dilution of the original PCR was reamplified with a nested GSP3 primer: 5′-CAGAAGGATGGAGTAGGCGCAAACTGT-3′. All reactions were carried out under the recommended conditions of the supplier. PCR products were electrophoresed on 1.0% agarose gels, and a target band of ~0.9 kb was gel purified and ligated into the pGEM-T vector (Promega), creating pZX-1. DNA sequencing of pZX-1 was performed on both strands by primer walking using an ABI sequencer and shown to unambiguously overlap with S48-1.

For Northern hybridization, 10 μg of total RNA was separated on a 1.0% formaldehyde agarose gel with a 0.4–9.5-kb RNA ladder (Life Technologies, Inc.). RNA was transferred onto Hybond-N+ membrane (Amersham Pharmacia Biotech) by capillary elution with 20× SSC and

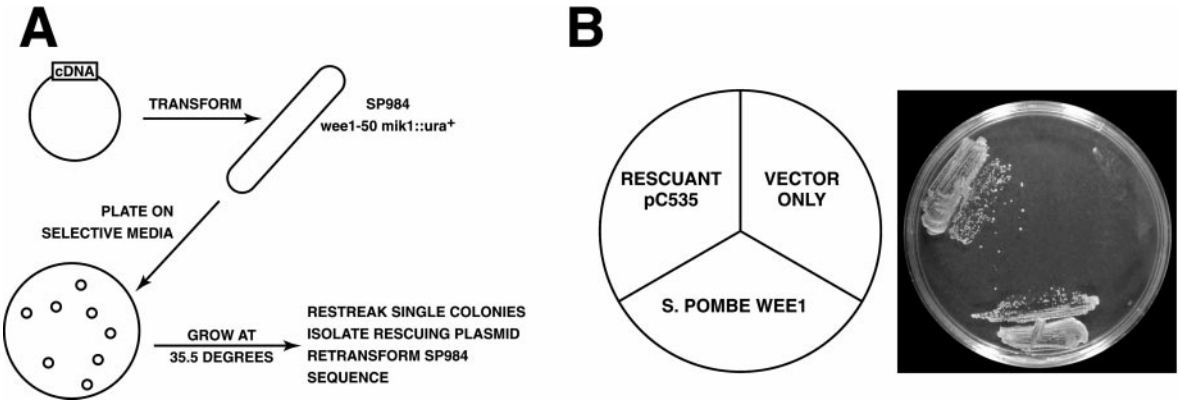

FIG. 1. **Rescuing of a mitotic catastrophe strain (SP984, *mik::ura4+*, *wee 1–50 leu1–32 ade6–210 ura4-D18 h*$^{+N}$) by an *X. laevis* cDNA encoding X53BP1.** *A*, schematic representation of the screen utilized to isolate pC535, a fission yeast/*E. coli* shuttle vector (pAX-NMT) containing a 1.9-kb *Apa*I/*Xho*I cDNA corresponding to the C-terminal region of X53BP1. See "Experimental Procedures" for details. *B*, suppression of mitotic catastrophe by pC535. SP984 was transformed with either the pAX-NMT vector only, rescuant pC535, or the wild type *wee1* gene cloned into pAX-NMT, grown for 6 days at the restrictive temperature of 35.5 °C, and streaked out on agar plates.

fixed with 0.5 N sodium hydroxide. The membrane was prehybridized at 65 °C for 15 min in rapid hybridization buffer (Amersham Pharmacia Biotech) and hybridized with a 0.9-kb probe corresponding to the extreme 5′-end of X53BP1 or a 1.2-kb fragment derived from *Xenopus* EF-1α (62) for 2 h at 65 °C in rapid hybridization buffer. The membrane was washed for 15 min at 65 °C for 15 min in 2× SSC, 1× SSC, and 0.1× SSC with 0.1% SDS and exposed to film.

Cell-free Extracts—Xenopus cell-free extracts were prepared by the method of Murray (53). Interphase extracts were generated by the addition of 0.4 mM calcium chloride to cytostatic factor (CSF)-arrested extracts in the presence of cycloheximide (100 μg/ml) and allowed to proceed for 30 min at 25 °C. The entry into interphase was verified through either H1 kinase assays or observation of nuclear envelope formation.

Recombinant Proteins and Antibody Production—To produce polyclonal rabbit antibodies against X53BP1, we generated a GST fusion protein between GST and residues 1652–2055 of X53BP1 by PCR amplification with a high fidelity DNA polymerase, creating GST-XBP1–5. This amplified X53BP1 fragment was ligated in frame into pGEX4T-1 (Amersham Pharmacia Biotech) on a *Bam*HI fragment. The resulting recombinant was confirmed and used to transform DH10B cells. Protein expression was induced by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 25 °C for 3 h. Soluble GST-XBP1–5 protein was affinity purified on glutathione-agarose. The purified protein was used as antigen for injection into rabbits (Covance). All other 53BP1 fusion proteins were generated in a similar manner. Various bleeds were tested for antibody production via immunoblotting with a chemiluminescent system (Amersham Pharmacia Biotech), and those that were positive were used to affinity-purify αX53BP1. Anti-GST antibodies were selectively removed from the serum by adsorption onto GST-loaded Sepharose beads. The unbound antibodies against X53BP1 were affinity-purified as described (72) with GST-XBP1–5 protein coupled to CNBr-Sepharose (Amersham Pharmacia Biotech) at a concentration of 1.0 mg/ml as per the manufacturer's instructions. For the generation of antibodies against Xp53, the full coding sequence to Xp53 was isolated from a *Xenopus* cDNA library (60), amplified with a high fidelity DNA polymerase, and ligated in frame into pGEX4T-1 for protein expression. Soluble GST-Xp53 was expressed in DH10B bacterial cells and affinity-purified as described above. The isolated protein was used as an antigen to generate polyclonal antibodies.

DNA Damage-inducible Phosphorylation of X53BP1—XTC cells were cultured in Liebovitz' L-15 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum and antibiotics. 36 plates were grown to 60% confluency. One half of the plates was treated with 10 Gy of γ-IR with a cesium irradiator, and the other half was left untreated. Samples were allowed to recover for 1 h post-treatment. After the recovery period, the cells were collected, washed with phosphate-buffered saline (PBS), and lysed on ice with PBS supplemented with 1% TX-100, 1% Nonidet P-40, 2 mM $CaCl_2$, and a mixture of protease inhibitors. Clarified supernatants were treated for 1 h with αX53BP1 (or αXp53 in some cases) that had been covalently coupled to protein A-Sepharose. The immunoprecipitates were subsequently washed four times with EB+ buffer (20 mM β-glycerol phosphate, 10 mM Hepes, 1% Nonidet P-40, and 25 mM sodium fluoride, pH 7.5). The beads were further washed twice in an EB+ buffer without detergent and sodium fluoride. Finally, the samples were washed twice in λ phosphatase buffer (50 mM Tris, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Brij 35, pH 7.5) and split in half. One half was treated with phosphatase buffer and the other was supplemented with 1.0 unit of λ-phosphatase (New England Biolabs), incubated at 30 °C for 30 min, and loaded onto Novex 3–8% acrylamide gels.

Indirect Immunofluorescence in XTC Cells—XTC cells were grown on polylysine-treated coverslips to 60% confluency in Liebovitz' L-15 medium and processed for indirect immunofluorescence with αX53BP1 as follows. Cells were fixed with 2% paraformaldehyde in 66% phosphate-buffered saline, pH 7.4, and permeabilized with 0.05% saponine, 1.5 mM NH_4Cl in phosphate-buffered saline. Permeabilized cells were treated with primary antibody for 1 h, washed three times in phosphate-buffered saline without paraformaldehyde, and incubated with an anti-rabbit IgG conjugated to fluorescein isothiocyanate for 1 h (Pierce). Mounted coverslips were viewed under oil at 100× magnification and photographed using an Olympus BX60 microscope equipped with a Spot digital camera (Diagnostics Instruments) interfaced. Images were transferred and processed into Adobe Photoshop 5.0.

ATM Kinase Assays—In vitro ATM kinase assays were performed essentially as described previously (7, 8). Briefly, 293T cells were transfected with constructs expressing either the full-length ATM gene or a kinase-defective version of the enzyme. The expressed proteins were immunoisolated with M2/M5 FLAG antibodies (Sigma) coupled to protein A/G-Sepharose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and incubated with candidate GST fusion proteins containing various segments of either the *Xenopus* or human 53BP1 proteins. Approximately 1 μg of affinity-purified, candidate substrate protein was incubated in ATM kinase buffer (8) with 10 μCi of [γ-^{32}P]ATP for 30 min at 30 °C with constant agitation. *In vivo* phosphorylation of 53BP1 was measured in a transient transfection assay using 293T cells. Transfections were performed with Lipofectin (Life Technologies, Inc.). Human 53BP1 residues 1–524 were fused in frame to pFLAG-NLS lox (11), creating plasmid pZX-12. pZX-12 encodes FLAGBP1–124. 2.5 μg of pZX-12 was transfected alone or cotransfected along with an equal amount of plasmid expressing either the wild type or kinase-dead version of ATM. After 48 h posttransfection, the cells were treated with either 0 or 50 Gy of ionizing radiation using a cesium irradiator. After a 1-h recovery, the cells were harvested, processed for immunoprecipitation, electrophoresed on 6% SDS gels, and transferred overnight in preparation for Western analysis with anti-FLAG M2/M5 antibodies (Sigma). FLAGBP1–1524 was visualized by Western blotting with αX53BP1 and the ECL detection system (Amersham Pharmacia Biotech).

RESULTS

Identification of X53BP1 as a Suppressor of Mitotic Catastrophe—To identify potential negative regulators of the cell cycle, we rescued the fission yeast mitotic catastrophe strain SP984 (55) with a *Xenopus* cDNA library prepared in the pAX-NMT *S. pombe* expression vector (56, 60). At the nonpermissive temperature of 35.5 °C, SP984 is deficient in Cdc2-specific tyrosine kinase activity, an inhibitory modification. This results in a precocious activation of Cdc2 during interphase, causing premature mitotic entry and cell death. We have previously used this method to identify Xorc2, a member of the *Xenopus*

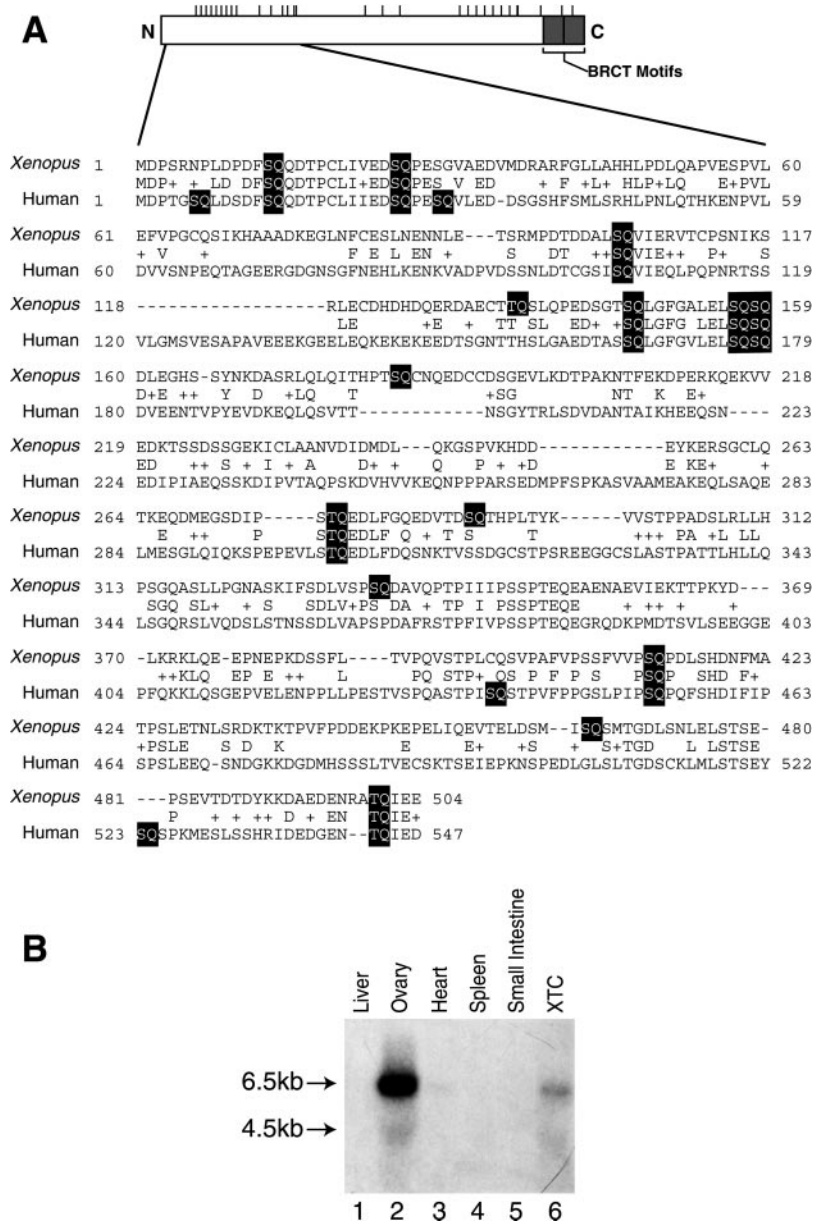

FIG. 2. **Characterization of the gene encoding X53BP1.** *A*, amino acid alignment of the N termini of *Xenopus* and human 53BP1 proteins. The frog and human 53BP1 proteins (GenBank™ accession numbers AF281071 and AF078776, respectively) were compared via a BLAST program (NCBI) as shown. Potential ATM family member phosphorylation sites (59) are highlighted in *black* and are also represented by the *vertical hatched lines*. *B*, Northern blot of X53BP1 transcripts in various *X. laevis* tissues. 10 μg of total RNA was isolated and processed for Northern blotting analysis as described under "Experimental Procedures." The major X53BP1 transcript of 6.5 kb is present in abundance in ovaries and to a lesser extent in the kidney-derived XTC cell line. Prolonged exposures also reveal a minor amount of transcript in the heart. A second, minor transcript migrating around 4.5 kb is also present.

origin recognition complex (56). A second rescuant described here, pC535 (Fig. 1), was found to contain a 1.9 kb *Apa*I/*Xho*I cDNA fragment encoding for the C-terminal region of the frog 53BP1 ortholog (X53BP1). 53BP1 was originally described as a human protein capable of binding to the central transactivation domain of murine p53 in a two-hybrid assay (48). The largest X53BP1 plasmid capable of rescuing SP984 was found to contain a 4-kb *Apa*I/*Xho*I fragment encoding for the C-terminal region of X53BP1. All rescuing cDNAs of X53BP1 possess the two repeating BRCT motifs found at the extreme C terminus (Fig. 2*A*). pC535-expressing mutant cells divided normally and exhibited neither an obvious *wee* nor elongated phenotype (not shown). Despite this, the rescued cells still entered mitosis inappropriately in the presence of 10 mM hydroxyurea at the restrictive temperature. Thus, despite its formal classification as a negative regulator of the cell cycle in fission yeast, the expression of X53BP1 is not sufficient to restore the replication checkpoint defect characteristic of SP984. The precise reasons for why X53BP1 rescues SP984 from mitotic catastrophe remain to be determined.

Cloning and Characterization of X53BP1—Because human 53BP1 is comprised of 1972 amino acids (58), we assumed that the frog ortholog would be of a similar size. If so, then it was clear that rescuant pC535 was only a partial cDNA clone. In an attempt to isolate the full coding sequence of X53BP1, we used the 1.9-kb *Apa*I/*Xho*I fragment from pC535 to screen a cDNA library derived from *X. laevis* oocytes (60). From 1 million library clones, 22 cDNAs encoding various portions of X53BP1 were isolated. The largest isolate, S48-1, was sequenced and found to be an incomplete cDNA, since it contained a 5.6-kb *Apa*I/*Xho*I fragment. To isolate the 5′-end of X53BP1, we performed a PCR RACE experiment (Life Technologies, Inc.) with RNA isolated from the XTC somatic cell line using sequences derived from the 5′-end of S48-1. A 0.9-kb fragment was successfully isolated, sequenced on both DNA strands, and shown to encode the N-terminal region of X53BP1. Importantly, this RACE product possesses an initiating methionine codon that properly aligns with the proposed first methionine of human 53BP1 (Ref. 58; Fig. 2*A*). This methionine residue is also preceded by an in-frame stop codon (UAG) six nucleotides upstream of the ATG codon (not shown). Moreover, this fragment contains a significant and unambiguous 3′ overlap to the 5′-end of S48-1 (not shown). The full-length cDNA for X53BP1 is ~6.5 kb long and contains 5′- and 3′-untranslated regions of 45 and

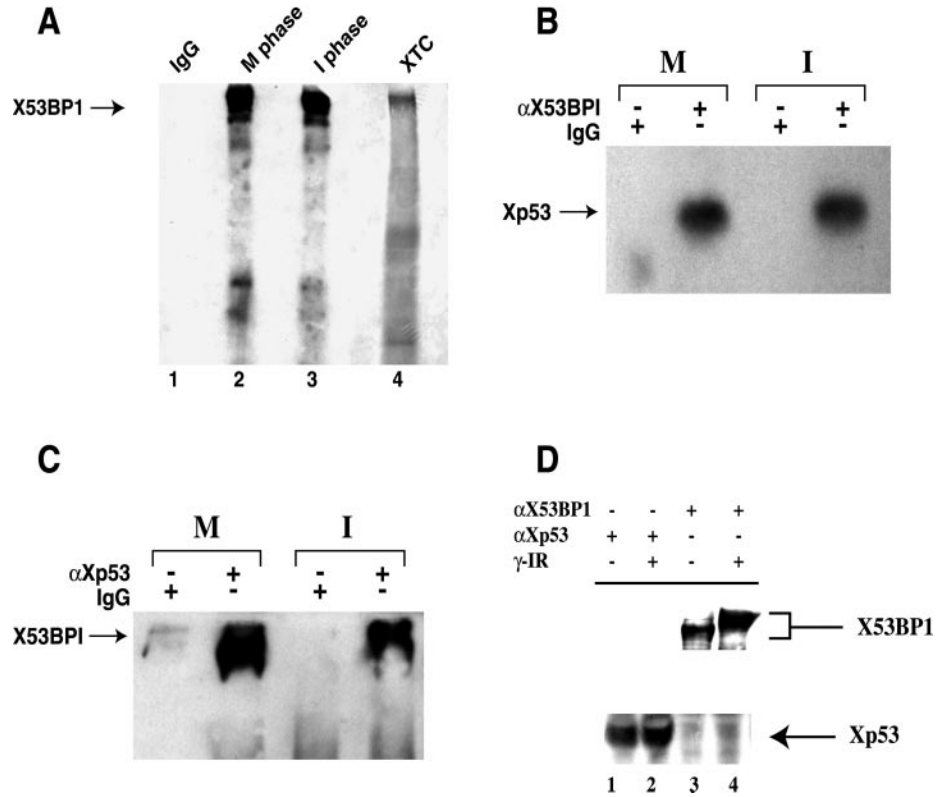

FIG. 3. **Analysis of X53BP1 in cell-free extracts and somatic XTC cells.** *A*, immunoblotting of cell cycle staged extracts or somatic XTC cells for X53BP1. 10 μl (~500 μg of protein) from either mitotic (CSF; *lanes 1* and *2*) or interphase extract (*lane 3*) was immunoprecipitated with either a control IgG (*lane 1*) or αX53BP1 (*lanes 2* and *3*), electrophoresed on 6% SDS gels, and immunoblotted with affinity-purified αX53BP1. X53BP1 migrates at ~300 kDa. *Lane 4*, XTC extract (~5–10 mg of protein) was immunoprecipitated with αX53BP1 and immunoblotted for X53BP1 with αX53BP1. *B*, coimmunoprecipitation of Xp53 with αX53BP1 in cell-free extracts. 50 μl of either CSF-arrested (*M*; *lanes 1* and *2*) or interphase-arrested (*I*; *lanes 3* and *4*) extracts was immunoprecipitated with a control IgG (*lanes 1* and *3*) or αX53BP1 (*lanes 2* and *4*) and processed for immunoblotting for Xp53 with either αXp53 or in some cases the *Xenopus* p53 antibody 2274 (66). *C*, coimmunoprecipitation of X53BP1 with αXp53. 50 μl of either CSF-arrested (*M*; *lanes 1* and *2*) or interphase-arrested (*I*; *lanes 3* and *4*) extracts were immunoprecipitated with affinity-purified αXp53 or a control rabbit IgG (*lanes 1* and *3*) and processed for immunoblotting with αX53BP1 (*lanes 2* and *4*). Visualization of target antigens was conducted with a chemiluminescent system (ECL; Amersham Pharmacia Biotech). *D*, coimmunoprecipitation of X53BP1 and Xp53 in XTC cells. XTC cells were treated with either 0 or 10 Gy of γ-IR and allowed to recover for 1 h prior to making extracts. XTC extracts were immunoprecipitated with αXp53 (*lanes 1* and *2*) or αX53BP1 (*lanes 3* and *4*) and immunoblotted for either X53BP1 or Xp53 as shown.

62 nucleotides (excluding the poly(A) tail), respectively. The complete sequence of X53BP1 encodes a protein of 2104 amino acids (predicted mass of 231.3 kDa) with significant homology to the 1972-residue human 53BP1 protein and has been deposited in GenBank™ (accession number AF281071).

Both 53BP1 proteins possess two repeating BRCT motifs (49, 50, 61) at their respective C termini. Additionally, the frog and human 53BP1 orthologs contain numerous "(S/T)Q" motifs, some of which are conserved, particularly in their N termini, where there is clustering (Fig. 2*A*). Such sequence elements have been shown to formulate strong recognition determinants for the ATM kinase and its related family members ATR and DNA-PK (59). In addition, X53BP1 contains four (S/T)P*X*(R/K) motifs that reside in potential Cdk recognition sites (not shown). Thus, both the frog and human 53BP1 proteins possess sequence features suggestive of functions in checkpoint control during the biological response to DNA damage.

To further understand the role of X53BP1 in *Xenopus*, we analyzed X53BP1 transcripts by Northern blotting (Fig. 2*B*). 10 μg of total RNA was isolated from various female frog tissues with Trizol and electrophoresed on 0.8% agarose gels. The gels were processed for Northern analysis with a 0.9-kb DNA probe specific for the 5′-end of X53BP1. As a loading control, a 1.1-kb *Pst*I probe specific for *Xenopus* EF-1α was used (not shown). These messages have been shown to be relatively constant in *Xenopus*. (62). Our results clearly indicate that X53BP1 transcripts appear to be abundant in ovaries but not in the other tissues that were sampled (Fig. 2*B*). Unlike *Xenopus*, the human 53BP1 transcripts were detected in all tissues sampled (48). As is also the case for human 53BP1, two transcripts can be detected (48). However, in contrast to the frog messages of ~7.0 and 4.5 kb (Fig. 2*A*), the human 53BP1 messages are 11.0 and 7.0 kb (48). X53BP1 mRNA is detectable, albeit at much reduced levels relative to the ovaries, in heart tissue and the somatic XTC cell line. The high X53BP1 transcript levels in ovarian tissue, as has also been demonstrated for Xp53 (63), are consistent with the maternal stockpiling that is seen for many proteins, including X53BP1 protein (see below).

Association of X53BP1 and Xp53 in Cell-free Extracts and Somatic Cells—To characterize X53BP1 further, we raised polyclonal antibodies that react against a glutathione *S*-transferase (GST) fusion protein encoding amino acids 1652–2055 (GST-XBP1–5), which reside near the C terminus of X53BP1. The soluble GST-XBP1–5 protein was purified on glutathione-agarose (Sigma), eluted with reduced glutathione as described (64), and injected into rabbits for polyclonal antibody production. Affinity-purified antibodies that react against X53BP1 (αX53BP1), but not against GST, were isolated as described under "Experimental Procedures" and used to examine the protein in cell-free extracts derived from *Xenopus* eggs (Fig. 3*A*). Such extracts have been shown to recapitulate the major events of the cell cycle including DNA replication and mitosis (53, 56, 60). *Xenopus* eggs are arrested in metaphase of meiosis II (M phase), a mitotic-like state, through the action of CSF.

X53BP1 is present in both M phase and interphase extracts in equivalent amounts as revealed by Western blotting (Fig. 3*A*). Like its human counterpart, X53BP1 migrates aberrantly on SDS gels (58). The predicted molecular mass of X53BP1 is 231 kDa, but both the M phase and interphase forms appear to migrate around 300 kDa (Fig. 3*A*). X53BP1 was often detected as a diffuse set of bands (Fig. 3*A*), consistent with the protein undergoing posttranslational modifications, possibly phosphorylation, throughout the cell cycle. To demonstrate that X53BP1 is present in somatic cells, in addition to embryonic extracts, we examined the protein in XTC cells, a somatic cell line (79). We immunoprecipitated X53BP1 from XTC cells with αX53BP1 and examined the presence of the protein by Western blotting. Our results show that X53BP1 is present in the XTC cell line but at much reduced levels relative to the embryonic protein (Fig. 3*A*). We estimate that the concentration of X53BP1 in *Xenopus* eggs is 100 nM and less than 10 nM in somatic cells. Unlike the diffuse banding pattern often seen for embryonic X53BP1, the somatic protein species appears to migrate as a much sharper band in 6% SDS gels (Fig. 3*A*).

Because 53BP1 was initially identified as a p53-binding protein, we investigated the relationship between X53BP1 and the *Xenopus* p53 protein (Xp53; Refs. 65–67) in cell-free extracts derived from frog eggs. Xp53 is highly expressed during oocyte development (68). *Xenopus* eggs contain an abundance of Xp53 protein, which has been shown to possess the same biochemical properties of its human counterpart (63, 66, 69). Despite this, there is no transcription occurring in eggs, since development relies upon maternal stores of protein until zygotic transcription occurs at the midblastula transition (70). Thus, the functional significance of Xp53 in early embryonic development is not clear, although roles for the protein during this period have been suggested. For example, microinjection of Xp53 mRNA into early cleavage stages interferes with normal frog development (67). In addition, inhibition of Xp53 function via ectopic expression of either a dominant negative Xp53 or its inhibitor X-dm2 results in an early block to differentiation (71).

To address the nature of any X53BP1/Xp53 interactions, we performed reciprocal coimmunoprecipitation experiments (Fig. 3*B*) from cell-free extracts derived from unfertilized eggs. For this purpose, we generated polyclonal antibodies against the frog p53 protein (αXp53). Coimmunoprecipitation experiments were performed with either αX53BP1, αXp53, or a control rabbit IgG. In some instances, we immunoblotted for Xp53 with antibody 2674 (α2674), a polyclonal Xp53 antiserum described previously (66). We found that αXp53 and α2674 gave similar results (not shown). For immunoprecipitations, all antibodies were covalently linked to protein A beads with dimethylpimelimidate as described previously (72). After a 1-h incubation at 4 °C in either CSF-arrested or interphase extracts, the beads were collected by brief centrifugation, thoroughly washed, and then resuspended in SDS sample prior to loading on SDS gels. The results clearly demonstrate that X53BP1 and Xp53 associate with each other throughout the cell cycle in cell-free extracts (Fig. 3, *B* and *C*). Xp53 can be detected in X53BP1 immunoprecipitates, and X53BP1 is present in Xp53 immunoprecipitates as shown. Neither of these two proteins are present with the control IgG, indicating that the association between X53BP1 and Xp53 is specific. Thus, consistent with previous studies in mammals (48, 58), X53BP1 and Xp53 form complexes in *Xenopus.* Interestingly, such complexes are present throughout the cell cycle in both M phase and interphase.

To further characterize the interaction between X53BP1 and Xp53, we performed reciprical coimmunoprecipitations in somatic XTC cells extracts. Immunoprecipitations from these extracts were processed in the same manner as described for the egg extracts. Unexpectedly, no interaction can be detected between X53BP1 and Xp53 in somatic cells (Fig. 3*D*). Because p53 is known to be involved in the response to γ-IR DNA damage, we assayed for a potential X53BP1/Xp53 interaction in the presence of this form of genotoxic stress. As observed before, no interaction between the two proteins can be seen. Similar observations have also been made for 53BP1 and p53 in human cell lines.[2] Thus, these data suggest that the association between X53BP1 and Xp53 is under developmental control or is restricted to germline tissue.

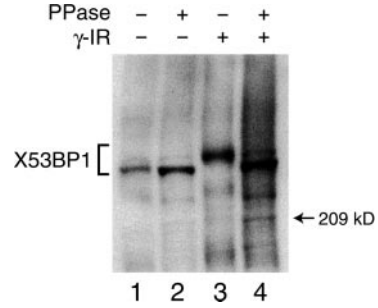

FIG. 4. **DNA damage-dependent phosphorylation of X53BP1 *in vivo*.** XTC cells were treated with either 0 (*lanes 1* and *2*) or 10 Gy of γ-IR (*lanes 3* and *4*). Lysed supernatants were immunoprecipitated with αX53BP1 and split into two equal halves. One half received buffer only (*lanes 1* and *3*), and the other was treated with 1.0 units of λ-phosphatase (*lanes 2* and *4*). Immunoprecipitates were electrophoresed on 3–8% gradient gels and immunoblotted with αX53BP1 as shown. Various electrophoretic forms of X53BP1 are indicated by the *brackets*. The position of the 209-kDa marker (myosin heavy chain) is indicated with an *arrow*.

DNA Damage-inducible Phosphorylation of X53BP1—Many proteins involved in checkpoint signaling are modified in response to DNA damage. These include the ATM-dependent phosphorylation of BRCA1, Cds1/Chk2, p53, and p95/Nbs1 (8, 9, 11–14). Given the relationship of 53BP1 to p53, the presence of its two-repeating BRCT domains, and its potential ATM phosphorylation sites, X53BP1 appeared as a strong candidate for modification by phosphorylation in response to DNA damage, particularly γ-IR. To address this issue, we examined the nature of X53BP1 in *Xenopus* somatic XTC cells that had been exposed to γ-IR. XTC cells, grown on plates to 60% confluency, were treated with either 0 or 10 Gy of γ-IR with the use of a cesium irradiator. After a brief recovery period, the cells were collected, lysed, and immunoprecipitated with αX53BP1 that had been linked to protein A beads. After thoroughly washing the pellet, the samples were split into two equal portions. One part was treated with buffer, and the second was treated with λ phosphatase. After incubation, the samples were processed for electrophoresis on 3–8% gradient gels. In the absence of γ-IR, X53BP1 migrates at ~300 kDa (Fig. 4, *lanes 1* and *2*). When exposed to 10 Gy of γ-IR, X53BP1 appears as even a slower migrating form (Fig. 4, *lane 3*). This retarded form of X53BP1 can be reversed back to its basal state through treatment with λ-phosphatase (Fig. 4), indicating that the γ-IR-induced modification of the protein is due to phosphorylation. Additionally, X53BP1 is also modified via phosphorylation in the absence of DNA damage, since it appears to have a slightly slower mobility in gradient gels relative to the λ-phosphatase-treated sample (Fig. 4, compare *lanes 1* and *2*). Other factors, such as cyclin-dependent kinases, may be responsible for this. Taken together, our results establish X53BP1 as a new member of the γ-IR DNA damage response pathway.

Subcellular Localization of X53BP1 in Somatic Cells by Indirect Immunofluorescence—Previous indirect immunofluores-

[2] T. Halazonetis, personal communication.

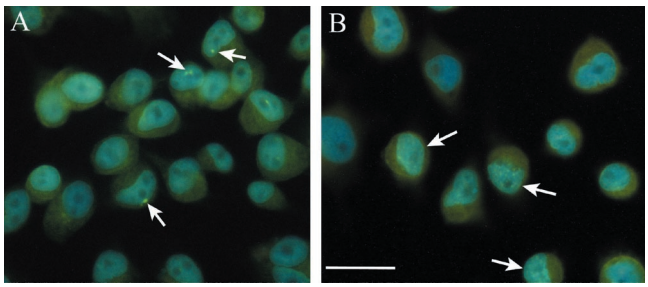

FIG. 5. **Subcellular localization of X53BP1 during the DNA damage response.** XTC cells were processed for indirect immunofluorescence with αX53BP1 as described under "Experimental Procedures" in the absence of DNA damage (*A*) or in the presence of 10 Gy of γ-IR (*B*). The *green* area (fluorescein isothiocyanate) represents staining specifically associated with αX53BP1, and the *blue* represents nuclear DNA staining with Hoescht 33258. The *scale bar* represents 25 μm.

cence (IF) experiments with human 53BP1 revealed that the protein can be found in both the cytoplasm and nucleus (58). The nuclear immunostaining observed for 53BP1 in these studies revealed that the protein could be found in a homogenous or punctate pattern. However, because these studies were done with cells that had been transfected with 53BP1, the subcellular localization of the endogenous protein could not be unambiguously determined. We used IF to determine the subcellular localization of the endogenous frog X53BP1 protein in XTC cells. Asynchronous cells were grown to 60% confluency on polylysine-coated coverslips and processed for IF analysis with affinity-purified αX53BP1 and a fluorescein-conjugated secondary IgG. Staining with either secondary antibody alone or with other nonspecific IgG molecules produced no signal (not shown). This, in conjunction with the fact that αX53BP1 recognizes one major band in XTC extracts (Fig. 3*A*), suggests that the immunostaining data specifically represents X53BP1. The staining pattern for X53BP1 agrees with the previously reported pattern for human 53BP1 (58). X53BP1 possesses a diffuse cytoplasmic staining pattern (Fig. 5, *A* and *B*). Moreover, X53BP1 appears to localize to a small number of large nuclear foci (1–3) in about 20% of the cells (Fig. 5*A*, see *arrows*). To observe any changes in the subcellular localization of X53BP1 that may occur in response to DNA damage, we exposed XTC cells to 10 Gy of γ-IR. After a recovery period, the cells were processed for IF. After examining a few hundred cells, it appears that many more, smaller foci are present in nuclei that have been exposed to the DNA-damaging agent (Fig. 5*B*, see *arrows*), suggesting that X53BP1 is redistributed within the nucleus in response to γ-IR. These foci are absent in nonirradiated samples. Since about 20% of the examined cells possess multiple nuclear foci, this suggests that the focal localization of X53BP1 may occur at a discrete point(s) within the cell cycle (*i.e.* S phase).

ATM-dependent Phosphorylation of 53BP1—The sequences of both X53BP1 and 53BP1 from humans suggest that the proteins may serve as effective substrates for members of the ATM kinase family (*e.g.* ATM, ATR, and DNA-PK). Both the frog and human 53BP1 proteins possess numerous (S/T)Q motifs, which are known to be the minimal, essential determinants for recognition by ATM family members (59). X53BP1 possesses 32 of these potential recognition motifs, whereas the human ortholog has 30 such sites. Several of these sites are clustered in the N-terminal region of X53BP1 (Fig. 2*A*). Such clustering is also evident in the N-terminal region of human 53BP1 (Ref. 58; Fig. 2*A*). Clustered (S/T)Q motifs have been suggested to formulate a strong recognition determinant for ATM and its related PI-like kinases (11, 59). Given these potential phosphorylation sites and the fact that X53BP1 is phosphorylated in response to γ-IR DNA damage, we asked if X53BP1 could serve as a substrate for the ATM kinase. It has been well established that ATM is activated in response to this type of genotoxic insult (7, 8). To show this, we generated a series of GST fusion proteins spanning X53BP1 (Fig. 5). Such an approach has been previously adopted to demonstrate the ATM and Chk2-dependent phosphorylation of BRCA1 (11, 77). To perform this assay, 293T cells were transfected with a FLAG-tagged cDNA encoding either the wild type ATM gene (WT) or a kinase-dead (KD) version of the enzyme (7, 8). After 48 h, the cells were harvested and lysed in TGN buffer (8). After a preclearing step with protein G-agarose beads, either WT or KD ATM protein was immunoisolated, extensively washed as described (8, 11), and incubated with candidate GST-X53BP1 fusion substrate proteins in the presence of radiolabeled ATP.

Our results demonstrate that GST fragment 1 (GST-XBP1–1) and GST-XBP1–4 serve as good *in vitro* substrates for ATM but not the KD version of the protein (Fig. 4). On the other hand, GST-XBP1–2, GST-XBP1–3, and GST-XBP1–5 appear to be essentially refractory to ATM-dependent phosphorylation in this assay despite the fact that these sequences contain several potential ATM recognition sites. GST-XBP1–1 encodes for aa residues 1–338 in X53BP1, a region of the protein that contains 11 putative ATM phosphorylation sites (Fig. 2*A*). Interestingly, GST-XBP1–1 migrates around 100 kDa on 6% SDS gels despite the fact that its predicted molecular mass is ~65 kDa. Such anomalous behavior in SDS gels has also been observed for human 53BP1 (Ref. 58; Fig. 6). X53BP1 has a predicted mass of 231 kDa but migrates at ~300 kDa (Figs. 3*A* and 4). Phosphorylation of GST-XBP1–1 by ATM appears to generate a doublet pattern, suggesting that the enzyme targets multiple residues within the first 338 amino acids of X53BP1 (Fig. 4). In contrast, we find that ATM-dependent phosphorylation of GST-XBP1–4, a fusion protein encoding X53BP1 residues 1201–1657, generates an apparent singlet, despite the fact that there are eight (S/T)Q motifs within this region. It is interesting to note that four of the potential phosphorylation sites of GST-XBP1–4 reside in a cluster spanning residues 1272–1327 (not shown). Taken together, these results clearly demonstrate that X53BP1 is phosphorylated *in vitro* at multiple places by the ATM kinase.

To further investigate the relationship between ATM phosphorylation and 53BP1, we created a GST fusion protein between human 53BP1 residues 1–524 (HBP1–524) and assayed for its potential to be recognized as a substrate by the ATM kinase. Human 53BP1 possesses 11 potential ATM kinase recognition motifs within this region of the protein (Ref. 58; Fig. 2*A*). Additionally, HBP1–524, like its amphibian counterpart, migrates much slower than its predicted molecular weight. Thus, N-terminal sequences of both the frog and human 53BP1 proteins contribute to the anomalous mobility that has been observed for these proteins in SDS gels (58). Nevertheless, HBP1–524 serves as a very good substrate for ATM in our *in vitro* assay and appears as a phosphorylated doublet (Fig. 7*A*). Because the N-terminal regions of both *Xenopus* and human 53BP1 proteins appear to be phosphorylated by ATM *in vitro*, we assessed the ability of the N-terminal region of human 53BP1 to serve as a potential substrate for ATM *in vivo*. Human 53BP1 residues 1–524 were cloned in frame into pFLAG-NLS lox (11), generating plasmid pZX-12. This plasmid expresses residues 1–524 fused to an N-terminal nuclear localization signal and to a FLAG epitope tag (FLAGBP1–524). As previously seen for this segment of 53BP1, H53BP1–524 also ran aberrantly on SDS gels. ATM-dependent phosphorylation was assessed in a transient transfection assay using 293T

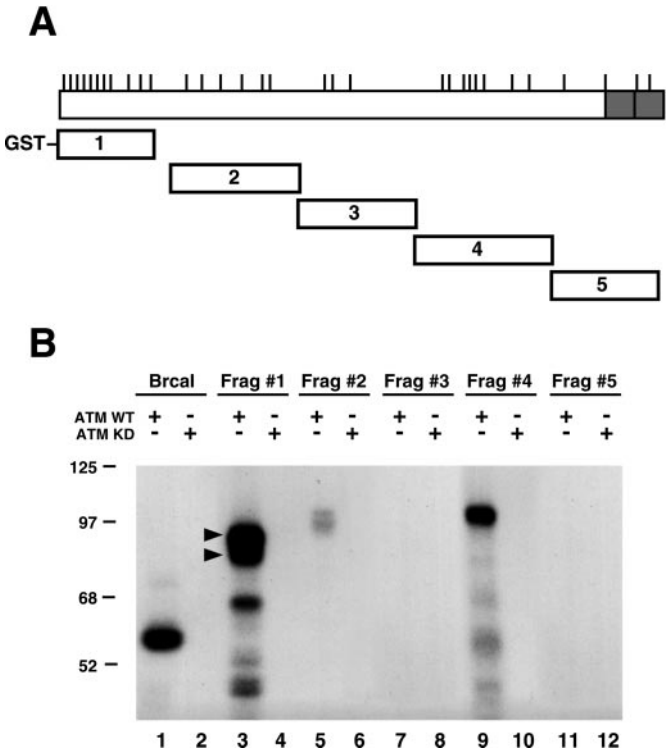

FIG. 6. **ATM-dependent phosphorylation of X53BP1 *in vitro*.** *A*, schematic representation of GST-XBP1 fusion substrates utilized in an assay for ATM kinase activity (7, 8). *Hatched lines* represent approximate positions of (S/T)Q motifs. Five fusion proteins as shown were created for the assay. The following aa residues are encoded for by the GST-XBP1 fusion proteins. GST-XBP1–1, aa 1–338; GST-XBP1–2, aa 393–821; GST-XBP1–3, 815–1206; GST-XBP4, 1201–1657; GST-XBP5, 1652–2,055. *B*, affinity-purified fusion proteins were processed for ATM kinase assays (7, 8) with either wild type ATM (*odd lanes*) or a KD version (*even lanes*) as shown. As a control, a GST fusion protein with Brca1 residues 1351–1552 was used (*lanes 1* and *2*; Ref. 11). The *arrows* in *lane 3* represent discrete phosphorylated species of GST-XBP1–1. Molecular mass markers are indicated on the *left* in kilodaltons. Lower molecular weight species represent degradation products that copurify from *E. coli* lysates.

cells. These cells were transfected with pZX-12 alone or in combination with plasmids encoding either the ATM WT or KD protein. After 48 h, the cells were exposed to either 0 or 50 Gy of γ-IR with a cesium irradiator, allowed to recover, and then processed for immunoprecipitation and immunoblotting analysis with the M2/M5 FLAG-specific antibodies. If 53BP1 is phosphorylated by γ-IR activated ATM, then slower mobility forms of 53BP1 might be expected to be seen in the form of shifts in the molecular weight of the protein. In the absence of γ-IR, pZX-12-transfected 293T cells were found to generate multiple forms of H53BP1–524 (Fig. 7*B*, *lane 1*), reminiscent of the behavior often observed for X53BP1 in cell-free extracts (Fig. 3*A*). Because these bands are reversible through treatment with λ phosphatase enzyme (Fig. 7*B*, *lane 2*), it appears that H53BP1–524 is phosphorylated in its basal state by endogenous enzymes within 293T cells. Despite this background level of phosphorylation for FLAGBP1–524, higher molecular weight, shifted forms of the protein are clearly apparent in γ-IR-treated samples that have been cotransfected with ATM WT but not with a plasmid encoding the kinase-defective enzyme (Fig. 7*B*, *lane 7*; see *arrow*). Such proteins often appear as diffuse species. We confirmed that these proteins are phosphorylated species of 53BP1, since treatment with λ-phosphatase completely reverses the induced mobility shift. Collectively, our results suggest that 53BP1 is a targeted by ATM during the DNA damage response.

DISCUSSION

The accurate replication and segregation of the genetic material is tightly controlled during the cell cycle. It has become increasingly clear that cells that cannot properly coordinate these processes within the framework of the cell cycle place themselves at great risks for developing chromosomal instabilities. Consequently, the faithful transmission of the genetic material will be compromised, leading to a number of potential pathologies including cancer (2). Cells respond to various forms of DNA damage by halting cell cycle progression and activating the machinery responsible for mending disabled DNA (1, 31, 32). One form of DNA damage is DSBs, which occur spontaneously during S phase, meiosis, and V(D)J recombination and through environmental agents such as γ-IR. As such, recent advancements into the mechanisms of checkpoint control in a range of organisms from yeast to humans have led to unexpected connections between DNA recombination/repair proteins and cell cycle control (22). The inability to repair DSBs poses a significant threat to genomic instability. Such genetic frailties have been observed in cells with mutations in a variety of genes encoding proteins that participate in the biological response to this type of DNA damage. Many of the proteins involved in the biological response to γ-IR are controlled in some manner through ATM-dependent phosphorylation. ATM phosphorylates numerous substrates, many of which participate in cell cycle control and repairing DSBs. These include BRCA1, Cds1/Chk2, p95/Nbs1, and p53 (7, 8, 10, 11, 13, 14, 77). In this study, we have characterized 53BP1 proteins from both frog and human. Our data classify 53BP1 as a component in the DNA damage response pathway.

We identified X53BP1 in a genetic screen designed to uncover suppressors of mitotic catastrophe. We observed that X53BP1-expressing cDNAs could rescue SP984 as effectively as *wee1*, the natural complementing gene. X53BP1 cDNAs were found to rescue SP984 only when expressed under the highly inducible *nmt1* promoter, implying that high levels of X53BP1 are responsible for the rescued phenotype. Additionally, all rescuing cDNAs encoded for X53BP1 derivatives possessing the C-terminal BRCT repeats. Thus, it is possible that overexpression of these repeated BRCT motifs leads to the titration of a cellular factor(s) that can either directly or indirectly influence the activity of Cdc2 and hence the ability of SP984 to undergo premature mitosis. Overexpression of X53BP1 in SP984, however, is not sufficient to restore the replication checkpoint defect associated with the strain as the rescued cells undergo a mitotic catastrophe when exposed to hydroxyurea. It is therefore unlikely that X53BP1 suppresses the growth defect of SP984 through the activation of the replication checkpoint. Thus, the precise reasons for the ability of X53BP1 to rescue SP984 remain unknown, although it is interesting to note that a yeast strain deficient in a checkpoint mechanism can be rescued by a protein that itself appears to function in a checkpoint response to DNA damage. Whatever the case, it is clear that X53BP1, when expressed in SP984, influences the growth of the yeast. Perhaps this accounts for its fortuitous isolation. Interestingly, it has been reported that the C-terminal BRCT repeats of BRCA1 can also confer growth-suppressing properties in budding yeast (73). Although the mechanism of this suppression is unknown, it was shown to require intact BRCT motifs. Despite these observations, the transformation of pC535 into wild type yeast cells does not impair the growth of the strain.

Unlike human 53BP1 transcripts, X53BP1 is highly expressed in ovarian tissue as revealed by Northern blotting with *Xenopus* total RNA. Consequently, X53BP1 is abundant in unfertilized eggs. The levels of X53BP1 do not appear to be cell

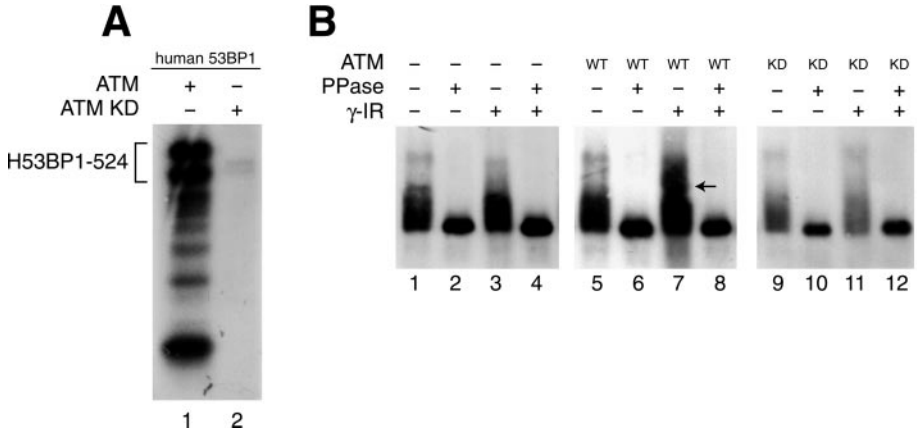

FIG. 7. **ATM-dependent phosphorylation of human 53BP1 *in vitro* and *in vivo*.** *A*, *in vitro* kinase assay of a GST fusion protein with GST and human 53BP1 aa 1–524 (HBP1–524). 1 μg of HBP1–524 was assayed as a substrate for either wild type ATM (*lane 1*) or a KD version of the enzyme (*lane 2*). Lower molecular weight species are the result of degradation products commonly associated with GST fusion proteins. *B*, *in vivo* assay for ATM-dependent phosphorylation of pZX-12-encoded FLAGBP1–524. 293T cells were transfected with 2.5 μg of pZX-12 alone (*left panel*) or with an equal amount of ATM-WT (*middle panel*) or ATM-KD (*right panel*) encoding plasmids. Some samples were treated with 50 Gy of γ-IR (*lanes 3*, *4*, *7*, *8*, *11*, and *12*). FLAGBP1–524 was isolated by immunoprecipitation and evaluated for modification by electrophoretic mobility shifts in 6% SDS gels in the absence (*odd lanes*) or presence of 1.0 units of λ-phosphatase (*even lanes*). The *arrow* represents FLAGBP1–524 shifted bands.

cycle-regulated during early development as judged by immunoblotting of cell-free extracts staged at either mitosis or interphase. We do observe, however, that the diffuse banding pattern of X53BP1 in egg extracts is quite different from the compact one seen for the somatic form of the protein. Whether this reflects alternate forms of X53BP1 (*i.e.* isoforms or post-translationally modified forms) remains to be determined. Because of the abundance of the protein, X53BP1 can be readily detected throughout the cell cycle in complexes with Xp53 through reciprocal coimmunoprecipitation experiments. It appears that a very large fraction of the embryonic X53BP1 is associated with Xp53 as judged by depletion analysis.[3] Previously, it has been observed that many proteins involved in early development are abundant in unfertilized eggs. This includes factors responsible for the rapid cell divisions that take place in early development, including DNA replication initiation proteins such as Xorc2, Xcdc6, and RPA, as well as the Xp53 transcription factor (56, 63, 74, 75). We anticipate that the large maternal stores of X53BP1 and Xp53 reflect a previously unappreciated role for these proteins during development. During early *Xenopus* development, zygotic transcription does not occur until the midblastula transition (70), eliminating any role for Xp53 as a transcription factor during this window of time. Checkpoint response mechanisms are not established until after midblastula transition, since treatment of embryos staged prior to this with DNA-damaging agents does not elicit a cell cycle arrest (76, 81). Nevertheless, previous studies have implicated Xp53 in frog development (67, 71). Based upon our findings that X53BP1 and Xp53 form complexes during this time period, it is possible that X53BP1 may influence the *Xenopus* developmental program and/or the biology of p53. Unexpectedly, we failed to observe an interaction between X53BP1 and Xp53 in somatic XTC cells. Similar observations have been made regarding 53BP1 and p53 in human cell lines.[2] Thus, for reasons presently unclear, the association between X53BP1 and Xp53 appears developmentally regulated or restricted to germline tissue.

Our findings establish X53BP1 as a protein that is phosphorylated in response to DNA damage. In particular, we have demonstrated that γ-IR, an agent known to create chromosomal breaks, can induce the phosphorylation of X53BP1 *in vivo*. We also provide evidence that the N-terminal region of human 53BP1 is phosphorylated in response to γ-IR *in vivo*. Our data suggest that at least two kinases phosphorylate X53BP1 as the protein is phosphorylated in both the absence and the presence of γ-IR DNA damage. Both the frog and human 53BP1 orthologs contain numerous phosphorylation sites for ATM kinase family members, particularly in their N-terminal regions (59). This includes ATM, ATR, and DNA-PK. Our evidence implicates ATM as one kinase that phosphorylates both the *Xenopus* and human 53BP1 proteins *in vitro*. The good *in vitro* specificity by ATM toward GST-XBP1–1 and GST-XBP1–4 suggests that these regions are promising targets of the kinase *in vivo*. Moreover, it appears that ATM can also phosphorylate an N-terminal domain of human 53BP1 in a DNA damage-dependent manner *in vivo*. Whether 53BP1 is also targeted by ATR or DNA-PK remains to be determined. Collectively, our data establish X53BP1 as a protein that participates in the response to γ-IR DNA damage.

Immunofluorescence experiments in asynchronous XTC cells reveal that X53BP1 is diffuse within the cytoplasm but present in the nucleus of a subset of cells in the form of a few large foci. Upon exposure to γ-IR, these foci appear to disperse and redistribute into smaller ones throughout the nucleus. We observed that this phenomenon occurred reproducibly in about 20% of the cells, perhaps indicating that the γ-IR-dependent dispersal of X53BP1 nuclear foci may occur at a discrete stage within the cell cycle, such as S phase. Presently, we cannot prove that X53BP1 phosphorylation and its dispersal into smaller nuclear foci in response to γ-IR are related. Such X53BP1 foci increase in number in response to γ-IR possibly as a result of the protein being localized to sites of newly created DNA damage. Previous studies with BRCA1 have demonstrated that the protein disperses from nuclear foci as a function of various DNA-damaging agents, including γ-IR (15). The DNA damage-dependent dispersal of BRCA1 foci has been shown to correlate very well with the phosphorylation of the protein (15). Notably, the Cds1/Chk2 effector kinase has been shown to be responsible, at least in part, for the dispersal of BRCA1 nuclear foci in response to DNA damage (77). We hypothesize that an analogous mechanism exists between 53BP1 phosphorylation and its subcellular localization and are currently designing experiments to test this notion.

In some respects, the general framework for the biological response to DNA damage appears conserved from fungal systems to human. DNA damage-sensing proteins activate PI

[3] J. C. Morales and P. B. Carpenter, unpublished data.

kinase-like enzymes that transduce the damaged-DNA signal to either effector kinases and/or transcription factors (1, 35). The end result is an attenuation in cell cycle progression and the activation of the DNA repair apparatus. Thus, many of the gene products identified through yeast genetics might also be expected to have conserved orthologs in higher eukaryotes. This appears to be the case for many of the DNA damage sensors (budding yeast/fission yeast Mec3/Hus1, Ddc/Rad9, and Rad17/Rad1), PI 3-kinase-like transducers Mec1/Rad3, effector kinases Chk1/Chk1 and Rad53/Cds1, and many aspects of the DNA repair machinery (1, 9, 25–30, 78, 80). Although budding yeast does not utilize p53-based mechanisms for cell cycle arrest, it activates analogous transcription-coupled repair processes through Rad53 and Dun1 (1). Given this, one might wonder how 53BP1 fits into any such generalized scheme, since no obvious orthologs of the protein appear in the yeasts. However, 53BP1 contains homology to previously identified yeast DNA damage response proteins only through its BRCT motifs. This most notably includes budding yeast Rad9 and Dbp11 and fission yeast Crb2 and Cut5 (1, 31, 52), all of which are thought of as upstream regulators in the DNA damage response (1, 52). If 53BP1 can be thought of in a similar manner, then it is conceivable that the protein is part of the sensing mechanism for DSBs that is transduced to ATM and/or its related family members. We have observed that X53BP1 becomes phosphorylated shortly after DNA damage (within 5 min),[3] consistent with the protein functioning as part of the initial biological response to γ-IR. Whatever the case, it appears that 53BP1 is an important player in the biological response to γ-IR DNA damage and possibly other types of genotoxic stress. The elucidation of its precise role in these processes awaits further investigation.

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