Frag1, a homolog of alternative replication factor C subunits, links replication stress surveillance with apoptosis

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We report the identification and characterization of a potent regulator of genomic integrity, mouse and human *FRAG1* gene, a conserved homolog of replication factor C large subunit that is homologous to the alternative replication factor C subunits Elg1, Ctf18/Chl12, and Rad24 of budding yeast. *FRAG1* was identified in a search for key caretaker genes involved in the regulation of genomic stability under conditions of replicative stress. In response to stress, Atr participates in the down-regulation of *FRAG1* expression, leading to the induction of apoptosis through the release of Rad9 from damaged chromatin during the S phase of the cell cycle, allowing Rad9–Bcl2 association and induction of proapoptotic Bax protein. We propose that the Frag1 signal pathway, by linking replication stress surveillance with apoptosis induction, plays a central role in determining whether DNA damage is compatible with cell survival or whether it requires cell elimination by apoptosis.

genomic integrity | Bcl2 | Rad9 | Atr | Rb

Replicative stress causes replication fork stalling or arrest, which can occur in yeast at naturally occurring sequences, such as replication fork barriers and replication slow zones (1). When damage is severe or the natural order of DNA replication is perturbed, DNA double-strand breaks can occur (2). Such events can trigger cellular checkpoints, allowing time for repair of damage before cell cycle progression (2). When the breaks are fixed or the damage is compatible with cell survival, double-strand breaks can give rise to the fixed chromosomal aberrations observed in cancer cells, such as translocations, inversions, amplifications, and deletions. Accumulated aberrations of caretaker pathways in concert with alterations of gatekeeper tumor suppressors give rise to transformed cells that acquire selective growth and survival advantages (3). Thus, the pathology of stalled or collapsed replication forks is important for understanding the role of faithful regulation of replication in preventing carcinogenesis.

Genotoxic stress-induced replication stalling activates checkpoint-signaling pathways that block cell cycle progression, control DNA repair, or trigger apoptosis (4) through membrane death receptors and the endogenous mitochondrial death pathways (5). Rad9 protein is involved in the control of the DNA damage-induced checkpoint (6). Studies in yeast and human cells have shown that Rad9 interacts with Hus1 and Rad1 in the 9-1-1 complex, which is a heterotrimeric complex and acts as a proliferating cell nuclear antigen-like sliding clamp (4, 7). In response to DNA damage, the 9-1-1 complex is loaded around DNA lesions by Rad17, which binds to chromatin before damage (8) and facilitates Atr-mediated phosphorylation and activation of Chk1 kinase to arrest cell cycle. Rad9 can participate in signaling apoptosis by interacting with antiapoptotic Bcl-2 family proteins Bcl-2 and Bcl-X_L but not with proapoptotic Bax and Bad (9). The interaction of Bcl2 with Bax prevents Bax from inducing cytochrome *c* release and cell death, and the Bax/Bcl2 ratio is crucial for regulation of apoptosis (10). Because the 9-1-1 clamp is also involved in DNA repair (7), the Rad9 complex is thought to play a key role in coordinating multiple functions of checkpoint activation, DNA repair, and apoptosis.

In this study, we report the identification and characterization of the *FRAG1* gene, which encodes a 1,820-aa mouse and 1,844-aa human conserved, uncharacterized protein homolog of the large replication factor C (RFC) subunit Rfc1 (861 aa) and the alternative RFC subunits Elg1 (791 aa), Ctf18/Chl12 (741 aa), and Rad24 (659 aa; Rad17 in human) in budding yeast. Elg1 (enhanced levels of genome instability), a RFC homolog, which forms an alternative RFC complex with Rfc2–Rfc5, was discovered through budding yeast genome-wide synthetic genetic interaction screening of mutants of replication fork-progression genes (11) and through the study of mutants exhibiting high levels of Ty recombination (12, 13). The Elg1 complex is distinct from RFCs for DNA replication, the DNA damage checkpoint, and sister chromatid cohesion (11–14). We have now isolated the mammalian *FRAG1* gene, characterized the function of Frag1 protein in higher eukaryotes, compared it with homologous DNA replication and damage response proteins of simpler organisms, and shown that it is involved in a Rad9-related damage checkpoint, a pathway that is important in determining whether DNA damage will be tolerated or whether the damaged cells will be eliminated by apoptosis.

Materials and Methods

Cell Culture. For synchronization by double thymidine block, after culture in medium with 10% FCS/DMEM containing 2.5 mM thymidine for 24 h (the first block), cells were washed with PBS, grown for 10 h in fresh DMEM/10% FCS, cultured 16 h in 2.5 mM thymidine (the second block) and then incubated as indicated without thymidine. Flow cytometric analysis after BrdUrd incorporation showed that >90% cells entered S phase 2–8 h after release. Cell viability was assessed by visualization of cell morphology, trypan blue, or erythrosine B exclusion, Hoescht 33342 vital staining, and flow-assisted cytometric analysis.

Genotoxic Stress and Colony Assay. For synchronized cells, 0.4 μM aphidicolin (Sigma) in 0.2% DMSO was included in the thymidine medium for 16 h of the second synchronization. Medium was exchanged for thymidine-free medium containing 2.2 μM caffeine (Sigma) and 0.4 μM aphidicolin for an indicated period. For DNA

Abbreviations: MEF, mouse embryonic fibroblast; MMS, methyl methanesulfonate; RFC, replication factor C; siRNA, short interfering RNA.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY557610 and AY557611).

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damage, the DNA alkylating agent methyl methanesulfonate (MMS) (15) was added in the medium at indicated conditions. For UV irradiation, 60–70% confluent monolayer cells were irradiated with UV-C emitted by germicidal lamps (GL-15, NIPPO Electronic, Tokyo, Japan) emitting at predominantly 254 nm. For colony assay, cells were cultured in medium with MMS for 1 h, washed, and plated in DMEM/10% FBS with 1.5% methylcellulose; colonies were counted 10 days later. For radiation, cells were exposed to ^{137}Cs [661 keV (1 eV = 1.602×10^{-19} J) at indicated doses] and assessed as indicated.

Plasmids and Small Interfering RNAs (siRNAs). pcDNA4V5 DNA (Clontech), was ligated in-frame with F1 (nucleotide positions from the first coding methionine, 1–1440), F2 (1400–1839), F3 (1794–3177), F4 (2697–3975), or FZ (3972–5535) DNA fragments of human Frag1 cDNA. Wild-type pBJF-FLAG-ATR (pBJF-FLAG-ATRwt), kinase-dead pBJF-FLAG-ATR (pBJF-FLAG-ATRkd) [from S. Schreiber (Harvard University, Cambridge, MA) and K. Cimprich (Stanford University, Stanford, CA)], HA-Rad9, Flag–N-terminally deleted Rad9 [Rad9-δN; from H-G. Wang (University of South Florida, Tampa)], and pCAGGS-hbcl-2 [from Y. Eguchi and Y. Tsujimoto (Osaka University, Osaka, Japan)] were used for transfection. GST-fusion (Amersham Pharmacia) was used for protein expression.

Construction of siRNA-expression plasmids was based on the U6 siRNA expression vector (Takara, Mie, Japan), which includes a mouse U6 promoter, a puromycin-resistance gene, and two BspMI sites. Two sets of the sense and antisense oligonucleotides (Table 1, which is published as supporting information on the PNAS web site) were annealed and ligated into the vector. U6 siRNA-Frag1 plasmids were transfected into cells by using TransIT-TKO transfection reagent (Mirus, Madison, WI) and selected in 1 μg/ml puromycin. Colonies were picked, and expression was evaluated by RT-PCR and immunoblot analysis. siRNA expression vectors with EGFP antisense or without inserts were used as controls (Takara). Oligo siRNAs for mouse p73, Atr, and luciferase were used as recommended (Santa Cruz Biotechnology).

cDNA Isolation and RNA Analysis. RNAs were extracted with a Qiagen (Valencia, CA) kit and cDNAs synthesized from 2 μg of poly(A)$^+$ RNA with Superscript II reverse transcriptase and oligo(dT) and random primers (Invitrogen). Differentially expressed genes were isolated with a cDNA subtraction kit (Clontech). After two rounds of hybridizations, cDNAs were amplified, ligated to vector, and sequenced.

For hybridization, 5-μg RNAs were fractionated by agarose gel electrophoresis, transferred to Nylon membrane, and hybridized with the following probes: cDNAs of the peptide coding region of *FRAG1* (N- and C-terminal), *RFC1*, *CTF18*, *DCC*, and *RAD17*, which were amplified by RT-PCR, subcloned, and sequenced. Filters were washed and exposed to x-ray film.

Protein Analysis and Fractionation. For immunoprecipitation, cells were harvested and 500-μg samples of cell lysates, after being precleared with protein G-Sepharose beads, were incubated with 3–4 μg of specific antibody overnight. Antigen–antibody complex was immobilized on protein G-Sepharose beads, and the beads were washed five times in lysis buffer. Bound proteins were eluted by boiling and subjected to SDS/PAGE and immunoblotting. Immunofluorescence staining and confocal analysis were performed by culturing cells in chambered slides, followed by methanol fixation, 0.05% Triton X-100 treatment, and staining with first and secondary antibodies. Primary antibodies used were anti-human p53 (BD Biosciences), phosphorylated p53 (Ser-15) (BD Biosciences), Mdm2 (Santa Cruz Biotechnology), Rb (BD Biosciences), Rad9 (Santa Cruz Biotechnology), phospho-H2AX (catalog no. 07-164; Upstate Biotechnology, Chicago), mitochondria (Chemicon), Bax (catalog no. 2772, Cell Signaling Technology; N-20, Santa Cruz Biotechnology), Atr (ab-2, EMD Biosciences, San Diego; catalog no. sc-1887, Santa Cruz Biotechnology), Orc2 (BD Biosciences), cytochrome *c* (Pharmingen), phospho-H2AX (catalog no. 05-636; Upstate Biotechnology), Grb2 (BD Biosciences), V5 (Invitrogen), Flag (Sigma), and actin (ICN, Irvine CA), which were detected with secondary antisera in an enhanced chemiluminescence system (ECL, Amersham Biosciences). Rabbit polyclonal anti-Frag1 antiserum was developed against peptide sequences mouse 345 CSLSDPENEQPVQKRKSN 362 and affinity-purified. *In vitro* transcription/translation was performed with a rabbit reticulocyte system (Amersham Biosciences) by labeling cDNAs cloned by RT-PCR amplification with [^{35}S]methionine (Amersham Biosciences). Proteins were incubated in 100 μl of binding buffer containing 150 mM NaCl, 0.1% Tween 20, 0.75 mg/ml BSA, 50 mM Tris·HCl (pH 8.0), 5 mM EDTA, and 10% (vol/vol) glycerol. For pulling down, glutathione–agarose bead-bound proteins were subjected to SDS/PAGE after being washed five times, and the gels were exposed to x-ray film. Cellular fractions were prepared as described in ref. 16.

Results and Discussion

Identification of FRAG1, a Gene Differentially Expressed After Replication Stress. DNA replication guarantees the duplication of the genome and requires concerted, dynamic changes of expression of specific gene products, which regulate the integrity of replication and surveillance of the genome for damage (17). When replication forks encounter damage in the DNA strands, stalling or arrest can result, leading to stimulation of the downstream checkpoint to initiate cell cycle arrest or apoptosis (1); however, the molecular mechanisms that sense stalled replication are not understood fully. To study differentially expressed genes in conditions of replication stress, synchronized mouse embryonic fibroblasts (MEFs) were exposed to aphidicolin, a DNA polymerase inhibitor, and harvested 4 h (in mid-S phase) after release from a double thymidine block. RNA was extracted from MEFs, and subtractive cDNA hybridization was performed to identify genes differentially expressed in the presence or absence of aphidicolin (Fig. 7*A*, which is published as supporting information on the PNAS web site). BLAST database searches indicated that 155 clones that we isolated and sequenced included 86 clones (55%) identical to mouse ESTs (>95% homologous over 200 bp). The 86 clones included redundant clones; 13 clones corresponded to an overlapped cDNA contig (denoted as FRAG1/N), seven clones corresponded to a contig (FRAG1/C), and five clones corresponded to *RFC1* cDNA. Interestingly, database searches indicated that FRAG1/N and FRAG1/C are located adjacent to each other (C130052G03Rik, GenBank accession no. XM_282980; Gm17, GenBank accession no. XM_111221) on mouse chromosome 11. Database searches for human orthologs of the mouse clones showed that the orthologs are parts of a continuous gene, FLJ12735 (GenBank accession no. NM_024857), at human chromosome 17q11.2. RT-PCR amplification indicated that those "two" mouse transcripts span a gene, suggesting that the two transcripts, FRAG1/N and FRAG1/C came from one gene, *FRAG1* (Ctf18/R*a*d24/Elg1-related gene 1). We have focused on characterization of the *FRAG1* gene.

Alteration of FRAG1 Expression. Northern blot analysis was performed with replication-related genes *RFC1, RAD17*, and *CTF18*, as well as FRAG1/N and FRAG1/C as probes. Synchronized MEFs were treated with aphidicolin or MMS (a DNA alkylating agent), agents that cause stalled DNA replication (15). Expression of FRAG1/N and FRAG1/C was markedly down-regulated by aphidicolin or MMS treatment, whereas the effect on *RFC1, Rad17*, and *CTF18* genes was less apparent after MMS treatment (Fig. 1*A*). RNA blot with cDNA probes of N- and C-terminal portions of *FRAG1* (FRAG1/N and FRAG1/C) (Fig. 1*C*) detected a predominant transcript of ≈9 kb expressed ubiquitously in 12 murine cell lines (Fig. 1*B*). To assess the stability of the *FRAG1* transcript,

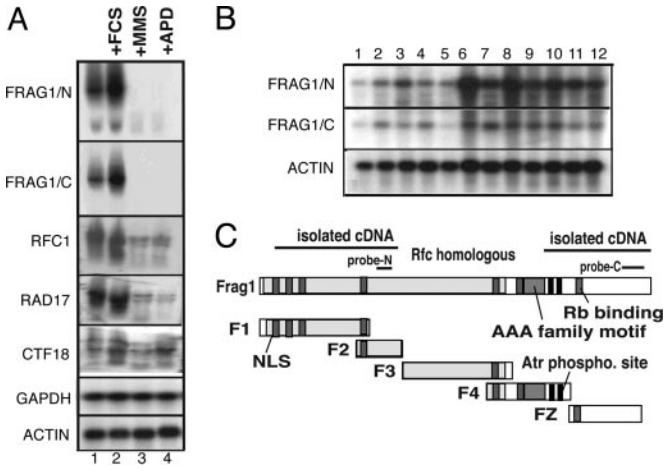

Fig. 1. Expression of the *FRAG1* gene. (*A*) RNA blot analysis. Synchronized MEFs were treated with aphidicolin, and 20 μg of each of the total RNAs were loaded on the gel, transferred to membrane, and hybridized to probes as indicated. (*B*) Frag1 expression in various mouse cell lines. Shown are poly(A)$^+$ RNA from the following sources: lane 1, PU5–1.8 (lymphoid tumor); lane 2, RAW264.7 (leukemia-virus induced tumor); lane 3, K-BALB (Kirsten murine leukemia virus-transformed fibroblast); lane 4, 3T3 (fibroblast); lane 5, L-M (murine L cells, transformed adipose connective tissue); lane 6, P19 (teratocarcinoma); lane 7, Hepa 1-6 (hepatoma); lane 8, R1.1 (T cell lymphoma); lane 9, L1210 (lymphocytic leukemia); lane 10, P388D1 (lymphoma); lane 11, P815 (mastcytoma); and lane 12, NB41A3 (neuroblastoma). (*C*) A map of Frag1 fragments. F1, F2, F3, F4, and FZ are cDNA fragments used in the study. Location of probes Frag1/N and Frag1/C in the cDNA are indicated. Rb binding motif LxCxE and two putative Atr-phosphorylation sites are located near a region homologous to the AAA family. Two locations of cDNA fragments, which were isolated through subtractive hybridization (nucleotide positions from the first methionine, 321–1835 and 3750–5388) are shown, and two probes for RNA blot analysis, which were synthesized by PCR amplification (nucleotide positions from the first methionine, 1580–1830 and 5130–5380) are shown. NLS, nuclear localization motifs.

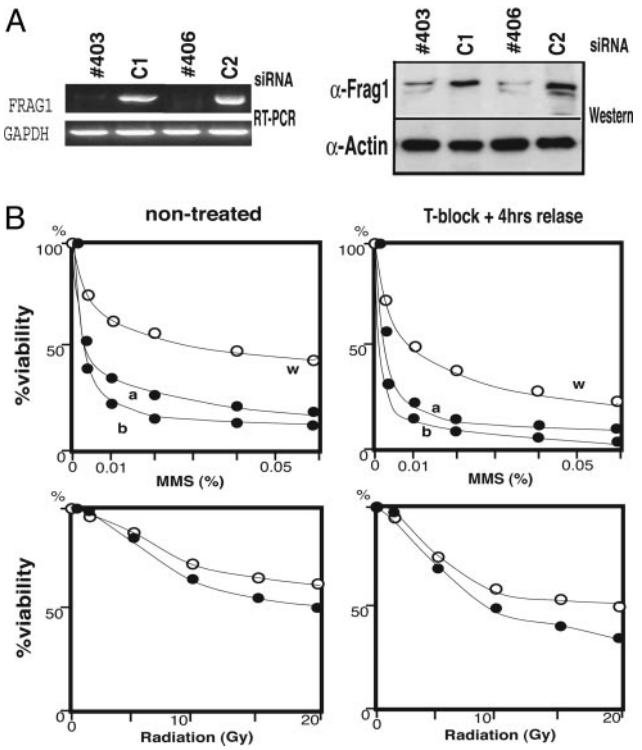

Fig. 2. Down-regulation of Frag1 expression sensitizes cells to replicative stress. (*A*) Down-regulation of *FRAG1* by siRNA. MEFs were transfected by U6 siRNA expression vector against FRAG1 and grown in selective medium. RNAs and protein lysates were extracted and analyzed by RT-PCR and immunoblot. Results of two independent experiments of *FRAG1* siRNA clones (#403 and #406) are shown. C, mock control siRNA. (*B*) Colony survival assay after exposure to MMS. Synchronized (*Right*) or asynchronized (*Left*) cells (1×10^6) were cultured in thymidine-free medium for 2 h to allow S-phase entrance. (*Upper*) MMS was added at the indicated concentrations for an additional 1 h, and cells were washed. (*Lower*) For radiation, cells were exposed at the indicated doses. Cells were plated in DMEM containing 1.5% methylcellulose, and colonies were counted 10 days after treatment. The percentage of survival was determined relative to the numbers of colonies from untreated cells. Lines labeled a and b indicate experiments with two independent siRNAs; lines labeled w indicate mock.

cells treated with actinomycin D to inhibit *de novo* RNA synthesis in medium with or without aphidicolin were harvested at serial time points and assessed for *FRAG1/N*, *FRAG1/C*, *CTF18*, *RFC*, and *RAD17* mRNA, indicating that the half-life of *FRAG1* mRNA appears to be <4 h after exposure to aphidicolin. In contrast, the half-life of *CTF18*, *RFC*, and *RAD17* mRNAs was >15 h, suggesting that *FRAG1* mRNA appeared less stable than transcripts of the other replication-related genes examined (Fig. 7 *B–E*).

Database searches indicated that the putative Frag1 protein has a conserved region homologous to a large subunit of RFC, which is considered an ortholog of the alternative RFC subunits, Elg1, Ctf18/Chl12, and Rad24 (Rad17 in fission yeast and human) of budding yeast (Fig. 1*C*) (11–14). Frag1 has a conserved AAA family motif, a hallmark of the ATPase family associated with various cellular activities, including chaperone-like functions that assist in the assembly, operation, or disassembly of protein complexes. Comparison of the mouse and human Frag1 amino acid sequences indicated that they conserve putative Atr-phosphorylation sites (mouse Frag1 at Ser-1150 and Ser-1168 and human Frag1 at Ser-1169 and Ser-1187) (18, 19), and a putative Rb binding site with a Leu-x-Cys-x-Glu (LxCxE) motif (amino acids 1409–1413 of mouse and 1428–1432 of human) (20).

Reduction of Frag1 Protein Increases Sensitivity to DNA Damage. Studies of budding yeast have shown that elg1Δ mutants are sensitive to DNA damage, suggesting that Elg1-RFC functions in the DNA damage response (11, 12). To study the effect of reduced expression of mammalian Frag1 protein, we performed siRNA experiments to inhibit expression of endogenous Frag1. RT-PCR and immunoblot study showed that cells transfected with the siRNA vector and selected in puromycin medium showed a marked reduction of Frag1 gene product (Fig. 2*A*). *FRAG1* siRNA transfectants were exposed to MMS or to γ-irradiation, and colony survival was assayed. Compared with control siRNA transfectants, two independent *FRAG1* siRNA transfectants showed enhanced sensitivity to replication stress, which was apparent in synchronized MEFs, suggesting that the damage activated the S phase checkpoint (Fig. 2*B*). The difference between siRNA knock-down and control cells after MMS treatment is more pronounced than differences observed after γ-irradiation. It is suggested that reduction of Frag1 increased the sensitivity to MMS.

Frag1 siRNA Inhibition Leads to Activation of Caspase and BAX. Immuunoblot analysis of Frag1 protein expression showed that Frag1 was reduced 2–6 h after exposure to aphidicolin or MMS, a reduction more rapid than for actin or Rad17 in MMS (Fig. 3*A*). Because involvement of Frag1 in cellular responses to DNA damage is suggested, we assessed the activation of proapoptotic proteins. Immunoblot analysis showed caspase 7 activation in *FRAG1* siRNA transfectants but not in control siRNA transfectants (Fig. 8*A*, which is published as supporting information on the PNAS web site). Bax protein expression with slow mobility was induced in two independent *FRAG1* siRNA transfectants 8 h after release from double thymidine cell cycle block and was markedly induced in

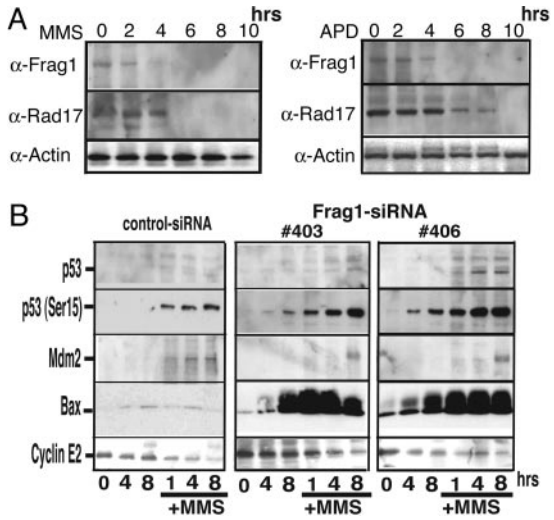

Fig. 3. Frag1 is involved in genotoxic response. (*A*) Frag1 down-regulation by genotoxic stress. MEFs were cultured in medium with 0.4 nM aphidicolin or 0.01% MMS for the indicated times. Cells were harvested, and lysates were subjected to SDS/PAGE and immunoblot analysis with antisera as indicated. (*B*) Frag1 knock-down sensitizes cells to genotoxic stress. Two independent Frag1 siRNA MEF clones (#403 and #406) synchronized in G_1 and grown in thymidine-free medium with or without exposure to MMS were harvested at the indicated times after release in thymidine-free medium. Protein lysates were immunoblotted with antibodies as indicated. Mismatched siRNA served as control.

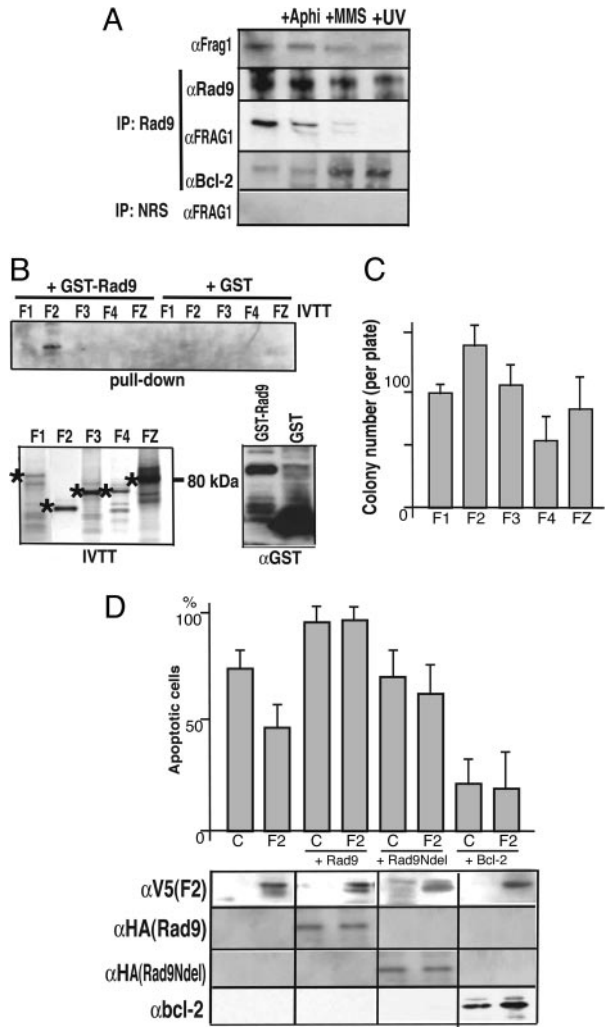

Fig. 4. Frag1 is involved in the Rad9–Bcl2 pathway. (*A*) Coimmunoprecipitation of Frag1, Rad9, and Bcl2. MEFs were grown in medium with 0.4 μM aphidicolin (Aphi) or 0.01% MMS for 24 h or exposed to 8 J/m^2 UV radiation and cultured for 24 h before harvesting. The leftmost lane is without treatment. Protein lysates were extracted and immunoprecipitated (IP) with anti-Rad9 or normal rabbit serum (NRS), followed by immunoblot with Frag1, Rad9, or Bcl2 antisera. (*B*) Pull down of *in vitro* transcribed and translated (IVTT) F1, F2, F3, F4, and FZ fragments of Frag1 by GST–Rad9 fusion protein. I*n vitro* transcribed and translated products were labeled with [^{35}S]methionine and incubated with GST–Rad9 fusion protein. The bound samples were pulled down with glutathione-agarose beads, which were subjected to SDS/PAGE, and gels were exposed to x-ray film. (*Upper*) Pull-down assay. (*Lower Left*) PAGE and exposure of *in vitro* transcribed and translated F1, F2, F3, F4, and FZ fragments (input), shown by asterisks. (*Lower Right*) Immunoblot with anti-GST. (*C*) Assay of colony survival of MEF transfectants after MMS exposure. MEFs transfected with pcDNA expression vector with F1, F2, F3, F4, or FZ cDNA and selected in G418 medium were subjected to colony survival assay, similarly to that shown in Fig. 2*B*. Error bars show standard deviations. (*D*) Cell death after MMS exposure. (*Upper*) Rad9, Rad9-δN (Rad9Ndel), and Bcl2 plasmids were introduced with selection plasmids in F2 transfectants and grown in selection medium for hygromycin resistance. Apoptotic cells were evaluated 48 h after MMS exposure by erythrosine B staining exclusion. (*Lower*) Immunoblot with anti-V5 tag (F2), anti-HA tag (Rad9), anti-HA tag (Rad9Ndel), and anti-Bcl2 antisera.

those *FRAG1* siRNA transfectants at all times after MMS exposure. In sharp contrast, Bax induction was not apparent in control siRNA transfectants in the conditions examined (Fig. 3*B*).

Upon activation by DNA damage-induced or oncogene-induced signaling pathways, phosphorylation of p53 at Ser-15 increases its half-life, accumulation, and tumor suppressing activity (21). Phosphorylation of p53 at Ser-15 leads to reduced interaction of p53 with its negative regulator, the oncoprotein Mdm2, and impairs the ability of Mdm2 to inhibit p53-dependent transactivation (21). Our analysis of two independent Frag1 siRNA transfectants showed that phosphorylation of p53 at Ser-15 was induced in cells after MMS exposure and at 4 (Fig. 3*B*, #406) and 8 h (Fig. 3*B*, #403 and #406) without MMS. In control siRNA transfectants, phosphorylation of p53 at Ser-15 was induced in cells after, but not before, exposure to MMS, showing that, even without MMS, the reduction of Frag1 can stimulate Bax induction in synchronized cells (at 4 or 8 h), emphasizing that reduction of Frag1 sensitizes cells to genotoxic response. Alteration of Mdm2 expression was less apparent. Taken together with the observation by microscopy that cytochrome *c* was released from mitochondria when Frag1 expression was inhibited by siRNA or when cells were exposed to MMS (Fig. 8*C*), it is suggested that reduction of Frag1 may be required for sensitizing cells to DNA damage and activating Bax-related cell death.

p53 translocates to mitochondria, where it directly induces Bax activation and cytochrome *c* release upon DNA damage (22). To determine whether p53 is involved in the induction of Bax expression in our siRNA transfectants, Trp-53-deficient MEFs were analyzed. Results of siRNA Frag1 inhibition showed that Bax was induced in Trp-53$^{+/-}$ and Trp-53$^{-/-}$ transfectants of MEFs and phosphorylation of p53 at Ser-15 was increased in Trp-53$^{+/-}$ transfectants after exposure to MMS, suggesting that Bax was activated regardless of p53 status and that p53 is dispensable for Bax induction in the Frag1 replication stress pathway (Fig. 8*B*). Recently, two p53 homologues have been identified, p73 and p63, that have high amino acid identity, suggesting shared function (23). Indeed, like p53, p73 can trigger several promoters, including Bax and p21 promoters, and is able to trigger cell death in response to the DNA damage. Introduction of p73 oligo siRNA into Frag1 siRNA vector transfectants of Trp-53$^{-/-}$, reduced Bax induction (data not shown), suggesting a role for p73 in the stimulation of the Frag1—Bax pathway.

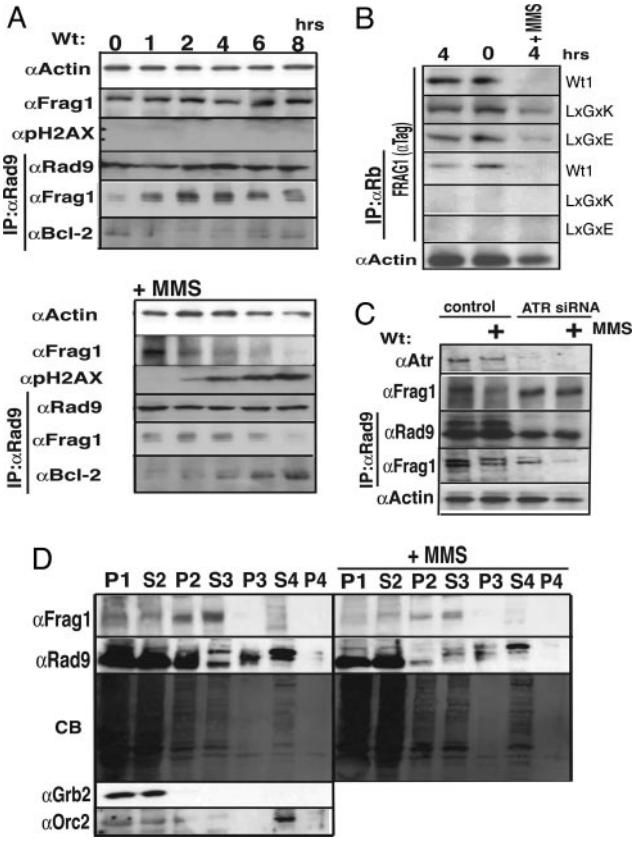

Fig. 5. Frag1 involvement in the DNA damage response. (*A*) Synchronized MEFs were cultured in growth medium with or without MMS (0.01%) for the indicated times, and cellular protein was extracted. Protein expression was studied by immunoblot with the indicated antibodies before and after immunoprecipitation (IP) with Rad9 antibody. (*B*) LxGxK, LxGxE, or wild-type (Wt) Frag1 transfectants that were synchronized at G_1 were released from G_1 in thymidine-free medium with or without MMS (0.01%) as indicated and subjected to immunoblot with anti-V5 (tag) or anti-actin antibody before and after immunoprecipitation with Rb antibody. (*C*) MEFs were transfected with Atr siRNA. A day after transfection, cells were cultured with or without MMS (0.01%) for 4 h, and protein lysates were immunoprecipitated and immunoblotted as indicated. (*D*) Subcellular localization of Frag1 and Rad9 after MMS treatment. Cellular components were fractionated from MEFs before and after exposure to MMS and subjected to immunoblot. Immunoblot with antibodies against Grb2 and Orc2, membranous and chromatin-bound proteins, are shown as controls. P1, whole-cell pellet; S2, cytosol and nucleosol; P2, detergent-insoluble nuclei; S3, DNase I-extracted nuclei; P3, DNase I-resistant fraction; S4, containing chromatin; P4, nuclear matrix. CB, Coomassie brilliant blue staining.

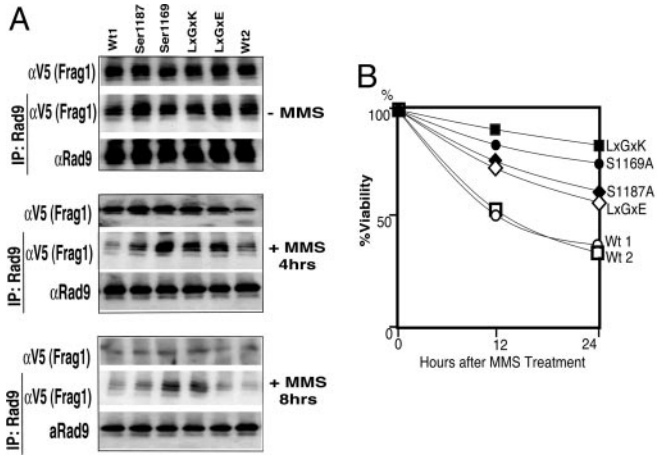

Fig. 6. The Frag1 response to DNA damage. (*A*) Association of Frag1 and Rad9 in response to DNA damage. Wild-type (Wt) and Frag1 mutant MEF transfectants synchronized at G_1 were released from G_1 in thymidine-free medium with 0.01% MMS for 4 or 8 h. Cellular lysates were extracted and subjected to immunoblot with anti-V5 (tag) or anti-actin antibody before and after immunoprecipitation (IP) with Rb antibody. (*B*) Viability of cells expressing wild-type and Frag1 mutants after MMS exposure. Wild-type and Frag1 mutant-expressing MEFs were cultured in medium with 0.01% MMS for the indicated times, and apoptotic cells were evaluated by erythrosine B staining exclusion.

Frag1 Associates with Rad9 and Is Involved in the Bcl2 Pathway. It was shown that proapoptotic Bax can form heterodimers with antiapoptotic Bcl2 in cells (24), which prevents Bax conformational changes required for apoptosis induction (10). Activated Bax proteins oligomerize and are stabilized in the mitochondrial membrane and induce cytochrome *c* release, an important process for the induction of cell death (10). After DNA damage, Rad9 plays a role in induction of apoptosis by associating with antiapoptotic Bcl2, which results in the inhibition of Bcl2 function (9). To investigate the Frag1 signal pathway, we have used coimmunoprecipitation analyses (Fig. 9*A*, which is published as supporting information on the PNAS web site) to define Frag1 associations with partner proteins involved in responses to replicative stress. Immunoblots probed with anti-Frag1 after immunoprecipitation with anti-Rad9 indicated their association in growing cells. Aphidicolin or MMS exposure resulted in reduced Frag1 expression and concomitant reduction of the association with Rad9. Conversely, an increase of Rad9 association with Bcl2 was observed after genotoxic stress (Fig. 4*A*). *In vitro* pull-down assay using recombinant GST–Rad9 fusion protein detected association with *in vitro* transcribed–translated Frag1-F2 fragment, corresponding to the RFC homologous region. Other Frag1 fragments did not associate with Rad9 (Figs. 1*C* and 4*B*), suggesting that the F2 fragment binds Rad9 and regulates apoptosis induction.

Colony formation assays of MEF transfectants expressing specific Frag1 peptides indicated that more colonies formed after F2 expression compared with other Frag1 peptides after genotoxic stress (Fig. 4*C*), suggesting that the F2 region of Frag1 functions to regulate apoptosis by interacting with Rad9. The stable F2 transfectants, in which MMS-induced apoptosis was inhibited, were transfected with Rad9 and selected for hygromycin resistance (Fig. 4*D Upper*, compare F2 and C). Overexpression of Rad9 in the F2 expressors caused an increase in apoptosis (Fig. 4*D*, +RAD9). Usage of Rad9-δN, which is defective in Bcl2 association (9), inhibited apoptosis (Fig. 4*D*, +Rad9Ndel). Furthermore, introduction of antiapoptotic Bcl2 into F2 transfectants resulted in profound inhibition of apoptosis (+Bcl2). Confocal microscopy showed that in F2 transfectants release of cytochrome *c* after exposure to MMS was inhibited (Fig. 9*B*). Results of these experiments strongly suggest that Frag1 modulates Rad9 association with Bcl2 and thereby induces DNA damage-induced apoptosis.

Atr Regulates Frag1–Rad9 Association and the Release of Rad9 from Frag1 in S Phase. To further define Frag1 function, we examined the cell cycle-dependence of Frag1 association with Rad9 in synchronized cells exposed to MMS. Association of Frag1 with Rad9 was weak in synchronized G_1 cells and increased in strength during progression into S phase. After exposure to MMS, the Frag1–Rad9 association was reduced, leading to an increase of Rad9–Bcl2 association (Fig. 5*A*). The data are consistent with the conclusion that Frag1 is involved in sensitizing Rad9 to genotoxic stress during S phase through their association. Confocal microscopic observation indicated that Frag1 and Atr are colocalized 8–12 h after exposure to MMS, whereas Frag1 seems to form foci before Atr focus formation (Fig. 10, which is published as supporting infor-

mation on the PNAS web site), suggesting a role for Frag1 in the Rad9 pathway via Atr response to DNA damage.

To study further the involvement suggested by the Frag1 motif search (Fig. 1*C*) of Atr and Rb in the Frag1–Rad9 pathway, we prepared wild-type and mutant human Frag1 expression vectors by substituting the putative Atr phosphorylation sites, Ser-1169 and Ser-1187 with Ala residues, and the Rb-binding site, LxCxE-1432 with LxGxK-1432 or LxGxE-1432. Transfected wild-type Frag1, but not LxGxE and LxGxK mutants, associated with Rb, as was more apparent in synchronized G_1 than S phase cells (Fig. 5*B*). After MMS, wild-type Frag1 expression was undetectable, whereas LxGxK, and to a lesser extent LxGxE, mutant proteins were detectable. The Frag1–Rb association was undetectable in wild type and two Rb-site mutants after MMS. In summary, it is suggested that Frag1 might play a role in pre-sensitizing cells to genotoxic stress during replication, i.e., in S phase, whereas Frag1 predominantly associates with Rb in G_1 phase.

To examine the role of Atr, endogenous Atr was inhibited by siRNA (Fig. 5*C*). Whereas MMS damage reduced endogenous Frag1 in control cells, reduction of Atr inhibited the down-regulation of endogenous Frag1 in response to DNA damage. Immunoprecipitation showed that, in response to MMS exposure, inhibition of Atr markedly reduced the association of Rad9 with Frag1, a reduction in siRNA ATR-treated cells that was more appreciable than in control cells. Thus, Atr stimulated two separable events: association of Rad9 with Frag1 and down-regulation of Frag1 in response to DNA damage.

Cellular components before and after MMS exposure were fractionated, and proteins were analyzed by immunoblot to study the translocation of Rad9 in response to DNA damage (Fig. 5*D*). After exposure to MMS, the amount of Rad9 in detergent-insoluble nuclei (P2) was significantly reduced, and the proportion of slow mobility Rad9 was increased in DNase I-extracted nuclei (S3), whereas reduction but not translocation of Frag1 was detected, suggesting that a predominant fraction of Rad9 translocated from chromatin to soluble fraction. These results suggest that Frag1 has a role in loading activated Rad9 onto damaged chromatin and stimulating its translocation.

To determine whether phosphorylation and Rb-binding of Frag1 are involved in the association and release of Rad9 (Fig. 6*A*), stable transfectants expressing Frag1 or Frag1 mutants were exposed to MMS, and protein lysates were analyzed by immunoblot. Association of Frag1 with Rad9 was reduced 4 and 8 h after cells were released from G_1 block and exposed to MMS; however, the reduction was inhibited in the Ser-1169A and LxGxK mutants and, to a lesser extent, in Ser-1187A and LxGxE mutants, suggesting that Atr phosphorylation stimulates the dissociation of Rad9 and that Rb binding is also involved, directly or indirectly, in Rad9 activation. The evaluation of apoptotic cells showed that the mutants, especially Ser-1169A and LxGxK, had DNA damage-resistant phenotypes compared with wild-type transfectants (Fig. 6*B*), emphasizing the importance of the Frag1–Rad9 association to apoptosis induction. We finally assessed cotransfectants with Frag1 and wild-type or kinase-dead ATR. Immunoblot analysis showed that, after MMS exposure, down-regulation of Frag1 was inhibited by kinase-dead ATR but not by wild-type ATR (data not shown), supporting the conclusion that phosphorylation by Atr plays a role in the Frag1–Rad9-regulated DNA damage response.

As for a mechanism, our data showed that Frag1 amino acids Ser-1169 and Ser-1187 play critical roles in the regulation of Rad9 release and cell death in response to DNA damage. Ser-1169 and Ser-1187 are putative phosphorylation sites for Atr, which is a sensor of stalled or collapsed replication forks at mid-S phase checkpoint (19). Overexpression of a Rad9-associated Frag1 polypeptide inhibited Bcl2 family-mediated apoptosis, suggesting that Frag1 functions as a platform for loading Rad9 to damaged lesions. As shown in the present study of *ATR* siRNA, after genotoxin exposure, reduction of Atr inhibited the down-regulation of endogenous Frag1 and markedly reduced association of Rad9 with Frag1, suggesting that the loading of Rad9 onto damaged chromatin by Frag1 may require Atr and that Atr could down-regulate Frag1 through phosphorylation sites Ser-1169 and Ser-1187. As for the activation of Rad9, earlier studies showed several mechanisms for recruiting Rad9 to damaged lesions, including Abl-mediated phosphorylation of Rad9, which induced binding of Rad9 to antiapototic BclxL (25); PKCδ phosphorylation of Rad9 after genotoxin exposure (26); and MEC1 and TEL1 of budding yeast, homologues of Atr and Atm, which regulate Rad9 hyperphosphorylation (27). Thus, Atr, in concert with those molecules, can play a direct or indirect role in recruiting Rad9 onto Frag1. Full execution of the steps could lead to the stimulation of the Rad9–Bcl2 cell death pathway. We propose a schema in which each step participates in sensing damage, activating checkpoint, and execution of apoptosis; the multisteps may compose the machinery for the pathway, which determines the fate of cells with perturbations in DNA replication progression, i.e., whether the DNA damage is compatible with cell survival or requires elimination by apoptosis (Fig. 11, which is published as supporting information on the PNAS web site).

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