Interaction between UV-damaged DNA Binding Activity Proteins and the c-Abl Tyrosine Kinase*

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The c-Abl tyrosine kinase is activated by some forms of DNA damage, including ionizing radiation, but not UV radiation. The functions of this activation in the damage response pathways remain obscure. To identify potential targets of c-Abl kinase, we utilized the yeast two-hybrid system to screen a murine cDNA library. One c-Abl binding protein of particular interest was the large subunit (DDB1) of the heterodimeric complex with UV-damaged DNA binding activity (UV-DDB). This complex binds with high specificity to DNA damaged by UV, is absent in a subset of xeroderma pigmentosum group E cells, and is required for global genomic repair of UV-induced damage. The association of c-Abl with DDB1 required the kinase domain of c-Abl and preserved the interaction between DDB1 and the small subunit (DDB2) of the UV-DDB complex. Significantly, overexpression of c-Abl increased tyrosine phosphorylation of DDB2 and suppressed UV-DDB activity. Conversely, a dominant negative, kinase-deficient allele of c-Abl decreased tyrosine phosphorylation of DDB2 and dramatically stimulated UV-DDB activity. These results suggest that one role of c-Abl may be to negatively regulate UV-DDB activity by phosphorylation of DDB2.

The c-abl gene encodes a ubiquitously expressed nonreceptor tyrosine kinase. Altered forms of the Abl kinase play pivotal roles in oncogenesis: the activated v-Abl kinase of the Abelson murine leukemia virus induces pre-B cell lymphoma in mice, and the Bcr-Abl fusion protein is responsible for the induction of chronic myelogenous leukemia in humans. However, the physiological functions of the wild type c-Abl kinase are far less clear. The N terminus of c-Abl contains Src homology domains 2 and 3 (SH2 and SH3) and a tyrosine kinase domain. The c-Abl tyrosine kinase is activated by some forms of DNA damage, including ionizing radiation, but not UV radiation. The functions of this activation in the damage response pathways remain obscure. To identify potential targets of c-Abl kinase, we utilized the yeast two-hybrid system to screen a murine cDNA library. One c-Abl binding protein of particular interest was the large subunit (DDB1) of the heterodimeric complex with UV-damaged DNA binding activity (UV-DDB). This complex binds with high specificity to DNA damaged by UV, is absent in a subset of xeroderma pigmentosum group E cells, and is required for global genomic repair of UV-induced damage. The association of c-Abl with DDB1 required the kinase domain of c-Abl and preserved the interaction between DDB1 and the small subunit (DDB2) of the UV-DDB complex. Significantly, overexpression of c-Abl increased tyrosine phosphorylation of DDB2 and suppressed UV-DDB activity. Conversely, a dominant negative, kinase-deficient allele of c-Abl decreased tyrosine phosphorylation of DDB2 and dramatically stimulated UV-DDB activity. These results suggest that one role of c-Abl may be to negatively regulate UV-DDB activity by phosphorylation of DDB2.

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1 The abbreviations used are: SH2 and SH3, Src homology domains 2 and 3; UV-DDB, UV-damaged DNA binding activity; XP, xeroderma pigmentosum complementation group E; GST, glutathione S-transferase; HA, hemagglutinin; DTT, dithiothreitol; PTK, protein-tyrosine kinase; EMSA, electrophoretic mobility shift assay; DNA-PKcs, catalytic subunit of DNA-dependent protein kinase; GGR, global genomic repair; c-Abl KR, kinase-deficient c-Abl.

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The interaction of c-Abl with the UV-DDB complex provides a new link between DNA damage and c-Abl activation. In this report we describe the nature of the interaction and provide evidence suggesting that c-Abl might inhibit UV-DDB activity by phosphorylating DDB2.

**MATERIALS AND METHODS**

**Cell Culture**—Human 293 cells, which are immortalized by adenovirus, were grown in Dulbecco’s modified Eagle’s medium (Specialty Media) supplemented with 10% fetal bovine serum (HyClone), 1% glutamine, and 1% penicillin/streptomycin (Invitrogen) at 37 °C in 5% CO₂. Human WI38 cells and Chinese hamster V79 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 1% glutamine, and 1% penicillin/streptomycin (Invitrogen) and maintained subconfluently.

**Plasmids**—The yeast two-hybrid screen was carried out with a vector expressing a fragment of kinase-deficient c-Abl encoding amino acids 4–1087. The vector AGP5 was generated by partially digesting kinase-deficient c-Abl cDNA with restriction enzyme BglII and subcloning into the yeast two-hybrid vector SH2-1.

Expression in mammalian cells was accomplished for wild type c-Abl, kinase-deficient c-Abl, various c-Abl truncation mutants, and Abi-1 by cloning the corresponding cDNAs into the expression vector pMT21.

GST-C, which expresses the C-terminal part of c-Abl fused with the GST protein, was generated from a c-Abl fragment encoding amino acids 603–959 that was cloned in-frame into pGEX2TK (Amer sham Biosciences). Other GST-Abl fusion protein constructs, GST-SH2, GST-SH3, GST-SH2, and GST-SH2-PTK, were the kind gifts of Dr. A. M. Pendergast (32).

The FLAG-DDB1, DDB1-HA, FLAG-DDB2 fusion proteins were all cloned into the mammalian expression vector pBJS with either the FLAG epitope fused to the region encoding the N terminus or the HA epitope fused to the region encoding the C terminus as previously described (37). Each of these DDB1 and DDB2 constructs was previously shown to be active in reconstituting UV-DDB activity upon transfection into mammalian cells (37).

**Yeast Two-hybrid Screening**—Yeast strain CTY10-5d (gift of Dr. R. Stern galz, State University of New York, Stony Brook) were transformed by the bait construct AGP5. CTY10-5d cells expressing AGP5 were then transformed with a murine WEHI-3 cDNA library by the yeast two-hybrid vector SH2-1.

GST-DDB1, DDB1-HA, and DDB2 fusion proteins were all transfected into mammalian cells expressing the bait construct AGP5. CTY10-5d cells expressing AGP5 were then transformed with a murine WEHI-3 cDNA library by the yeast two-hybrid vector SH2-1.

**Immunoprecipitation**—After transfection with a combination of different plasmids, 293 cells were extracted with either EBC buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM MgCl₂, 4 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of pepstatin, leupeptin, and aprotinin) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue.

**Human 293 cells expressing appropriate proteins were solubilized with TNE1 buffer. Proteins extracted from 293 cells were mixed with 50 μg of purified GST fusion protein immobilized on glutathione-agarose beads. The mixture was incubated at 4 °C for 2 h with gentle rocking, and the beads were washed four times with TNE1 buffer. Bound materials were eluted by boiling in Laemmli sample buffer, fractionated by SDS-PAGE (7.5% polyacrylamide), blotted onto a nitrocellulose membrane, and probed with appropriate antibodies.

**Immunoprecipitation**—After transfection with a combination of different plasmids, 293 cells were extracted with either EBC buffer (50 mM Tris, pH 7.6, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 10 mM ε-amino caproic acid, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μg/ml leupeptin, and 10 μg/ml apro tinin) or radioimmuneprecipitation assay buffer (10 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxy cholate, 0.1% SDS, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μg/ml leupeptin, and 10 μg/ml apro tinin). Cell extracts were incubated with appropriate antibodies for 1 h at 4 °C. Proteins bound to antibodies were collected by incubation with protein A-agarose beads (Santa Cruz Biotechnology) for 3 h. After washing, bound proteins were eluted by boiling in Laemmli sample buffer. Anti-FLAG M2 affinity gel (IBI-Kodak) was used to immunoprecipitate proteins with the FLAG epitope.

**Northern Blot Analysis**—Total RNA was extracted from 293 cells transfected with different constructs by RNAzol B kit (Tel-Test), according to the manufacturer’s instructions. A probe for DDB1 was generated by digesting DDB1 cDNA with EcoRI and EagI. A probe for DDB2 was generated by digesting the DDB2 expression construct with XhoI and XbaI. DNA fragments were isolated from 1% agarose gel and labeled with [32P]dCTP with an oligo labeling kit (Amer sham Biosciences). DNA was denatured, resolved by 1.2% formaldehyde agarose gel, transferred to Nytran Plus (Schleicher & Schuell), and cross-linked with UV (Stratagene). Total RNA (30 μg) was loaded on each lane. The membrane was prehybridized in QuickHyb (Stratagene) at 65 °C for 4 h and hybridized with [32P]-labeled probe in QuickHyb overnight. The membrane was washed twice for 30 min with 2× SSC, 0.1% SDS at room temperature, washed once with 0.1× SSC, 0.1% SDS at 65 °C for 20 min, and then exposed to x-ray film at −80°C.

**Cytoplasmic RNA was harvested from WI38 cells using the RNeasy Mini kit (Qiagen). RNA was quantitated using a RiboGreen RNA quantitation kit (Molecular Probes). RNA was run on a 1% agarose gel and blotted onto Hybond N+ nitrocellulose using the NortherMax-Gly blotting kit (Ambion). Radiolabeled RNA probes and DNA probes were synthesized using the Strip-EZ kit (Ambion). Blots were stripped and re-probed sequentially.

**Preparation of Nuclear and Cytoplasmic Extract**—Confluent cells from 60-mm plates were harvested in 1 ml of ice-cold phosphate-buffered saline. Cells are washed once with phosphate-buffered saline, and once with buffer A (10 mM Hepes-KOH, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of pepstatin, leupeptin, and aprotinin). This supernatant was collected as the cytoplasmic extract. The cell pellet was resuspended in 200 μl of buffer A plus 1% Nonidet P-40, and the cell suspension was incubated at 4°C for 10 min with gentle shaking. Cell lysates were microcentrifuged at 13,000 × g for 5 s. The cell pellet was washed with 300 μl of buffer A by gentle pipetting and microcentrifuged at 13,000 × g for 5 s. The cell pellet was resuspended in 150 μl of buffer C (20 mM Hepes-KOH, pH 7.4, 20% glycerol (v/v), 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of pepstatin, leupeptin, and aprotinin). This supernatant was collected as the cytoplasmic extract. The cell pellet was resuspended in 200 μl of buffer A plus 1% Nonidet P-40, and the cell suspension was incubated at 4°C for 10 min with gentle shaking. Cell lysates were microcentrifuged at 13,000 × g for 5 s.

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were assayed for UV-DDB activity by electrophoretic mobility shift assay as described previously (21). Briefly, a 148-bp DNA probe (f148) was labeled with [32P]dCTP with Klenow enzyme and either left intact or damaged with UV at a dose of 4000 J/m². A 16-μl reaction included 2 μl of 5× binding buffer (12 mM Hepes-KOH, pH 7.9, 60 mM KCl, 5 mM MgCl₂, 4 mM Tris, 0.6 mM EDTA, 1 mM DTT, and 12% glycerol), 1 ng of f148 probe, 3 μg of DS-PAGE (7.5% polyacrylamide), blotted onto a nitrocellulose membrane, and probed with appropriate antibodies.

**Immunoprecipitation**—After transfection with a combination of different plasmids, 293 cells were extracted with either EBC buffer (50 mM Tris, pH 7.6, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 10 mM ε-amino caproic acid, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μg/ml leupeptin, and 10 μg/ml apro tinin) or radioimmuneprecipitation assay buffer (10 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxy cholate, 0.1% SDS, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μg/ml leupeptin, and 10 μg/ml apro tinin) and probed with appropriate antibodies.
proteins containing different fragments of c-Abl (Fig. 1). Abi-1, a previously identified Abl binding protein that binds to both the SH3 domain and the C terminus (35, 36), was used as a control. GST fusion proteins were generated, and a comparable amount of each protein was bound to glutathione-agarose beads and incubated with cell extracts of 293 cells expressing Myc-tagged Abi-1 or FLAG-tagged DDB1, which was previously shown to be active in reconstituting UV-DDB activity (37). Materials bound to beads were eluted with SDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose, and examined by immunoblot. As expected, Abi-1 was bound by Abl constructs containing the SH3 or C-terminal region of Abi but not other constructs (Fig. 1A). By contrast, DDB1 was bound only by a GST fusion containing the protein-tyrosine kinase (PTK) domain but not other GST fusion proteins (Fig. 1A). In particular, GST-SH2PTK, but not GST-SH2, associates with DDB1, suggesting that the PTK domain of c-Abl might be responsible for the binding between c-Abl and DDB1. The structures of GST-Abl fusion proteins and their abilities to associate with either DDB1 or Abi-1 are summarized in Fig. 1B.

**DDB1 Associates with c-Abl in Vitro**—To test whether the interaction between c-Abl and DDB1 occurs in vivo, human 293 cells were cotransfected with constructs expressing c-Abl and either FLAG-tagged DDB1, FLAG-tagged DDB2, or HA-tagged DDB1. Each of the tagged constructs was shown to be active in reconstituting UV-DDB activity upon transfection into mammalian cells (37). Indeed, FLAG-DDB2 was shown to be equivalent to DDB2 in conferring global genomic repair to intact cells (31). Cell lysates were immunoprecipitated with anti-FLAG antibodies, resolved by SDS-PAGE, and immunoblotted with anti-Abi antibodies. As seen in Fig. 2, c-Abl was specifically coimmunoprecipitated with FLAG-DDB1 but not FLAG-DDB2 (lanes 3 and 4). However, c-Abl was coimmunoprecipitated with FLAG-DDB2 if DDB1-HA was also present (lane 5), suggesting that DDB1 can act to bridge the binding between c-Abl and DDB2. This finding is consistent with the finding that DDB1 associates with DDB2 as a heterodimer (22, 37). It also suggests that the association of c-Abl with DDB1 preserves the interaction between DDB1 and DDB2 and that these three molecules are able to form a stable complex in vivo.

To further map the region of c-Abl interacting with DDB1 in vivo, we generated various c-Abl mutants and tested them in a coimmunoprecipitation assay. The nuclear localization signal was retained in each of these c-Abl mutants. In fact, overexpressed c-Abl and FLAG-DDB1 localized to both the cytoplasm and the nucleus (data not shown). The c-Abl mutants and FLAG-DDB1 were coexpressed in 293 cells, lysates were prepared, and the proteins were immunoprecipitated with anti-c-Abl antibodies, resolved by SDS-PAGE, and analyzed by immunoblot. Kinase-deficient c-Abl (c-Abl KR) and wild type c-Abl interacted equally well with DDB1 (Fig. 3A, lanes 2 and 3), consistent with the fact that DDB1 was isolated with a c-Abl KR fragment as bait in the original yeast two-hybrid screen. A c-Abl mutant with a deletion of the PTK domain failed to associate with FLAG-DDB1 (Fig. 3A, lane 8), whereas a minimal c-Abl construct containing only the PTK domain and the nuclear localization signal coimmunoprecipitated with FLAG-DDB1 (Fig. 3A, lane 7). Fig. 3B shows the c-Abl constructs and summarizes the ability of each to bind FLAG-DDB1. We conclude that the PTK domain was necessary and sufficient for the interaction between c-Abl and DDB1. Taken together, these results demonstrate that c-Abl associates with DDB1 both in vitro and in vivo and that the PTK domain of c-Abl is responsible for the binding between c-Abl and DDB1.

**c-Abl Inhibits UV-DDB Activity**—We wished to determine
whether changes in c-Abl might affect the levels of UV-DDB activity in intact cells. Human 293 cells were transiently transfected with expression vectors for wild type c-Abl and various c-Abl mutants and then analyzed for UV-DDB activity. Transfection efficiencies were over 80% as measured by /H9252-galactosidase staining of cells expressing a control /H9252-galactosidase cDNA (data not shown). Cell extracts were prepared 48 h after transfection and assayed for UV-DDB activity with an electrophoretic mobility shift assay.

The constitutive level of UV-DDB was established in mock transfected 293 cells (Fig. 4, lanes 1–4). As previously reported (37), the binding activity indicated as bands B1 and B2 was specific for UV-damaged DNA and thus identified as UV-DDB. In particular, UV-DDB was absent for the undamaged probe (lane 1), present for the UV-damaged probe (lane 2), resistant to competition from undamaged DNA (lane 3), and sensitive to competition from undamaged DNA (lane 4).

The effects of c-Abl on UV-DDB activity were determined in 293 cells transfected with expression vectors for wild type c-Abl and various c-Abl mutants and then analyzed for UV-DDB activity. Transfection efficiencies were over 80% as measured by β-galactosidase staining of cells expressing a control β-galactosidase cDNA (data not shown). Cell extracts were prepared 48 h after transfection and assayed for UV-DDB activity with an electrophoretic mobility shift assay.

The constitutive level of UV-DDB was established in mock transfected 293 cells (Fig. 4, lanes 1–4). As previously reported (37), the binding activity indicated as bands B1 and B2 was specific for UV-damaged DNA and thus identified as UV-DDB. In particular, UV-DDB was absent for the undamaged probe (lane 1), present for the UV-damaged probe (lane 2), resistant to competition from undamaged DNA (lane 3), and sensitive to competition from undamaged DNA (lane 4).

The effects of c-Abl on UV-DDB activity were determined in 293 cells transfected with expression vectors for wild type or mutant c-Abl. Overexpression of c-Abl caused a reproducible decrease in UV-DDB (compare lanes 5 and 6). A kinase-deficient mutant of c-Abl, K298R, which has been used by other investigators to produce a dominant negative effect on endogenous c-Abl activity (38), dramatically increased UV-DDB activity (compare lanes 5 and 7). This increased binding activity was specific for UV-damaged DNA, because it was sensitive to competition from UV-damaged DNA (lane 10) but unaffected by undamaged DNA (lane 9). The changes in UV-DDB activity induced by wild type and kinase-deficient c-Abl required interaction with DDB1, because expression of c-Abl ΔPTK, a c-Abl mutant that fails to bind DDB1, had no effect on UV-DDB activity (compare lanes 5 and 8). The successful expression of the mutant Abl alleles was confirmed in each case by immunoblot (Fig. 4B).

In principle, the effect of c-Abl on UV-DDB could be mediated by changes in the transcription of DDB1 or DDB2. To test this possibility, total RNA from transfected 293 cells was analyzed for levels of DDB2 and DDB1 mRNA by Northern blot. The expression of either wild type or kinase-deficient c-Abl had no effect on the levels of DDB2 and DDB1 mRNA (Fig. 4C).

To demonstrate that c-Abl regulates UV-DDB via its kinase activity, we treated WI38 cells with the c-Abl kinase inhibitor, STI571. Fig. 5 shows that endogenous UV-DDB levels increase up to 3.4-fold upon addition of STI571. Thus, endogenous levels of c-Abl inhibit UV-DDB activity by a post-transcriptional mechanism that has no effect on DDB2 or DDB1 mRNA levels and that requires the tyrosine kinase activity of c-Abl. Inhibition of c-Abl results in UV-DDB activation.

DDB1 and DDB2 Are Substrates of c-Abl Kinase—To test whether c-Abl affects the tyrosine phosphorylation of DDB1, cultures of 293 cells were cotransfected with expression vectors

FIG. 2. c-Abl associates with DDB2 in vivo through its interaction with DDB1. Human 293 cells were cotransfected with: 1) expression vectors based on pMT21 containing no insert or cDNA for c-Abl (4 μg) and 2) expression vectors based on pBJ5 containing no insert (4 μg) or cDNAs for FLAG-DDB1 (4 μg), FLAG-DDB2 (4 μg), or DDB1-HA (2 μg). Cell lysates were immunoprecipitated with anti-FLAG affinity gel, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Ab antibodies (top panel). The expression of different proteins was shown by probing the total lysates with appropriate antibodies (bottom panels).

FIG. 3. The association between c-Abl and DDB1 in vivo is mediated by the c-Abl kinase domain. A, human 293 cells cotransfected with expression vectors for FLAG-DDB1 (5 μg) and different c-Abl constructs (5 μg). The c-Abl constructs and the blank vector were based on plasmid pMT21. Cell lysates were immunoprecipitated with anti-Ab antibodies, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FLAG antibodies (top panel). The expression of FLAG-DDB1 and different c-Abl constructs was determined by probing total lysates with anti-FLAG or anti-Ab antibodies (bottom panels). B, summary of the c-Abl constructs and the ability of each to bind DDB1 in vivo.
for DDB1-HA and either wild type or kinase-deficient c-Abl. The phosphotyrosine level of DDB1-HA was determined by immunoprecipitation with anti-HA antibodies, and immunoblotting with anti-phosphotyrosine antibodies. When DDB1-HA was expressed alone, there was no detectable tyrosine phosphorylation on DDB1-HA (Fig. 6A, lane 1). Coexpression of wild type c-Abl produced significant tyrosine phosphorylation of DDB1 (Fig. 6A, lane 2). By contrast, coexpression of kinase-deficient c-Abl failed to produce tyrosine phosphorylation of DDB1 (Fig. 6A, lane 3).

These results failed to explain how kinase-deficient c-Abl dramatically stimulated UV-DDB activity, because there was no detectable tyrosine phosphorylation of DDB1-HA either in...
Indian 293 cells were cotransfected with FLAG-DDB2 (3 μg) and empty vector (6 μg) (lane 1), DDB1-HA (3 μg) and c-Abl (3 μg) (lane 2), and DDB1-HA (3 μg) and c-Abl KR (3 μg) (lane 3). Cell lysates were immunoprecipitated with anti-HA antibodies, resolved by SDS-PAGE, and probed with anti-FLAG antibodies (top panel). The membrane was then reprobed with anti-HA antibodies (middle panel). The expression of FLAG-DDB2 in total lysates was checked with anti-FLAG antibodies (bottom panel).

The presence or absence of kinase-deficient c-Abl. Furthermore, expression of kinase-deficient c-Abl failed to increase and may have decreased DDB1-HA protein levels (Fig. 6A, lane 3).

To determine the effect of c-Abl on the phosphotyrosine level of DDB2, cells were cotransfected with expression vectors for c-Abl, FLAG-DDB2, and DDB1-HA. Tyrosine phosphorylation of DDB2 was determined by immunoprecipitation with anti-FLAG antibodies and probing with anti-phosphotyrosine antibodies. In contrast to DDB1, DDB2 was highly phosphorylated on tyrosine residues even in the absence of c-Abl overexpression (Fig. 6B, lane 1). Expression of a kinase-deficient c-Abl mutant, acting as a dominant-negative allele, dramatically decreased the tyrosine phosphorylation of DDB2 (Fig. 6B, lane 3), whereas expression of Abl ΔPTK had no effect (Fig. 6B, lane 4). At moderate levels of c-Abl expression, DDB2 phosphorylation level did not change (Fig. 6B, lane 2). However, when the ratio of transfected c-Abl to DDB2 was increased from 1:1 to 2:1, the added c-Abl now induced increased tyrosine phosphorylation of DDB2 (Fig. 6B, lanes 5 and 6). Therefore, overexpression of c-Abl leads to hyperphosphorylation of DDB2, and expression of the kinase defective allele decreases this phosphorylation, suggesting that this may be the mechanism that leads to inhibition of UV-DDB activity. Direct analysis of the phosphorylation of endogenous DDB2 by c-Abl could not be performed, because there are no specific antibodies against DDB2.

The Interaction of DDB1 and DDB2 Is Not Affected by c-Abl—UV-DDB activity is dependent on interaction between the DDB1 and DDB2 proteins (37), and inhibition of UV-DDB by c-Abl might be due to disruption of this interaction. To test this possibility, DDB1-HA, FLAG-DDB2, and c-Abl were coexpressed in 293 cells, and coimmunoprecipitation assays were performed. Lysates were immunoprecipitated with anti-HA antibodies. The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-FLAG antibodies to detect coimmunoprecipitation of FLAG-DDB2, or anti-HA antibodies to confirm precipitation of DDB1-HA (Fig. 7). Expression of wild type or kinase-deficient c-Abl had no effect the interaction between DDB1-HA and DDB2. Thus, wild type c-Abl preserved the interaction between DDB1 and DDB2.

UV-DDB Activity in Human Cells Exposed to UV and IR—We wished to determine whether the interaction of c-Abl with DDB1 is physiologically relevant in cells exposed to DNA damage. We previously reported that both UV and IR lead to p53-dependent increases in DDB2 transcription in intact cells (39). In addition, the tyrosine kinase activity of c-Abl is stimulated by IR but not UV light (12, 13).

In 293 cells, we have shown that c-Abl interacts with DDB1 (Figs. 1–3), that transfection of c-Abl inhibits UV-DDB activity (Fig. 4), and that transfection of c-Abl increases tyrosine phosphorylation of DDB2 in a manner that correlates with inhibition of UV-DDB activity (Fig. 6). Although the results of the transfection experiments should be interpreted cautiously because they involve forced expression of c-Abl, the results make testable predictions for how intact cells would respond to DNA damage. To confirm the physiological relevance of the interaction between c-Abl and DDB1, we tested the effect of UV and IR on UV-DDB in WI38 human cells, which have a normal p53 response (39).

Because UV activates DDB2 transcription but fails to activate c-Abl, we predicted that WI38 cells exposed to UV should show increased levels of UV-DDB activity. This prediction was confirmed in an EMSA, which shows increased nuclear UV-DDB activity (Fig. 8A, lanes 5 and 6) associated with increased DDB2 transcription (Fig. 8B).

Because IR activates both DDB2 transcription and the c-Abl tyrosine kinase, we supposed that the subsequent effects on UV-DDB activity might offset each other. Indeed, when cells were exposed to IR, DDB2 transcription increased 2.6-fold after 4 h and 2.3-fold after 9 h (Fig. 8B). However, the levels of nuclear UV-DDB remained unchanged after IR (Fig. 8A). The total amount of UV-DDB also remained unchanged, because there was no detectable increase in cytoplasmic UV-DDB. Thus, increased DDB2 transcription after IR was offset by post-transcriptional inhibition of UV-DDB activity.

We next wanted to test if UV and IR damage affected the phosphotyrosine levels of DDB2. We used hamster V79 cells, which fail to express endogenous DDB2 (37). The hamster cells were cotransfected with FLAG-DDB2 and DDB1-HA and exposed to UV or IR. Tyrosine phosphorylation on FLAG-DDB2 was increased 2.3-fold after IR, as judged by densitometry, but not after UV (Fig. 9). This effect was reproducible in multiple experiments and is consistent with the conclusion that IR activated c-Abl phosphorylates DDB2 and decreases UV-DDB activity. By contrast, UV does not activate c-Abl, leaving DDB2 phosphotyrosine levels unchanged. Thus, UV-DDB activity increases after UV due to the p53-dependent increase in transcription of DDB2 and the lack of inhibition by c-Abl.

UV-DDB Activity in c-Abl Mouse Knockout Cells—Hamster cells and wild type mouse cells are significantly different from human cells, expressing at least 30-fold lower levels of UV-DDB activity, normally undetectable (31). Nevertheless, we attempted to measure UV-DDB activity in c-Abl knockout cells (6), and we could not detect activities in wild type or mutant cells (data not shown).

**DISCUSSION**

In this study, we utilized the yeast two-hybrid assay to identify DDB1, the large subunit of UV-DDB, as a new c-Abl-interacting protein. The association between c-Abl and DDB1 occurred both in vitro and in vivo. The protein-tyrosine kinase (PTK) domain of c-Abl was responsible for its interaction with DDB1, but binding did not require an active kinase. UV-DDB activity requires the interaction of two subunits, DDB1 and DDB2, and c-Abl formed a complex with DDB1 that preserved
this interaction. Thus, c-Abl, DDB1, and DDB2 are capable of forming a ternary complex.

Strikingly, overexpression of wild type c-Abl inhibited UV-DDB activity, whereas expression of a kinase-deficient c-Abl strongly stimulated UV-DDB activity. The c-Abl-mediated regulation of UV-DDB was post-transcriptional, because the effects occurred in the absence of any change in the levels of DDB1 or DDB2 mRNA. Furthermore, regulation by c-Abl was dependent on its tyrosine kinase activity, because the specific tyrosine kinase inhibitor STI571 produced an increase in UV-DDB activity. Although tyrosine phosphorylation of DDB1 was observed in cells overexpressing wild type c-Abl, it was undetectable both in untransfected cells and in cells expressing kinase-deficient, dominant negative c-Abl. Thus, the dramatic stimulation of UV-DDB by dominant negative c-Abl could not be explained in terms of tyrosine phosphorylation of DDB1. On the other hand, tyrosine phosphorylation of DDB2 was detected in intact cells, increased upon overexpression of wild type c-Abl, and decreased with expression of dominant negative c-Abl. In this case, the effects of c-Abl on UV-DDB activity correlated with its effects on DDB2 tyrosine phosphorylation. These findings suggest that c-Abl might act as a negative regulator of UV-DDB by phosphorylating DDB2. There are nine tyrosine residues in DDB2, and two of them fall into the consensus c-Abl phosphorylation sequence (YXXP). However, mutating these nine tyrosine residues individually did not result in a reduction in DDB2 tyrosine phosphorylation, indicating redundant phosphorylation sites.

In addition to c-Abl, mammalian cells express the highly homologous Abl-related gene, Arg (40). It is possible that Arg may regulate DDB2 by tyrosine phosphorylation, and part of the dramatic stimulation of UV-DDB activity induced by kinase-deficient c-Abl could possibly be due to its inhibition of Arg. However, Arg is confined to the cytoplasm in cells (41), whereas c-Abl is found in both the cytoplasm and the nucleus. The DDB2 protein is restricted to the nucleus (42), and the presence of c-Abl but not Arg in the same cellular compartment suggests that c-Abl will prove to be critical for the regulation of DDB2. DDB2 is tyrosine-phosphorylated in vivo. c-Abl is a nuclear protein and binds to the DDB1-DDB2 complex. Therefore, c-Abl is a good DDB2 kinase candidate. However, our study cannot rule out the possibility that other kinases might also contribute to the phosphorylation of DDB2.

There is also evidence that c-Abl regulates other repair proteins. Rad51, a human homolog of bacterial Rec A, functions in homologous recombination for the repair of DNA double-strand breaks induced by ionizing radiation. Rad51 interacts with c-Abl, treatment of cells with ionizing radiation induces c-Abl-dependent phosphorylation of Rad51, and phosphorylation by c-Abl inhibits the ability of Rad51 to catalyze ATP-dependent strand exchange (43). DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase, functions in a parallel pathway for double-strand break repair by nonhomologous end-joining. DNA-PKcs interacts with c-Abl and undergoes c-Abl-dependent phosphorylation after ionizing radiation. Phosphorylation of DNA-PKcs by c-Abl inhibits the ability of DNA-PKcs to form a complex with DNA (44).

UV-induced DNA lesions are removed by nucleotide excision repair via two interdependent pathways: transcription-coupled repair and global genomic repair (GGR) (45). Transcription-coupled repair removes lesions from the DNA strand transcribed by RNA polymerase II, whereas GGR removes lesions from the nontranscribed strand as well as nontranscribed regions of the genome. Xeroderma pigmentosum (XP) is an autosomal recessive human disease characterized by hypersensitivity to UV radiation and extreme susceptibility to skin cancer, due to defects in nucleotide excision repair (46). XP consists of seven genetic complementation groups, and cells from group E have a specific defect in GGR of UV-induced cyclobutane pyrimidine dimers (39). UV-DDB is absent in a subset of XP-E cells, due to inactivating mutations in DDB2 (37, 47).

Several observations indicate that DDB2 is the target for regulating UV-DDB in response to DNA damage. First, DDB2
is rate-limiting for the expression of UV-DBB activity, because overexpression of DDB2 (but not DDB1) in wild type cells leads to increased UV-DBB activity (37). Second, transcription of DDB2 is p53-dependent, and increases in a p53-dependent manner after cells are exposed to UV or ionizing radiation (37). Transcription of DDB2 also increases after exposure of cells to cisplatin (48). The data presented here on the interaction between c-Abl and the UV-DBB complex suggest a new form of DDB2 regulation, at the level of post-transcriptional protein modification.

What might be the physiological significance for the regulation of UV-DBB by c-Abl? The c-Abl kinase is activated by many forms of DNA damage, including ionizing radiation, mitomycin C, methyl methanesulfonate, and cisplatin (12, 13). Similar activation of c-Abl kinase by UV radiation would appear to be detrimental to the cell, because the subsequent inhibition of UV-DBB would interfere with GGR of the UV-damaged DNA. Interestingly, previous investigators failed to detect c-Abl activation 1 h after cells were exposed to the high dose of 40 J/m² UV radiation (13). In addition, we found no c-Abl activation at later time points: 4, 8, 16, and 24 h after UV doses of 10 and 20 J/m² (data not shown).

What is the advantage for c-Abl inhibition of UV-DBB after ionizing radiation? One possibility is that c-Abl suppresses interference by UV-DBB with other DNA repair pathways. Interference between repair pathways has been noted previously. Mismatch repair proteins bind nonproductively to DNA damaged by cisplatin and alkylating agents (49). Mutations in mismatch repair eliminate this nonproductive binding. This leads to more efficient binding by nucleotide excision repair proteins and increased resistance to cisplatin and alkylating agents. Similarly, yeast photolyase binds nonproductively to DNA damaged by cisplatin, methyl methanesulfonate, and mitomycin C, interfering with nucleotide excision repair (50). Ionizing radiation and mitomycin C, which activate c-Abl, produce lesions that are repaired by pathways other than nucleotide excision repair (51). For example, thymine glycols, which are induced by ionizing radiation, are efficiently removed through base excision repair. Interstrand cross-links induced by mitomycin C are repaired by homologous recombination. The UV-DBB complex binds to a broad spectrum of different lesions (52) such as apurinic sites and interstrand cross-links induced by nitrogen mustard or psoralen, which are repaired by other pathways. It is possible that activation of c-Abl permits these lesions to be repaired more efficiently by regulating adverse interference by UV-DBB with the appropriate repair pathways.

A second possibility is that c-Abl inhibition of UV-DBB occurs because phosphorylation of DDB2 remodels the UV-DBB complex to recognize lesions induced by ionizing radiation more efficiently. It is noteworthy that a genome-wide search for genes induced by ionizing radiation yielded several nucleotide excision repair genes: DDB2, XPC, gadd45, and PCNA (53). Although tyrosine phosphorylation of DDB2 remodels the UV-DBB complex so that it binds less effectively to UV-damaged DNA, the remodeled UV-DBB proteins may then bind with higher affinity to forms of base damage produced by ionizing radiation. Thus, ionizing radiation induces increased transcription of the DDB2 gene, mediated by p53, and post-transcriptional phosphorylation of DDB2 protein, mediated by c-Abl. These regulatory mechanisms might produce increased levels of a modified form of the UV-DBB complex that recognizes ionizing radiation-specific lesions more efficiently and targets those lesions for repair.

In conclusion, we have presented evidence that the c-Abl kinase physically interacts with UV-DBB proteins and potentially regulates their activity by affecting the tyrosine phosphorylation level of DDB2. Tyrosine phosphorylation of DDB2 may prevent UV-DBB from interfering with other repair pathways or, alternatively, increase the ability of UV-DBB to direct the repair of lesions induced by agents that activate c-Abl. Indeed, these two possibilities are not mutually exclusive. A full understanding of how c-Abl regulates UV-DBB will require further study.

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