Genotoxic Drugs Induce Interaction of the c-Abl Tyrosine Kinase and the Tumor Suppressor Protein p53*

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The function of the c-Abl protein tyrosine kinase is unknown. The present studies demonstrate that the antimitabolite 1-beta-D-arabinofuranosylcytosine (ara-C) induces binding of c-Abl and p53. Ara-C treatment of cells that express wild type or a dominant negative, kinase-inactive c-Abl(K-R) was associated with formation of c-Abl-p53 complexes and increased expression of the cyclin-dependent kinase (Cdk) inhibitor p21. However, down-regulation of Cdk2 by ara-C was found in cells expressing wild type c-Abl and not in cells expressing c-Abl(K-R) or those deficient in p53. Similar findings were obtained following treatment of cells with the alkylating agent methyl methanesulfonate (MMS). Cells that express the c-Abl dominant negative or are null for c-Abl exhibited partial abrogation of Cdk2 down-regulation and G1 arrest in response to MMS exposure. Cells lacking the c-abl gene also responded to ara-C and MMS with increases in p53 levels and induction of p21. These findings indicate that the cellular response to certain genotoxic drugs involves binding of c-Abl to p53 and down-regulation of Cdk2 by a c-Abl kinase/p53-dependent mechanism.

The c-abl gene encodes a ubiquitously expressed nonreceptor tyrosine kinase that is primarily localized to the nucleus. The c-Abl SH3 domain binds to Abl interactor (Abi) proteins 1 and 2 (1, 2). The finding that the SH3 domain of c-Abl also binds constitutively to the protein tyrosine phosphatase SHPTP1 has supported the existence of distinct nuclear c-Abl pools (3). The carboxyl-terminal region of c-Abl contains a nuclear localization signal (4), a DNA-binding domain (5), an actin-binding domain (6, 7), and proline-rich sequences that bind to SH3 domain-containing proteins (8, 9). Substrates of c-Abl include the c-Crk adaptor protein (8, 9), RNA polymerase II (10, 11), and proline-rich sequences that bind to SH3 domain-containing proteins (8, 9). Substrates of c-Abl include the c-Crk adaptor protein (8, 9), RNA polymerase II (10, 11), and proline-rich sequences that bind to SH3 domain-containing proteins (8, 9).

Overexpression of c-Abl causes an arrest of growth in G1 phase (12, 13). Growth suppression by c-Abl requires tyrosine kinase activity, nuclear localization, and the SH2 domain (12).

Other studies have shown that c-Abl requires p53 to suppress growth (14). c-Abl binds to p53 in vitro and enhances the transactivation function of p53 (14). More recent work has suggested that the cytostatic effect of c-Abl requires both p53 and the retinoblastoma (Rb) protein (15). The involvement of c-Abl in cell cycle control has also been supported by the finding that c-Abl is phosphorylated by p34cdc2 and that such modification inhibits DNA binding (5, 16). Recent studies have demonstrated that c-Abl kinase activity is stimulated by certain DNA-damaging agents (17). Taken together with the potential interactions of c-Abl with p53, Rb, and Cdc2, these findings have suggested that c-Abl may play a role in the cell cycle response to DNA damage.

The present results demonstrate that c-Abl associates with p53 in the cellular response to the antimitabolite 1-beta-D-arabinofuranosylcytosine (ara-C) and the alkylating agent methyl methanesulfonate (MMS). Ara-C misincorporates into cellular DNA (18, 19) and blocks replication by site-specific termination of DNA strands (20–22). MMS is a monofunctional alkylating agent that induces DNA base damage and strand breaks (23). The results indicate that the c-Abl kinase function is involved in the growth arrest response induced by these and certain other genotoxic agents.

MATERIALS AND METHODS
Cell Culture—MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% heat-inactivated fetal bovine serum (Sigma), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Null pSR, MSV-Kneo, pSR-MSV-Abl/K(R)-RtKNeo (12), or E6Neo (24) vectors were stably introduced into cells by LipofectAMINE (Life Technologies, Inc.) and selection in G418. The cells were treated with 10 μM ara-C (Sigma) or 10 μg/ml MMS (Sigma). Cell cycle analysis was performed as described (25).

Immunoprecipitations and Immunoblot Analysis—Cell lysates were prepared as described (25) and incubated with rabbit anti-Cdk2 (sc-163, Santa Cruz Biotechnology, Santa Diego, CA), mouse anti-c-Abl (Ab-3, Oncogene Science, Cambridge, MA), mouse anti-p53 (Ab-6, Oncogene Science) antibodies for 6–12 h at 4 °C and then for 60 min with protein A-Sepharose. For mouse antibodies, 10 μg/sample of rabbit anti-mouse IgG was added and incubated for 60 min before the addition of protein A beads. The immune complexes were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and analyzed by immunoblotting using an ECL (Amersham Corp.) detection system.

Kinase Assays—Cdk2 kinase assays were performed as described (25). For c-Abl kinase assays, recombinant SHPTP1 protein (100 μg/ml) was used as substrate (3), and the reaction was incubated for 30 min at 30 °C.

RESULTS AND DISCUSSION
Treatment of NIH3T3 cells with Ara-C is associated with activation of the c-Abl kinase (26). To determine whether the activation of c-Abl by genotoxic drugs is associated with growth arrest, we prepared MCF-7 cells that stably express a dominant negative, kinase-inactive c-Abl (K290R) mutant (12). Treatment of control MCF-7/pSR cells with ara-C was associated with increases in c-Abl kinase activity (Fig. 1A). By contrast, there was no detectable c-Abl activity in untreated or ara-C-treated MCF-7/c-Abl(K-R) cells (Fig. 1A). Other studies have demonstrated that c-Abl binds to p53 in vitro (14). Consequently, we asked whether ara-C induces an interaction between c-Abl and p53 in vitro. As a control, we also prepared cells

1 The abbreviations used are: ara-C, 1-beta-D-arabinofuranosylcytosine; MMS, methyl methanesulfonate; MEF, mouse embryo fibroblast; PCNA, proliferating cell nuclear antigen.
that express the human papillomavirus E6 protein to promote degradation of p53 (24). The MCF-7/E6 cells responded to ara-C with induction of c-Abl activity (Fig. 1A). In coimmunoprecipitation studies, there was no detectable p53 in the c-Abl immunoprecipitates from control MCF-7/pSR cells (Fig. 1B). However, binding of c-Abl and p53 was detectable when MCF-7/pSR cells were treated with ara-C for 1 h (Fig. 1B). The association of c-Abl and p53 was also increased by treatment of MCF-7/c-Abl(K-R), but not MCF-7/E6, cells with ara-C (Fig. 1B). These findings indicated that ara-C induces a c-Abl-p53 interaction independent of the c-Abl kinase function. Treatment of MCF-7/pSR and MCF-7/c-Abl(K-R), but not MCF-7/E6, cells with ara-C was also associated with increases in p53 expression, while there was no apparent effect of this agent on p53 levels in MCF-7/E6 cells (Fig. 1C). As transcription of p21 is regulated by p53 (27), the MCF-7/pSR and MCF-7/c-Abl(K-R) cells expressed p21 (Fig. 1D). However, while p21 was induced in both MCF-7/pSR and MCF-7/c-Abl(K-R) cells and was bound to Cdk2 in both cell types (data not shown), Cdk2 activity was down-regulated by ara-C treatment in only the MCF-7/pSR cells (Fig. 1D).

As observed with ara-C, MMS exposure resulted in increased c-Abl activity in the MCF-7/pSR and MCF-7/E6, but not the MCF-7/c-Abl(K-R), cells (Fig. 2A). MMS increased binding of c-Abl and p53 in MCF-7/pSR and MCF-7/c-Abl(K-R), but not MCF-7/E6, cells (Fig. 2B). MMS also induced the expression of p53 and p21 in only the MCF-7/pSR and MCF-7/c-Abl(K-R) cells (Fig. 2C). The MMS-induced increases in p21 levels were associated with binding of p21 to Cdk2 (Fig. 2D). However, while Cdk2 activity was down-regulated in MMS-treated MCF-7/pSR cells, there was little effect of MMS on Cdk2 activity in the MCF-7/c-Abl(K-R) cells (Fig. 2E).

The role of c-Abl in genotoxic drug-induced growth arrest was assessed by the ability of MMS to decrease populations of S phase cells. The number of MCF-7/pSR cells in S phase at 9 h after MMS treatment was 67% of that obtained for untreated cells (Table I). By contrast, MMS-treated MCF-7/c-Abl(K-R) cells exhibited partial inhibition of the G1 response with an S phase population after treatment of over 80% of that for untreated cells (Table I). Similar findings were obtained with two...
independently isolated MCF-7/c-Abl(K-R) clones, while the G1 arrest response was almost completely abrogated in the MCF-7/E6 cells (Table I). These findings suggest that, as found for the down-regulation of Cdk2, c-Abl kinase activity is involved in MMS-induced G1 arrest by a p53-dependent mechanism.

To substantiate the link between c-Abl and DNA damage-induced growth arrest, we studied the effects of MMS on mouse embryo fibroblasts (MEFs) deficient in c-Abl (Abl−/−) (30). Wild type MEFs responded to MMS with induction of the c-Abl kinase, while there was no detectable c-Abl activity or protein in MMS-treated Abl−/− cells (data not shown). Both wild type and Abl−/− MEFs responded to MMS with increases in p53 and p21 levels (Fig. 3A). These findings indicated that c-Abl is not necessary for DNA damage-induced increases in the transactivation function of p53. However, despite induction of p21 in both cell types, Cdk2 activity was significantly down-regulated in MMS-treated wild type, but not Abl−/−, MEFs (Fig. 3B). Wild type MEFs also responded to MMS with G1 arrest. The number of wild type MEFs in S phase at 9 h after MMS treatment was 51% of that obtained for untreated cells (Fig. 3C). By contrast, MMS-treated Abl−/− cells had an S phase population which was 71% of that for untreated cells (Fig. 3C). These findings suggest that c-Abl is involved in the down-regulation of Cdk2 and that the G1 arrest response involves mechanisms other than activation of p21.

The present results demonstrate that treatment of cells with ara-C or MMS is associated with binding of c-Abl and p53. Similar findings have been obtained with the genotoxic topoisomerase inhibitors camptothecin and etoposide (data not shown) and with ionizing radiation exposure (25). Thus, diverse types of DNA damage activate c-Abl (17, 26) and the mechanisms dependent on activation of c-Abl kinase and binding of c-Abl to p53, but independent of p21.

The present results also indicate that activation of the c-Abl kinase is necessary for the complete G1 arrest response to genotoxic drugs. Induction of p21 expression and binding of this Cdk inhibitor to Cdk2 by genotoxic stress is insufficient for the complete down-regulation of Cdk2. Rather, our findings indicate that induction of c-Abl kinase activity is also involved in the down-regulation of Cdk2 and growth arrest. Recent work has shown that overexpression of kinase active, but not inactive, c-Abl in p21−/− fibroblasts is associated with down-regulation of Cdk2 and growth arrest (25). In concert with these results is the demonstration that p53-dependent events can inhibit cell growth through p21-independent mechanisms (31–33). Taken together, these findings indicate that genotoxic agents induce the down-regulation of Cdk2 and G1 arrest by a mechanism dependent on activation of c-Abl kinase and binding of c-Abl to p53, but independent of p21.

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**TABLE I**

*Interaction of c-Abl with p53*

**Percentage of cells entering S phase at 9 h of MMS treatment was determined relative to untreated cells. Results are expressed as the mean ± S.E. of six experiments for MCF-7/pSR, two independently selected MCF-7/c-Abl (K-R) clones (a and b), and MCF-7/E6 cells.**

| Transfectant | Control | Treated | Treated/Control |
|--------------|---------|---------|-----------------|
| MCF-7/pSR    | 38.8 ± 3.2 | 26.2 ± 2.8 | 67.5            |
| MCF-7/c-Abl (K-R) Clone a | 38.4 ± 3.9 | 32.2 ± 4.6 | 83.9            |
| MCF-7/c-Abl (K-R) Clone b | 37.9 ± 3.0 | 30.6 ± 2.8 | 80.7            |
| MCF-7/E6     | 36.6 ± 4.8 | 34.5 ± 3.1 | 94.3            |

**FIG. 3. Effects of MMS on down-regulation of Cdk2 activity and growth arrest in Abl-deficient cells.** C57Bl/6 wild type (Abl+/+) and Abl−/− MEFs were treated with MMS. A, lysates obtained at the indicated times were immunoblotted with anti-p53, anti-p21, and anti-PCNA. B, lysates were subjected to immunoprecipitation with anti-Cdk2. The immunoprecipitates were analyzed by histone H1 kinase assay (top) and immunoblotting with anti-Cdk2 (bottom). C, percentage of cells in S phase at 9 h of MMS treatment relative to untreated cells. Results are expressed as the mean ± S.E. of six experiments.
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