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Accessibility
c-Abl Activation Regulates Induction of the SEK1/Stress-activated Protein Kinase Pathway in the Cellular Response to 1-β-d-Arabinofuranosylcytosine*

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Previous work has shown that treatment1 of cells with the antimetabolite 1-β-d-arabinofuranosylcytosine (ara-C) is associated with induction of the c-jun gene. The present studies demonstrate that ara-C activates the c-Abl non-receptor tyrosine kinase. We also demonstrate that activity of the stress-activated protein kinase (SAP kinase) is increased in ara-C-treated cells. Using cells deficient in c-Abl (Abl−/−) and after introduction of the c-abl gene, we show that ara-C-induced c-Abl activity is necessary for the stimulation of SAP kinase. Other studies using cells transfected with a SEK1 dominant negative demonstrate that ara-C-induced SAP kinase activity is SEK1-dependent. Furthermore, we show that overexpression of truncated c-Abl results in activation of the SEK1/SAP kinase cascade.

1-β-d-Arabinofuranosylcytosine (ara-C)2 is the most effective agent used in the treatment of acute myelogenous leukemia (1). This agent misincorporates into cellular DNA (2, 3) and inhibits replication by site-specific termination of DNA strands (4–6). Although the precise mechanisms responsible for the lethal effects of this agent remain unclear, recent studies have supported the activation of nuclear signaling cascades in ara-C-treated cells. Exposure of human myeloid leukemia cells to ara-C is associated with induction of c-jun and other early response genes (7, 8). The induction of c-jun transcription is positively autoregulated by its product c-jun in cells treated with phorbol esters (9). Treatment with ara-C is also associated with post-translational modification of c-jun and enhancement of J un/AP-1 activity (10). Moreover, binding of activated c-jun to the AP-1 site in the c-jun gene promoter confers ara-C inducibility of this gene (10). Two serines (Ser-63 and Ser-73) in the transactivation domain of c-jun that are phosphorylated in response to phorbol ester and UV light have been identified as substrates for the mitogen-activated and stress-activated protein (SAP) kinases (11–13). Other studies have demonstrated that the SAP kinase/extracellular signal-regulated kinase 1 (SEK1) is responsible for activation of SAP kinase (14, 15).

The product of the c-abl gene is a non-receptor tyrosine kinase (15). c-Abl is localized to the nucleus and cytoplasm (17, 18) and shares structural features with Src family tyrosine kinases. In addition, c-Abl contains C-terminal actin binding and DNA binding domains (17, 18). The finding that c-Abl associates with the retinoblastoma (Rb) protein has supported a role for c-Abl in regulating the cell cycle (19). Other work has demonstrated that overexpression of c-Abl is associated with the arrest of growth in the G1 phase (20, 21). Overexpression of a dominant negative c-Abl results in deregulation of withdrawal from or reentry into the cell cycle (21). These findings have suggested that c-Abl negatively regulates growth. Other studies have demonstrated that c-Abl is phosphorylated on multiple sites by p34cdc2 and that such modification inhibits DNA binding (17, 22). c-Abl phosphorylates the C-terminal domain of RNA polymerase II (23, 24) and stimulates transcription (19). Despite these insights into a potential role for c-Abl, the precise function of this tyrosine kinase remains unclear.

The present studies demonstrate that c-Abl is activated by ara-C treatment. We also demonstrate that c-Abl is required for ara-C-induced SAP kinase activity and that c-Abl activates SAP kinase through SEK1.

MATERIALS AND METHODS

Cell Culture—NIH3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, and 2 mM L-glutamine. NIH3T3 cells that stably express the SEK1 dominant negative were prepared by electroporation of pC3DNA SEK1 AL (14), and individual clones were selected by limiting dilution in the presence of 500 μg/ml G418. Abl-deficient fibroblasts (Abl−/−) were isolated from fetal tissue of a mouse homozygous for a disrupted c-abl gene (25). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (26). Abl−/− cells were infected with a helper-free retrovirus expressing the c-abl gene and selected in puromycin (designated Abl−/− cells). 293 cells were grown as described (14). NIH3T3 cells were infected with a helper-free retrovirus expressing the SH3 domain deleted mutant of c-Abl (AXB) and selected in neomycin (27). Cells were treated with 10 μM ara-C (Sigma).

Subcellular Fractionation—Nuclear proteins were isolated as described (28). In brief, cells were washed three times with phosphate-buffered saline and suspended in 3 cell volumes of hypotonic solution (10 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.1 mM sodium vanadate, 2 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin, and 10 μM leupeptin). After incubation on ice for 30 min to allow swelling, the cells were disrupted in a Dounce homogenizer (15–20 strokes). The homogenate was layered on a cushion of 1 M sucrose in hypotonic solution and
subjected to centrifugation at 1,600 × g for 15 min to pellet nuclei. The nuclear pellet was washed twice by resuspension in 1 ml sucrose-hypotonic solution, and centrifugation was done at 1,600 × g for 10 min. The nuclei were then suspended in lysis solution (0.5% Nonident P-40, 0.1% sodium deoxycholate, and 0.1% Brij 35 in hypotonic solution). After incubation at 4 °C for 30 min, the suspension was centrifuged at 12,000 × g for 15 min, and the supernatate was used as the nuclear fraction.

**c-Abl Immunoprecipitation and Immune Complex Kinase Assays—**

Equal amounts of nuclear proteins were subjected to immunoprecipitation with anti-c-Abl (K-12, Santa Cruz Biotechnology, San Diego, CA) as described (28). Immune complexes were recovered by incubation with protein A-Sepharose for 2 hr at 4 °C. Precipitated rabbit serum (PIRS) was used as a negative control. Immune complex kinase assays were performed by incubating protein complexes in kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol) with 5 μg of GST-Crk(120–225) or GST-Crk(120–212) and 5 μCi of [γ-32P]ATP for 30 min at 28 °C. Phosphorylation was analyzed by 10% SDS-PAGE and autoradiography. In peptide phosphorylation assays, immune complexes were incubated in kinase buffer with 20 μM peptide (EAYAAPFACKK) (29), 10 μM ATP, and 5 μCi of [γ-32P]ATP for 4 min at 25 °C. After incubation, 25 μl were spotted onto P81 phosphocellulose discs (Life Technologies, Inc.), followed by washing with 1% phosphoric acid and then distilled water. The incorporated [32P]phosphate was determined by scintillation counting.

**RESULTS AND DISCUSSION**

Previous studies have demonstrated that ara-C induces a stress response that includes activation of Jun/AP-1 and c-jun transciption (7). In order to determine whether c-Abl is involved in the cellular response to ara-C, we treated NIH3T3 cells with this agent and prepared anti-Abl immunoprecipitates from nuclear lysates. In vitro kinase assays were performed with the Crk protein as substrate. c-Abl binds to the N-terminal SH3 domain of Crk and phosphorylates Tyr-221 (26, 31). Analysis of the anti-Abl immunoprecipitates with a GST-Crk(120–225) fusion protein demonstrated increased (≈3–4-fold) Crk phosphorylation as a consequence of ara-C treatment (Fig. 1A). The finding that there was little if any phosphorylation of a GST-Crk(120–212) fusion protein, which lacks the critical Tyr-221 for c-Abl phosphorylation, supported detection of c-Abl activity (data not shown). The ara-C-induced tyrosine kinase activity was also studied with a peptide (EAYAAPFACKK) recently identified as a specific substrate for c-Abl (29). Anti-Abl immunoprecipitates from ara-C-treated cells phosphorylated this peptide at a level approximately 3-fold higher than that obtained with similar immunoprecipitates from untreated cells (Fig. 1B). These results and the finding that immunoprecipitates with PIRS fail to demonstrate ara-C-induced peptide phosphorylation (Fig. 1B) support activation of c-Abl by ara-C.

Previous work has shown that SAP kinase is activated in cells treated with tumor necrosis factor α, anisomycin, ionizing radiation, and UV light (11, 12, 32). To determine whether ara-C induces SAP kinase, we analyzed anti-SAP kinase precipitates for phosphorylation of the transactivation domain of jun (15). Using this approach, ara-C treatment was associated with increased phosphorylation of a GST-Jun(2–100) fusion protein (Fig. 2A). In contrast, cells deficient in c-Abl (Abl-sh/−) failed to respond to ara-C with stimulation of SAP kinase activity (Fig. 2A). In order to confirm the involvement of c-Abl in ara-C-induced SAP kinase activity, we used Abl-sh/− cells that had been infected with a c-Abl containing retrovirus (designated Abl+). Immunoblot analysis of the Abl+ cells demonstrated expression of c-Abl (33). While ara-C failed to induce c-Abl activity in Abl+ cells, the Abl+ cells responded to ara-C with stimulation of c-Abl activity (Fig. 2B). Moreover, the Abl+ cells responded to ara-C exposure with increases in SAP kinase activity (Fig. 2C). These findings suggested that c-Abl is necessary for activation of SAP kinase in cells treated with ara-C.

SAP kinase is activated by SEK1 (14, 15). In order to determine whether c-Abl is involved in the cellular response to ara-C, we treated NIH3T3 cells with this agent and prepared anti-Abl immunoprecipitates from nuclear lysates. In vitro kinase assays were performed with the Crk protein as substrate. c-Abl binds to the N-terminal SH3 domain of Crk and phosphorylates Tyr-221 (26, 31). Analysis of the anti-Abl immunoprecipitates with a GST-Crk(120–225) fusion protein demonstrated increased (≈3–4-fold) Crk phosphorylation as a consequence of ara-C treatment (Fig. 1A). The finding that there was little if any phosphorylation of a GST-Crk(120–212) fusion protein, which lacks the critical Tyr-221 for c-Abl phosphorylation, supported detection of c-Abl activity (data not shown). The ara-C-induced tyrosine kinase activity was also studied with a peptide (EAYAAPFACKK) recently identified as a specific substrate for c-Abl (29). Anti-Abl immunoprecipitates from ara-C-treated cells phosphorylated this peptide at a level approximately 3-fold higher than that obtained with similar immunoprecipitates from untreated cells (Fig. 1B). These results and the finding that immunoprecipitates with PIRS fail to demonstrate ara-C-induced peptide phosphorylation (Fig. 1B) support activation of c-Abl by ara-C.
little if any phosphorylation of the GST-Crk(120–212) fusion protein (Fig. 3A, lane 3). While these results supported activation of c-Abl, the NIH3T3 SEK1 AL cells failed to respond to ara-C with activation of SAP kinase (Fig. 3B). Similar results were obtained with other clones stably expressing the SEK1 AL dominant negative protein (data not shown). Taken together, these findings demonstrate that ara-C-induced SAP kinase activity is c-Abl- and SEK1-dependent.

In order to confirm and extend our findings in ara-C-treated NIH3T3 cells, we asked whether other cell types respond similarly to this agent. Indeed, treatment of 293 kidney cells with ara-C was associated with activation of c-Abl (Fig. 4A). These cells also responded to ara-C with increases in SAP kinase activity (Fig. 4B). To further analyze the relationship between c-Abl and SEK1/SAP kinase, we transfected 293 cells with pEBG-SEK1 and pGNG Abl (SH3-deleted abl gene) and assayed glutathione-agarose protein complexes for in vitro phosphorylation of GST-J un(2-100). Similar results were obtained following transfection of pEBG-MEK1 and pGNG Abl (Fig. 4C). While transfection of SAP kinase was associated with detectable GST-J un phosphorylation, there was little if any effect on the intensity of this signal by cotransfection with pGNG Abl or pEBG-SEK1 (Fig. 4C). However, transfection of SAP kinase with both pGNG and pEBG-MEK1 resulted in a marked increase in GST-J un phosphorylation.
pE8G-SEK1 resulted in pronounced GST-Jun phosphorylation as evidenced by an increase in signal and a decrease in electrophoretic mobility (Fig. 4C). Moreover, the finding that transfection of the pE8G SEK1 K→R dominant negative completely blocks Abl stimulation of SAP kinase activity provided further support for c-Abl involvement in activation of SEK1/SAP kinase. The results also support the inability of MEK1 to substitute for SEK1 in stimulation of SAP kinase by pGNG Abl cotransfection (Fig. 4C). Other studies were performed with pGNG Abl-transfected NIH3T3 cells that stably express the SH3-deleted and activated Abl mutant (designated pGNG Abl-transfected NIH3T3 cells that stably express the C terminus following ara-C incorporation and thereby slowing or termination of DNA chain elongation. The incorporation of ara-C into DNA results in inhibition of replication forks and the accumulation of DNA fragments (35). While the event(s) responsible for activation of c-Abl remains unclear, DNA fragmentation may represent an initial signal. In this context, treatment with certain other agents that damage DNA, such as ionizing radiation, is also associated with c-Abl activation (33). These findings and the present studies suggest that c-Abl is involved in SEK1-dependent activation of SAP kinase in response to DNA damage.

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