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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 treatment[1] 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/JNK) 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)[1] is the most effective agent used in the treatment of acute myelogenous leukemia (1). This agent misincorporates into cellular DNA (2, 3) and inhibits its 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 Jun/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 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 (16). 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 G_1 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 $p34^{cdc2}$ 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, 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).[2] 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 (ΔXB) 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 $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml 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

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[1] The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; SAP, stress-activated protein; SEK1, SAP kinase/extracellular signal-regulated kinase kinase 1; PIRS, preimmune rabbit serum; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase.

[2] R. Ren, unpublished data.

subjected to centrifugation at 1,600 × *g* for 15 min to pellet nuclei. The nuclear pellet was washed twice by resuspension in 1 M sucrose/hypotonic solution, and centrifugation was done at 1,600 × *g* for 10 min. The nuclei were then suspended in lysis solution (0.5% Nonidet 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 supernatant 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 h at 4 °C. Preimmune 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 $MnCl_2$, 1 mM dithiothreitol) with 5 μg of GST-Crk(120–225) or GST-Crk(120–212) and 5 μCi of [γ-^{32}P]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 (EAIYAAPFAKKK) (29), 10 μM ATP, and 5 μCi of [γ-^{32}P]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 [^{32}P]phosphate was determined by scintillation counting.

c-Jun Immune Complex Kinase Assays—The GST-Jun(2–100) fusion protein was prepared and purified by affinity chromatography using glutathione-Sepharose beads as described (30). Cells were washed with phosphate-buffered saline and lysed in 1 ml of lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 μg/ml leupeptin and aprotinin). After incubation on ice for 30 min, insoluble material was removed by centrifugation at 14,000 rpm for 10 min at 4 °C. Lysates were incubated with PIRS or anti-p54 SAP kinase (12) and protein A-Sepharose for 2 h at 4 °C. The immune complexes were washed three times with lysis buffer and once with kinase buffer and resuspended in kinase buffer containing 1 μCi/μl [γ-^{32}P]ATP (6000 Ci/mmol, DuPont NEN) and 5 μg of GST-Jun. The reaction was incubated for 15 min at 30 °C and terminated by the addition of SDS sample buffer. The proteins were analyzed by SDS-PAGE and autoradiography.

Transient Transfection Studies—Using the calcium phosphate method, cells were transfected with 5 μg of pEBG, pEBG-SEK1, pEBG-SEK1 K→R, pEBG-MEK1, or pEBG-SAP kinase (14). 293 cells were also transfected with a retroviral vector (pGNG) encoding an SH3-deleted transforming *abl* gene (14). After incubation for 16 h, the cells were lysed in ice-cold lysis buffer. Lysates were incubated with glutathione-agarose for 30 min, and the complexes were suspended in kinase buffer containing [γ-^{32}P]ATP and GST-Jun. The reactions were incubated for 15 min and the proteins separated by SDS-PAGE for analysis by autoradiography and Coomassie Blue staining.

RESULTS AND DISCUSSION

Previous studies have demonstrated that ara-C induces a stress response that includes activation of Jun/AP-1 and c-*jun* transcription (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. 1*A*). 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 (EAIYAAPRAKKK) 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. 1*B*). These results and the finding that immunoprecipitates with PIRS fail to demonstrate

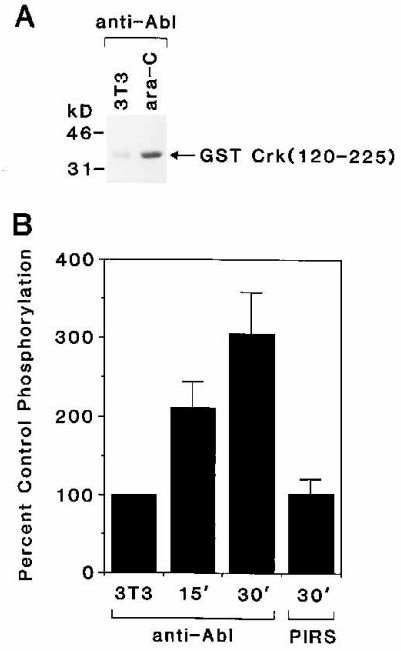

FIG. 1. **Activation of c-Abl by ara-C in NIH3T3 cells.** *A*, NIH3T3 cells were treated with 10 μM ara-C for 30 min. Nuclei were isolated and the nuclear proteins subjected to immunoprecipitation with anti-Abl. *In vitro* immune complex kinase assays were performed using a GST-Crk(120–225) fusion protein as substrate. GST-Crk(120–212) fusion protein (which lacks the critical Tyr-221) was used as a negative control (data not shown). *B*, NIH3T3 cells were treated with 10 μM ara-C and harvested at the indicated times. Nuclear proteins were then subjected to immunoprecipitation with anti-Abl antibody. Immunoprecipitations were also performed with PIRS from cells exposed to 10 μM ara-C and harvested at 30 min. *In vitro* immune complex kinase assays were performed using the c-Abl substrate EAIYAAPFAKKK. The data (percent control phosphorylation) represent the mean ± S.E. of two separate experiments.

ara-C-induced peptide phosphorylation (Fig. 1*B*) 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 c-Jun. Using this approach, ara-C treatment was associated with increased phosphorylation of a GST-Jun(2–100) fusion protein (Fig. 2*A*). In contrast, cells deficient in c-Abl (Abl$^{-/-}$) failed to respond to ara-C with stimulation of SAP kinase activity (Fig. 2*A*). In order to confirm the involvement of c-Abl in ara-C-induced SAP kinase activity, we used Abl$^{-/-}$ 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. 2*B*). Moreover, the Abl^{+} cells responded to ara-C exposure with increases in SAP kinase activity (Fig. 2*C*). 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 ara-C-induced SAP kinase activity is SEK1-dependent, we prepared NIH3T3 cells that stably express a dominant negative SEK1 in which the critical phosphorylation sites are mutated as Ser → Ala and Thr → Leu (SEK1 AL mutant) (provided by James Woodgett) (14). Treatment of the NIH3T3 SEK1 AL transfectants with ara-C was associated with stimulation of c-Abl activity as determined by GST-Crk(120–225) phosphorylation (Fig. 3*A*, *lanes 1* and *2*). In contrast, there was

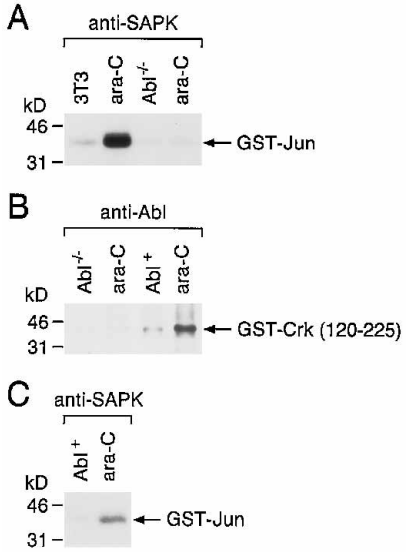

FIG. 2. **Activation of SAP kinase activity by ara-C.** *A*, NIH3T3 and Abl$^{-/-}$ cells were treated with 10 μM ara-C and harvested at 2 h. Total lysates were immunoprecipitated with anti-SAP kinase (*anti-SAPK*) antibody, and *in vitro* immune complex kinase reactions containing GST-Jun(2–100) fusion protein were analyzed by 10% SDS-PAGE and autoradiography. *B*, Abl$^{-/-}$ and c-Abl^{+} cells were treated with 10 μM ara-C for 30 min. Nuclear proteins were subjected to immunoprecipitation with anti-Abl. *In vitro* immune complex kinase assays were performed using GST-Crk(120–225) fusion protein. *C*, total cell lysates from control and ara-C-treated c-Abl^{+} cells were subjected to immunoprecipitation with anti-SAP kinase, and *in vitro* immune complex kinase reactions were performed with GST-Jun(2–100) fusion protein as substrate.

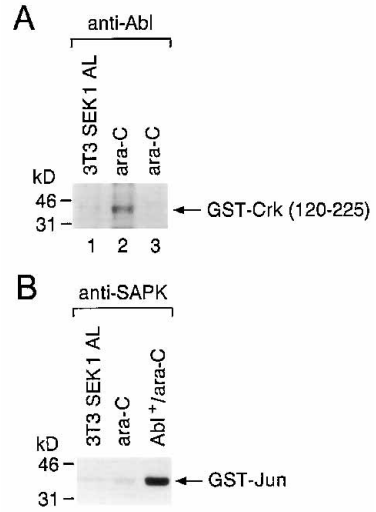

FIG. 3. **Activation of SAP kinase by ara-C is blocked in cells overexpressing SEK1 AL dominant negative mutant.** NIH3T3 SEK1 AL cells were treated with 10 μM ara-C for 30 min (*A*) or 2 h (*B*). A. Nuclear proteins were analyzed for c-Abl activity using GST-Crk(120–225) fusion protein as substrate (*lanes 1* and *2*). GST-Crk(121–212) fusion protein was used as a negative control (*lane 3*). *B*, total proteins were analyzed for SAP kinase (*SAPK*) activity. As a positive control, Abl^{+} cells were treated with 10 μM ara-C for 2 h, and total protein was analyzed for phosphorylation of GST-Jun(2–100).

little if any phosphorylation of the GST-Crk(120–212) fusion protein (Fig. 3*A*, *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. 3*B*). 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

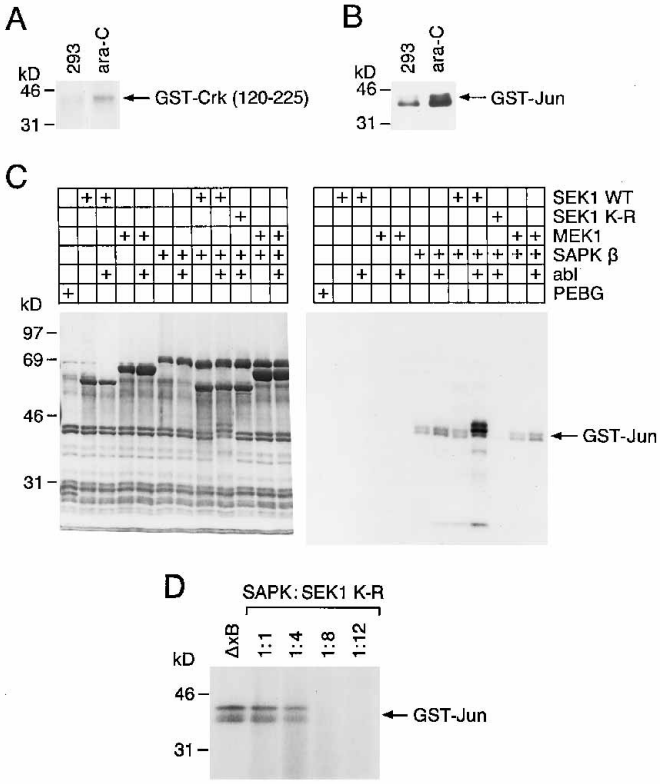

FIG. 4. **Role of Abl in activating SAP kinase in 293 cells.** *A*, 293 cells were treated with 10 μM ara-C and harvested at 30 min. Nuclei were isolated and the nuclear proteins subjected to immunoprecipitation with anti-Abl. *In vitro* immune complex kinase assays were performed using a GST-Crk(120–225) fusion protein as substrate. *B*, 293 cells were treated with 10 μM ara-C for 2 h. Total lysates were immunoprecipitated with anti-SAP kinase, and *in vitro* immune complex kinase assays were performed using GST-Jun(2–100) as substrate. *C*, 293 cells were transiently transfected with the indicated vectors and after 16 h, cell lysates were incubated with glutathione-agarose for 30 min. *In vitro* immune complex kinase assays were performed in the resulting protein complexes by using GST-Jun as a substrate. Proteins were separated by SDS-PAGE and analyzed by Coomassie Blue staining (*left panel*) and autoradiography (*right panel*). *WT*, wild type; *SAPK*, SAP kinase. *D*, pEBG-SAPK (2 μg/plate) was transiently transfected into NIH3T3 ΔXB cells together with the pEBG SEK1 K→R dominant negative. The molar ratio of the SAPK vector to the dominant negative plasmid is indicated, with the total DNA concentration kept constant by supplementation with pEBG vector. Total cell lysates were incubated with glutathione-agarose for 30 min at 4 °C. *In vitro* immune complex kinase assays were performed on the resulting protein complexes using GST-Jun as substrate. Proteins were separated by SDS-PAGE and analyzed by autoradiography.

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. 4*A*). These cells also responded to ara-C with increases in SAP kinase activity (Fig. 4*B*). 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-Jun. Using these experimental conditions, there was no detectable phosphorylation of GST-Jun (Fig. 4*C*). Similar results were obtained following transfection of pEBG-MEK1 and pGNG Abl (Fig. 4*C*). While transfection of SAP kinase was associated with detectable GST-Jun phosphorylation, there was little if any effect on the intensity of this signal by cotransfection with pGNG Abl or pEBG-SEK1 (Fig. 4*C*). However, transfection of SAP kinase with both pGNG and

pEBG-SEK1 resulted in pronounced GST-Jun phosphorylation as evidenced by an increase in signal and a decrease in electrophoretic mobility (Fig. 4*C*). Moreover, the finding that transfection of the pEBG 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. 4*C*). Other studies were performed with pGNG Abl-transfected NIH3T3 cells that stably express the SH3-deleted and activated Abl mutant (designated ΔXB) (27). Transfection of ΔXB cells with pEBG SAP kinase and different molar ratios of SEK1 K→R resulted in complete abrogation of SAP kinase activity (Fig. 4*D*). Taken together, these results in 293 and NIH3T3 cells demonstrate that activation of c-Abl is upstream to the SEK1/SAP kinase cascade. The finding that SEK1 and SAP kinase are detectable in the nucleus[3] also suggests that this cascade may be activated independently of cytoplasmic proteins.

Ara-C acts as an efficient but not absolute DNA chain terminator (4). The conformational and hydrogen bonding differences of the arabinose sugar moiety (34) are consistent with decreased reactivity of the 3′ 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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