1-β-D-Arabinofuranosylcytosine Activates Tyrosine Phosphorylation of p34^{cdc2} and Its Association with the Src-like p56/p53^{lyn} Kinase in Human Myeloid Leukemia Cells†

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ABSTRACT: Recent studies have demonstrated that treatment of human myeloid leukemia cells with 1-β-D-arabinofuranosylcytosine (ara-C) is associated with activation of serine/threonine protein kinases and early response gene expression. The present work has examined the involvement of protein tyrosine phosphorylation in ara-C-induced responses of HL-60 myeloid leukemia cells. The results of immunoprecipitation studies demonstrate that HL-60 cells respond to ara-C with tyrosine phosphorylation of the cell cycle regulatory protein p34^{cdc2} and a decrease in the activity of this kinase. This effect was detectable at 15 min of ara-C exposure. Coimmunoprecipitations with anti-p34^{cdc2} support binding of this protein to the Src-like p56/p53^{lyn} tyrosine kinase in ara-C-treated, but not untreated, cells. The results further demonstrate that ara-C treatment is associated with a dose-dependent activation of p56/p53^{lyn} and that ara-C-induced p56/p53^{lyn} activity is blocked by the protein tyrosine inhibitors herbimycin A and genistein. Studies with a glutathione *S*-transferase−Lyn fusion protein confirm interaction of p34^{cdc2} and p56/p53^{lyn} in lysates of ara-C-treated cells. Moreover, we demonstrate that (1) p56/p53^{lyn} phosphorylates Tyr-15 of p34^{cdc2} in vitro and (2) phosphorylation of p34^{cdc2} by p56/p53^{lyn} inhibits p34^{cdc2} activity. These findings indicate that the cellular response to ara-C includes activation of p56/p53^{lyn} and that association of p56/p53^{lyn} with p34^{cdc2} may contribute to regulation of the cell cycle progression in ara-C-treated cells.

1-β-D-Arabinofuranosylcytosine (ara-C)[1] is one of the most effective chemotherapeutic agents used in the treatment of acute myelogenous leukemia (Frei et al., 1969). While certain insights are available regarding the inhibitory effects of ara-C on DNA replication, the precise mechanisms that contribute to the lethal events induced by this agent remain unclear. Ara-C incorporates into leukemic cell DNA (Kufe et al., 1980; Major et al., 1981) and slows chain elongation by altering the reactivity of the 3′ terminus (Kufe et al., 1984; Major et al., 1982). The inhibitory effects of ara-C are related to both incorporation into DNA and the sequence of the DNA template (Ohno et al., 1988; Townsend & Cheng, 1987). Other studies have demonstrated that inhibition of DNA synthesis by ara-C is associated with DNA fragmentation and endonucleolytic cleavage (Fram & Kufe, 1982; Gunji et al., 1991).

Recent work has shown that ara-C induces certain signal transduction pathways, particularly activation of early response genes. Treatment of myeloid leukemia cells with ara-C is associated with induction of the c-jun, jun-B, jun-D, and c-fos genes (Datta et al., 1990; Kharbanda et al., 1990, 1993a,b). The finding that activation of c-jun gene transcription by ara-C is dependent on induction of a serine/threonine protein kinase C (PKC)-like activity has supported the involvement of both cytoplasmic and nuclear signaling mechanisms (Kharbanda et al., 1991). Moreover, nuclear factor κB (NF-κB), which resides in the cytoplasm in an inactive form, undergoes an increase in DNA binding activity in ara-C-treated cells (Brach et al., 1992). Other studies have shown that ara-C treatment is associated with induction of the serine/threonine protein kinase pp90^{rsk} and activation of the early growth response gene 1 by a CArG motif-mediated mechanism (Kharbanda et al., 1993b). These findings have indicated that certain signal transduction pathways involving serine/threonine protein phosphorylation are activated in the cellular response to this agent.

The present studies have asked whether protein tyrosine phosphorylation also contributes to the signaling events induced by ara-C. The results demonstrate that treatment of HL-60 myeloid leukemia cells with ara-C is associated with tyrosine phosphorylation of the cell cycle regulatory protein p34^{cdc2} and that p34^{cdc2} associates with the Src-like p56/p53^{lyn} tyrosine kinase in ara-C-treated cells.

MATERIALS AND METHODS

Cell Culture. HL-60 leukemia cells (American Type Culture Collection, Bethesda, MD) were grown in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 1 mM nonessential amino acids. The cells were treated with varying concentrations of ara-C (Sigma Chemical Co., St. Louis, MO), 10 μM genistein (GIBCO/BRL, Gaithersburg, MD), and 10 μM herbimycin A (GIBCO/BRL).

Immune Complex Formation. Cells (2 × 10^7) were washed with ice-cold phosphate-buffered saline (PBS) and then lysed in 2 mL of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM sodium vanadate, 1 mM PMSF, 1 mM DTT, and 10 μg/mL each of leupeptin and aprotinin) for 30 min on ice. The insoluble material was removed by centrifugation at 14 000 rpm for 10 min at 4

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[1] Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; GST, glutathione *S*-transferase; GAP, GTPase activating protein; PI 3-K, phosphatidylinositol 3-kinase.

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°C. Soluble proteins in the supernatant were precleared by incubation with 5 μg/mL rabbit anti-mouse IgG for 1 h at 4 °C and then addition of protein A−Sepharose for 30 min. The supernatants were incubated with a mouse anti-phosphotyrosine monoclonal antibody (MAb 4G10, anti-P-Tyr; UBI, Lake Placid, NY), a mouse anti-p34^{cdc2} MAb (sc-54; Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit anti-human Lyn polyclonal antibody (sc-15, Santa Cruz), a rabbit anti-Fyn polyclonal antibody (Santa Cruz), and a mouse anti-Src monoclonal antibody (UBI) for 1 h at 4 °C followed by 30 min with protein A−Sepharose.

Immunoblot Analysis. Immune complexes were washed 3 times with lysis buffer and boiled for 5 min in SDS sample buffer (0.35 M Tris-HCl, pH 6.8, 10% SDS, 35% glycerol, and 5% 2-mercaptoethanol). The complexes were then separated by electrophoresis in 10% SDS−polyacrylamide gels and transferred to nitrocellulose paper. After being blocked in 5% dry milk in PBST (PBS/0.05% Tween 20) for 1 h at room temperature, the filters were incubated for 1 h with appropriate antibody, washed twice with PBST, and incubated with anti-mouse or anti-rabbit IgG peroxidase conjugate (Sigma). The antigen−antibody complexes were visualized by chemiluminescence (ECL detection system; Amersham, Arlington Heights, IL).

Immune Complex Kinase Assays. Immune complexes were washed 3 times with lysis buffer and once with kinase buffer (20 mM HEPES, pH 7.0, 10 mM $MnCl_2$, and 10 mM $MgCl_2$). The beads were resuspended in 30 μL of kinase buffer containing 1 μCi/μL [γ-^{32}P]ATP (3000 Ci/mmol; NEN, Boston, MA) and 5 μg of acid-treated enolase (Sigma). Alternatively, kinase reactions (20 μL) contained beads, 1 μCi/μL [γ-^{32}P]ATP, and 0.1 mg/mL histone H1 (GIBCO/BRL). The reaction was incubated for 10 min at 30 °C and terminated by the addition of SDS sample buffer. The proteins were separated in 10% SDS−PAGE and analyzed by autoradiography.

Immune complexes were also resuspended in 30 μL of kinase buffer containing 1 μCi/μL [γ-^{32}P]ATP and either 100 μM cdc2 peptide (amino acids 7−20; IEKIGEGTYGVVYK) or 100 μM mutated cdc2 peptide with Phe-15 substituted for Tyr-15 (IEKIGEGTFGVVYK). The reactions were incubated for 10 min at 30 °C and terminated by spotting on P81 phosphocellulose disks (GIBCO/BRL). The disks were washed twice with 1% phosphoric acid and twice with water before analysis by liquid scintillation counting.

Fusion Protein Binding Assays. The plasmid encoding a glutathione *S*-transferase (GST)−Lyn (amino acids 1−243) fusion protein was provided by J. Cambier, Denver, CO (Pleiman et al., 1993). The pGEX construct was transfected into *Escherichia coli* DH5α, and expression of the fusion protein was induced by treating log phase cells with isopropyl β-D-thiogalactopyranoside. The cell pellets were lysed by sonication. The fusion protein was purified by affinity chromatography using glutathione−Sepharose beads (Pharmacia) as described (Pleiman et al., 1993) and equilibrated in lysis buffer.

Cell lysates were incubated with 50 μg of immobilized GST or GST−Lyn for 2 h at 4 °C. The protein complexes were washed 3 times with lysis buffer and boiled for 5 min in SDS sample buffer. The complexes were then separated by 10% SDS−PAGE and subjected to immunoblot analysis with anti-p34^{cdc2}.

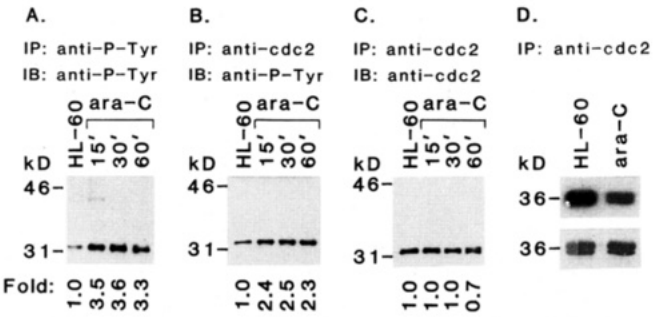

FIGURE 1: Effects of ara-C treatment on tyrosine phosphorylation of p34^{cdc2}. HL-60 cells were exposed to 10^{-5} M ara-C and harvested at the indicated times. (A) Cell lysates were subjected to immunoprecipitation with anti-P-Tyr. The immunoprecipitates were analyzed by SDS−PAGE and immunoblotting with anti-P-Tyr. (B) Cell lysates were subjected to immunoprecipitation with anti-p34^{cdc2} and immunoblot analysis with anti-P-Tyr. (C) Cell lysates were subjected to immunoprecipitation with anti-p34^{cdc2} and immunoblot analysis with anti-p34^{cdc2}. The fold increase in the intensity of the signals, as determined by densitometric scanning, is indicated at the bottom. (D) Lysates from control and ara-C-treated cells were subjected to immunoprecipitation with anti-p34^{cdc2}. The immune complexes were assayed for phosphorylation of histone H1 by SDS−PAGE and autoradiography (upper panel). Equal loading of histone H1 was assessed by Coomassie blue staining (bottom panel).

RESULTS

In order to determine whether treatment of HL-60 cells with ara-C is associated with increases in tyrosine phosphorylation, we incubated cell lysates with anti-P-Tyr and then assayed the immunoprecipitates by immunoblotting with anti-P-Tyr. Using this approach, a 34 kDa species detectable in untreated HL-60 cells exhibited an increase in anti-P-Tyr reactivity following ara-C exposure (Figure 1A). This increase in tyrosine phosphorylation was apparent at 15 min (3.5-fold as determined by densitometric scanning) and persisted through 60 min (3.3-fold) of treatment (Figure 1A). Another protein of approximately 42 kDa also exhibited increases in tyrosine phosphorylation at 15 min of ara-C exposure, although this effect was transient and undetectable at 30 min (Figure 1A). In contrast, these ara-C-induced increases in tyrosine phosphorylation were not apparent when whole cell extracts were subjected to immunoblotting with anti-P-Tyr. Since recent studies have demonstrated that p34^{cdc2} undergoes increases in tyrosine phosphorylation following exposure of HL-60 cells to ionizing radiation (Kharbanda et al., 1994a), we asked whether similar results are obtained as a result of ara-C treatment. Immunoprecipitation of p34^{cdc2} and then immunoblotting of the precipitates with anti-P-Tyr demonstrated an increase in tyrosine phosphorylation at 15 (2.4-fold) to 60 (2.3-fold) min of ara-C exposure (Figure 1B). Reprobing the same filter with the anti-p34^{cdc2} antibody demonstrated no detectable difference in levels of p34^{cdc2} protein (Figure 1C). Analysis of anti-p34^{cdc2} immunoprecipitates from ara-C-treated cells also demonstrated decreased phosphorylation of histone H1 as compared to that from control cells (Figure 1D). These findings indicated that ara-C treatment of HL-60 cells is associated with increased tyrosine phosphorylation of p34^{cdc2} and decreased p34^{cdc2} activity.

The ability of p34^{cdc2} to associate with a protein tyrosine kinase following ara-C treatment was next examined by coimmunoprecipitation experiments. Lysates of control and ara-C-treated HL-60 cells were incubated with anti-p34^{cdc2}

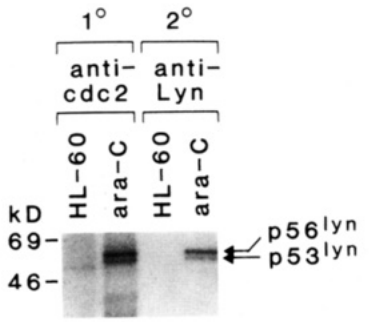

FIGURE 2: Coimmunoprecipitation of $p34^{cdc2}$ and $p56/p53^{lyn}$ in ara-C-treated cells. HL-60 cells were exposed to 10^{-5} M ara-C and harvested at 1 h. Lysates from control (labeled HL-60) and ara-C-treated cells were subjected to immunoprecipitation with anti-$p34^{cdc2}$ (1°). The immune complexes were assayed for in vitro kinase activity by incubation with [γ-^{32}P]ATP. One aliquot of the kinase reaction was analyzed by SDS−PAGE and autoradiography (left panel; anti-$p34^{cdc2}$). The other aliquot was washed to remove free ATP and boiled in SDS buffer to disrupt complexes. A secondary (2°) immunoprecipitation was then performed with anti-Lyn (right panel; anti-Lyn). The anti-Lyn immunoprecipitates were separated by SDS−PAGE and analyzed by autoradiography.

and the immunoprecipitates assayed for phosphorylation in the presence of [γ-^{32}P]ATP. An aliquot of the in vitro kinase reaction was assayed by SDS−PAGE and autoradiography. There was no detectable phosphorylation in anti-$p34^{cdc2}$ immunoprecipitates from control HL-60 cell lysates (Figure 2). However, analysis of similar immunoprecipitates from lysates of ara-C-treated HL-60 cells revealed phosphorylated proteins of approximately 56 and 53 kDa (Figure 2). Since the Src-like $p56/p53^{lyn}$ protein tyrosine kinase has similar physical characteristics, the other aliquot of the in vitro kinase reaction was treated to disrupt protein complexes and then subjected to immunoprecipitation with anti-Lyn. Analysis of these secondary immunoprecipitates demonstrated the presence of phosphorylated $p56/p53^{lyn}$ when assaying protein from ara-C-treated but not control cells (Figure 2). These findings indicated that $p34^{cdc2}$ associates with $p56/p53^{lyn}$ as a consequence of ara-C treatment.

The interaction between $p34^{cdc2}$ and $p56/p53^{lyn}$ prompted other studies to determine whether this Src-like tyrosine kinase is activated during ara-C exposure. Analysis of anti-Lyn immunoprecipitates by blotting with anti-P-Tyr indicated an increase in tyrosine phosphorylation of $p56/p53^{lyn}$ after treatment with ara-C for 15 min (data not shown) through 1 h (Figure 3A). This effect was not related to increases in $p56/p53^{lyn}$ protein since comparable levels were observed in immunoprecipitates from control and ara-C-treated cells when reprobing the filter with anti-Lyn (Figure 3B). Activation of $p56/p53^{lyn}$ was further studied by autophosphorylation assays in which anti-Lyn immunoprecipitates are incubated in the presence of [γ-^{32}P]ATP and phosphorylation is assessed by autoradiography. Using this approach, $p56/p53^{lyn}$ activity was increased at 1 h of ara-C exposure as compared to that in untreated cells (Figure 3C). Other studies have demonstrated that $p59^{fyn}$ and $pp60^{c\text{-}src}$ are also expressed in HL-60 cells (Barnekow & Gessler, 1986; Gee et al., 1986; Katagiri et al., 1991). However, treatment with ara-C for 1 h was associated with little if any change in $p59^{fyn}$ or $pp60^{c\text{-}src}$ activity (Figure 3C). Similar findings were obtained with additional time points. These results indicated that $p56/p53^{lyn}$ is selectively activated by ara-C treatment of HL-60 cells.

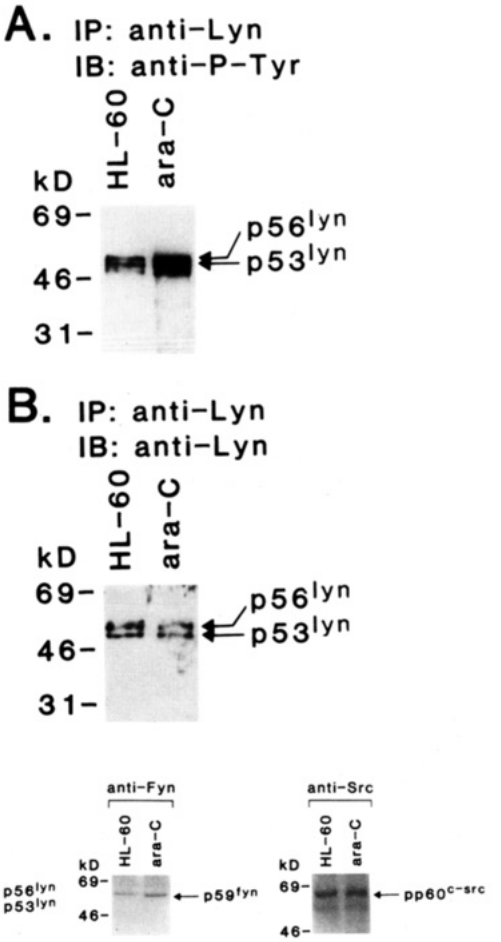

FIGURE 3: Effects of ara-C treatment on tyrosine phosphorylation of $p56/p53^{lyn}$. HL-60 cells were treated with 10^{-5} M ara-C for 1 h. (A) Cell lysates were subjected to immunoprecipitation with anti-Lyn. The immunoprecipitates were analyzed by SDS−PAGE and immunoblotting with anti-P-Tyr. (B) Cell lysates were subjected to immunoprecipitation with anti-Lyn and immunoblot analysis with anti-Lyn. (C) Cell lysates were subjected to immunoprecipitation with anti-Lyn, anti-Fyn, and anti-Src. Autophosphorylation reactions were performed by adding [γ-^{32}P]ATP for 10 min at 30 °C. Phosphorylated protein was analyzed by 10% SDS−PAGE and autoradiography.

Similar findings of $p56/p53^{lyn}$ activation by ara-C were obtained when using enolase as substrate (Figure 4A). Exposure of HL-60 cells to concentrations of ara-C as low as 10^{-7} M demonstrated increases in phosphorylation of both $p56/p53^{lyn}$ and enolase (Figure 4A). Moreover, the finding that exposure to higher concentrations of ara-C is associated with further increases in $p56/p53^{lyn}$ and enolase phosphorylation supported a concentration-dependent effect (Figure 4A). These results indicated that treatment with ara-C results in activation of $p56/p53^{lyn}$ kinase. In order to confirm the induction of protein tyrosine kinase activity, we used the inhibitors herbimycin A (Uehara et al., 1989) and genistein (Akiyama et al., 1987). Stimulation of $p56/p53^{lyn}$ and enolase phosphorylation by ara-C was completely blocked when cells were pretreated with these inhibitors (Figure 4B).

The present findings suggest that activation of $p56/p53^{lyn}$ in ara-C-treated cells and its association with $p34^{cdc2}$ could contribute to increased tyrosine phosphorylation of $p34^{cdc2}$. In order to further examine this possibility, experiments were performed with a GST−Lyn fusion protein. Lysates from ara-C-treated cells were incubated with immobilized GST or GST−Lyn fusion proteins, and the adsorbates were

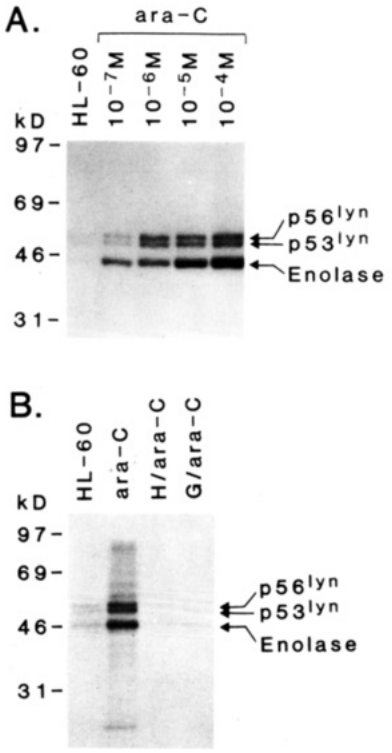

FIGURE 4: Concentration-dependent effects of ara-C on p56/p53^{lyn} tyrosine kinase activity. (A) HL-60 cells were exposed to the indicated concentrations of ara-C and then harvested at 1 h. Cell lysates were subjected to immunoprecipitation with anti-Lyn. Phosphorylation reactions were performed in the presence of [γ-^{32}P]-ATP and 5 μg of enolase for 10 min. Phosphorylated protein was analyzed by SDS−PAGE and autoradiography. (B) HL-60 cells were pretreated with either 10 μM herbimycin A (H) or 10 μM genistein (G) for 1 h and then exposed to 10^{-5} M ara-C for an additional 1 h. Cell lysates were immunoprecipitated using anti-Lyn, and immune complex kinase assays were performed in the presence of enolase as a substrate.

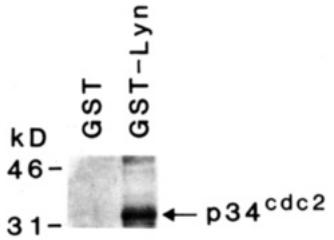

FIGURE 5: Association of p56/p53^{lyn} and p34^{cdc2}. HL-60 cells were treated with 10^{-5} M ara-C for 1 h. Cell lysates were incubated with GST or GST−Lyn proteins immobilized on beads. The resulting complexes were separated by SDS−PAGE and analyzed by immunoblotting with anti-cdc2 antibody.

analyzed by immunoblotting with anti-p34^{cdc2}. There was no detectable reactivity of the anti-p34^{cdc2} antibody when assaying adsorbates obtained from immobilized GST (Figure 5). However, p34^{cdc2} was detectable in adsorbates obtained with the GST−Lyn fusion protein (Figure 5). Taken together with the results of anti-p34^{cdc2} coimmunoprecipitation studies (Figure 2), these findings further supported an association between p56/p53^{lyn} and p34^{cdc2}.

Since the serine/threonine kinase activity of p34^{cdc2} is regulated by phosphorylation on Tyr-15 in vivo (Atherton-Fessler et al., 1993), we also asked whether this site can serve as a substrate for p56/p53^{lyn}. In exploring this issue, we used as substrates a synthetic peptide derived from amino acids 7−20 of p34^{cdc2} and another with substitution of Tyr-15 by Phe-15. While anti-Lyn immune complexes from control HL-60 cells phosphorylated the cdc2 peptide, this activity was increased nearly 2-fold when similar complexes from ara-C-treated cells were used (Figure 6A). In contrast, anti-Lyn immune complexes from both control and ara-C-treated cells displayed little activity with the mutated cdc2 peptide (Figure 6A). In order to determine whether phosphorylation of p34^{cdc2} by p56/p53^{lyn} decreases p34^{cdc2} activity, we incubated anti-p34^{cdc2} immune complexes with purified p56/p53^{lyn} and assayed for phosphorylation of histone H1. The results demonstrate that p56/p53^{lyn} decreases p34^{cdc2} activity (Figure 6B). These findings suggest that interaction of p56/p53^{lyn} with p34^{cdc2} is associated with phosphorylation of p34^{cdc2} on Tyr-15 and a decrease in serine/threonine kinase activity.

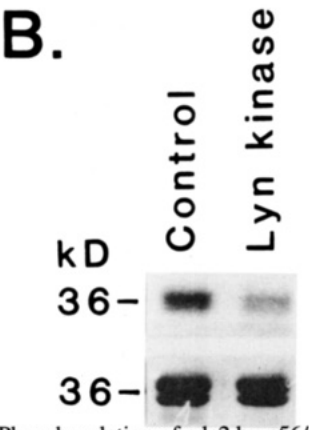

FIGURE 6: (A) Phosphorylation of cdc2 by p56/p53^{lyn}. HL-60 cells were treated with 10^{-5} M ara-C for 1 h. Cell lysates were subjected to immunoprecipitation with anti-Lyn. The immunoprecipitates were assayed for phosphorylation of either a cdc2 (IEKIGEGT YGVVYK) or a mutated cdc2 (mcdc2; Y-15 to F-15) peptide. The results represent the means ± SD of two independent experiments each performed in duplicate and are normalized to control phosphorylation of the cdc2 peptide. Control cells (hatched bars); ara-C-treated cells (cross-hatched bars). (B) Anti-p34^{cdc2} immune complexes were suspended in kinase buffer with 10 mM ATP and incubated in the absence and presence of purified p56/p53^{lyn} (10 units; UBI) for 10 min at 30 °C. After being washed, the beads were incubated with histone H1 and [γ-^{32}P]ATP, and phosphorylation was assessed by SDS−PAGE and autoradiography (upper panel). Equal loading of histone H1 was assessed by Coomassie blue staining (lower panel).

DISCUSSION

The response of human leukemic cells to ara-C includes inhibition of growth, induction of a more differentiated phenotype, and activation of programmed cell death (Gunji et al., 1991; Kufe et al., 1980; Luisi-DeLuca et al., 1984;

Major et al., 1981). The signaling events that regulate these responses, however, have remained unclear. Protein tyrosine phosphorylation−dephosphorylation contributes to the control of cell growth and differentiation. The protein tyrosine kinases have been classified as receptor-type and nonreceptor or Src-like kinases. The Src-like kinases are for the most part associated with the inner surface of the plasma membrane and transduce signals from cell surface receptors (Cantley et al., 1991) by inducing tyrosine phosphorylation and/or activation of effectors such as phospholipase C (Carter et al., 1991; Hempel et al., 1992), mitogen-activated protein kinase (Casillas et al., 1991), GTPase activating protein (GAP) (Gold et al., 1992a), and phosphatidylinositol 3-kinase (PI 3-K) (Gold et al., 1992b; Yamanashi et al., 1992). The different Src-like kinases vary in their ability to associate with these effectors and may preferentially activate distinct sets of downstream signals (Pleiman et al., 1993).

The lyn gene is a member of the src family and encodes, as a result of alternate splicing, two forms of the Lyn kinase, $p56^{lyn}$ and $p53^{lyn}$ (Yamanashi et al., 1987, 1989). In B lymphocytes, $p56/p53^{lyn}$ associates with the B-cell antigen receptor complex and participates in signaling that includes activation of PI 3-K (Yamanashi et al., 1992). Activation of platelets with thrombin induces the association of $p56/p53^{lyn}$ with GAP (Cichowski et al., 1992). While $p56/p53^{lyn}$ is also expressed in other hematopoietic lineages (Katagiri et al., 1991), the functional role of this kinase in myeloid cells is unknown. The results of the present studies in ara-C-treated HL-60 leukemia cells demonstrate activation of $p56/p53^{lyn}$, but not certain other Src-like kinases such as $p59^{lyn}$ or $pp60^{c\text{-}src}$. The finding that similar effects are observed with other anticancer agents, such as ionizing radiation and mitomycin C (Kharbanda et al., 1994b,c), suggests that DNA damage may be an early signal in this pathway. Other studies have shown that oxidative reagents, such as H_2O_2, induce the Src-like $p56^{lck}$ in T cells (Nakamura et al., 1993). However, the finding that H_2O_2 has no effect on $p56/p53^{lyn}$ activity in HL-60 cells (data now shown) suggests that induction of this kinase by ara-C is not related to alterations in redox regulation.

The available evidence indicates that the antiproliferative effects of ara-C are related to the incorporation of this nucleoside analog into DNA. Conformational and hydrogen-bonding differences of the incorporated arabinose sugar moiety alter chromatin structure and slow DNA chain elongation (Cozzarelli, 1977). More recent studies have demonstrated that exposure to this agent is associated with induction of serine/threonine protein kinases and specific gene transcription. Induction of PKC-like activity (Kharbanda et al., 1991), NF-κB (Brach et al., 1992), and $pp90^{rsk}$ (Kharbanda et al., 1993b) has supported the involvement of cytoplasmic signaling events. The present results further support intracytoplasmic signal transduction in ara-C-treated cells and particularly the involvement of protein tyrosine phosphorylation. Since ara-C slows DNA replication, recognition of premature strand termination could conceivably activate signals that are transduced from the nucleus. In support of this possibility is the finding that the state of DNA replication is associated with regulation of $p34^{cdc2}$ activity by tyrosine phosphorylation (Smythe & Newport, 1992).

The $p34^{cdc2}$ serine/threonine protein kinase forms complexes with cyclins A or B and is required for the initiation of cell division (Nurse, 1990). The kinase activity of $p34^{cdc2}$ in fission yeast *Schizosaccharomyces pombe* is inhibited by phosphorylation of Tyr-15 in the ATP-binding site of the catalytic subunit (Gould & Nurse, 1989). The available evidence indicates that the $p107^{wee1}$ dual specificity kinase is responsible for phosphorylation of $p34^{cdc2}$ on Tyr-15 at the replication-dependent mitotic checkpoint (Featherstone & Russell, 1991; Parker et al., 1992; Russell & Nurse, 1987). In human cells, the Wee1 kinase is located in the nucleus and through phosphorylation of $p34^{cdc2}$ coordinates the completion of DNA replication before mitotsis (Heald et al., 1993). However, distinct mitotic checkpoints appear to monitor for completion of DNA synthesis and the presence of DNA damage. For example, recent studies with *S. pombe* in which the wee1 gene is defective or missing have demonstrated that $p107^{wee1}$ is not required for the checkpoint that monitors for DNA damage induced by ionizing radiation (Barbet & Carr, 1993). These findings have indicated that a kinase other than $p107^{wee1}$ is responsible for tyrosine phosphorylation of $p34^{cdc2}$ and mitotic arrest in cells with DNA damage. The present findings that $p56/p53^{lyn}$ associates with $p34^{cdc2}$ and that $p34^{cdc2}$ exhibits increases in tyrosine phosphorylation, as well as decreases in activity, in ara-C-treated cells suggest that $p56/p53^{lyn}$ contributes to the regulation of $p34^{cdc2}$ in the presence of ara-C-induced DNA damage. Moreover, the findings that (1) $p56/p53^{lyn}$ phosphorylates $p34^{cdc2}$ in vitro at the same Tyr-15 site which is phosphorylated in vivo and (2) purified $p56/p53^{lyn}$ inhibits $p34^{cdc2}$ activity would lend further support to this possibility.

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