Communication

Cells Arrested in G₁ by the v-Abl Tyrosine Kinase Do Not Express Cyclin A Despite the Hyperphosphorylation of RB*

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The v-Abl tyrosine kinase encoded by the Abelson murine leukemia virus (A-MuLV) can either stimulate or inhibit cell proliferation, depending on the cell context. In a NIH-3T3-derived cell line, N3T3, v-Abl blocks the serum-induced entry into S phase. In these G₁-arrested cells v-Abl does not interfere with the activation of cyclin D₁ or cyclin E-dependent kinases. As a result, v-Abl does not block the hyperphosphorylation and inactivation of the retinoblastoma protein RB. However, activation of cyclin A-dependent kinase is inhibited due to a v-Abl-induced block in the accumulation of cyclin A mRNA and protein. Ectopic expression of cyclin A enabled the v-Abl-arrested cells to enter S phase, whereas cyclins E and D₁, or E2Fs 1 and 4 could not overcome the v-Abl arrest. Taken together, these results suggest that v-Abl tyrosine kinase arrests cell cycle progression in G₁ by inhibiting the expression of cyclin A.

Progression through the eukaryotic cell division cycle is regulated by cyclin-dependent kinases (1–3). A number of cyclins have been identified and can be divided into at least three groups, the G₁-cyclins, the S-cyclins and the G₂/M-cyclins. Activation of the G₁-cyclin-dependent kinases (Cdks) drives the commitment to enter S phase. The function of cyclins can be deduced by (a) the timing of their expression, (b) the effect of anti-cyclin antibodies on the cell cycle, and (c) the effect of ectopic expression of cyclins. Cyclin D₁ (CycD₁), cyclin E (CycE), and cyclin A (CycA) are characterized as G₁ cyclins. Consistent with this, antibodies to CycD₁, -E, or -A can block entry into S phase (4–6), and the ectopic expression of CycD₁, -E, or -A can advance S phase entry (6–9).

In mammalian cells, the identification of G₁-cyclins has also been aided by the study of agents that cause G₁ arrest. For example, transforming growth factor-β can block cells in G₁ by inhibiting both the cyclin D (CycD)- and CycE-dependent kinases through the activation of inhibitors such as p15INK4B and p27Kip1 (10–12). DNA-damaging agents, e.g. γ-irradiation, also block cells in G₁ by inducing p21Cip, an inhibitor of CycD-, CycE-, and CycA-associated kinases (13, 14). A major target of phosphorylation by the cyclin-dependent kinases at the G₁/S transition is the retinoblastoma protein, RB (15). The initiation of RB phosphorylation during G₁ is most likely catalyzed by the CycD-dependent kinases, Cdk4 and Cdk6 (15, 16). The maintenance of RB phosphorylation from G₁ to M phase is cyclin-dependent. For example, the major RB-kinase in M phase cells is cyclin B/Cdc2 (17). Phosphorylation of RB is generally inhibited in G₁-arrested cells, including those blocked by transforming growth factor-β or DNA damage (15, 16). Thus, RB phosphorylation is likely to be a key event in the progression through G₁. However, it is unclear if RB phosphorylation is sufficient to drive entry into S phase.

It has been shown previously that the oncogenic v-Abl tyrosine kinase can either transform or growth arrest NIH-3T3 cells (18). Based on their response to v-Abl, two genetically stable subclones of NIH-3T3 cells (P3T3 and N3T3) were isolated. In the P3T3 cells v-Abl acts as an oncogene causing anchorage and growth factor-independent growth. In contrast, the N3T3 cells are growth-arrested by v-Abl. Using a temperature-sensitive allele of v-abl, we have shown that v-Abl blocks N3T3 cells in the G₁ phase of the cell cycle. N3T3 cells stably expressing temperature-sensitive v-Abl (D4 cells) grow normally at 39 °C, the restrictive temperature for kinase activity, but become G₁-arrested at 32 °C, the permissive temperature (18). When v-Abl is activated in S phase cells, no effect on the cell cycle progression is observed until the subsequent G₂, showing that v-Abl acts only to block G₁, but not other phases of the cell cycle. The N3T3 cells are also growth-inhibited by the Bcr-Abl oncogene, the phosphol ester 12-O-tetradecanoylphorbol-13-acetate, and the immunosuppressant rapamycin (19, 20). Determining the basis of the G₁ block in N3T3 cells may define a cell cycle checkpoint, which must be overcome for v-Abl to have oncogenic potential.

MATERIALS AND METHODS

Cell Culture and Synchronization—Cells were cultured at 39 °C (D411XA) or 37 °C (N3T3) in Dulbecco’s modified Eagle’s medium with 10% defined bovine calf serum (CS, HyClone). D411XA (D4) cells expressed the temperature-sensitive v-Abl and had previously been characterized (18). Synchronization in G₁ was made by serum deprivation (Dulbecco’s modified Eagle’s medium with 0.1% CS) for 48-h stimulation with 10% CS was used to induce synchronous cell cycle entry. For the activation of v-Abl in the synchronized D4 cells, the culture temperature was shifted from 39 to 32 °C 2 h before serum stimulation.

In Vitro Kinase Assay—Fifty micrograms of protein lysate was used in each in vitro kinase assay as described previously (21). The lysate was incubated with the appropriate antibody in 1× SDS-RIPA buffer plus protein A-Sepharose beads at 4 °C for 4–5 h. The immunoprecipitate was washed three times with 1× SDS-RIPA buffer, once with the 1× SDS-RIPA buffer minus SDS, and 2 times with 1× kinase buffer. The immunoprecipitate was incubated at room temperature for 30 min in 2× kinase buffer supplemented with 0.5 μg of GST-RB/SIE protein as the substrate (23). 0.2 μl of [γ-32P]ATP (approximately 30 μCi, 7000 Ci/mmol), 50 μM cold ATP, and 1 μM dithiothreitol. The kinase reaction was stopped by resuspending in 1× SDS sample loading buffer, boiled,

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‡ The abbreviations used are: Cdk, cyclin-dependent kinase; CycA, cyclin A; CycE, cyclin E; CycD, cyclin D; RB, protein product of the retinoblastoma susceptibility gene; pRB, hypophosphorylated RB; p27Kip1, hyperphosphorylated RB; T-Ag, SV40 large T-antigen; PBS, phosphate-buffered saline; Cs, bovine calf serum; RIPA, radiimmune precipitation buffer; FACs, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate.
The protein level was quantitated and plotted (NaCl, 1 mM EDTA, 100 mM Tris-Cl 8.0, 0.5% Nonidet P-40) supplementing functional proteins. For CD20 staining, the transfected cells and pRC/CMV (Invitrogen). Each of these cyclin plasmids has been cyclin D1, from Dr. C. J. Sherr (25); cyclin E, from Dr. J. M. Roberts (9); include CD20, from Dr. E. Harlow; cyclin A, from Dr. S. I. Reed (24);

tion of N3T3 and D4 cells were performed with lipofectAMINE (Life technology).

electrophoresis, the gel was stained with Coomassie Blue, dried with addition.

(v-Abl-active). Samples were collected at the indicated hour after serum

blotting in synchronized D4 cells at 39°C (v-Abl-inactive) or 32°C (v-Abl-active).

Kip1, the level of Cdk2, CycE, or CycA was determined by immunoblotting in synchronized D4 cells. The protein level was quantitated and plotted (C, 39°C; ●, 32°C) with the level at 0 h set to 1. E, the steady state mRNA level of CycA was determined by Northern blot with total RNA prepared from synchronized D4 cells at two different temperatures.

and separated by 12% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with Coomassie Blue, dried with vacuum, and the phosphorylated RB was detected by autoradiography.

Cell Transfection, CD20 Staining, and Flow Cytometry—Transfection of N3T3 and D4 cells were performed with lipofectAMINE (Life Technologies, Inc.). The expression plasmids used in the transfection include CD20, from Dr. E. Harlow; cyclin A, from Dr. S. I. Reed (24); cyclin D1, from Dr. C. J. Sherr (25); cyclin E, from Dr. J. M. Roberts (9); E2F-1, from Dr. W. K. Kaelin, Jr. (26); E2F-4, from R. Bernards (27); and pRCCMV (Invitrogen). Each of these cyclin plasmids has been shown to drive the hyperphosphorylation of RB, confirming that they encode functional proteins. For CD20 staining, the transfected cells were trypsinized and washed twice with 1 × PBS. The cells were resuspended in 1 × PBS containing 0.1% bovine serum albumin, 10% FITC-conjugated anti-CD20 antibody (PharMingen), and incubated on ice for 30 min. Cells were then washed with PBS, fixed in 80% ethanol, and stained with propidium iodide. The cell cycle profile was determined by FACS analysis on a FACScan (Becton Dickinson), and the consequent reduction in Cdk2 activity.

With D4 cell at 39°C, RB was shifted to the hyperphosphorylation form by 12 h after serum stimulation, correlating with G1/S transition (Fig. 2A, lanes 1–4). The phosphorylation of RB also occurred at 32°C, beginning at 6 h and reaching completion by 18 h (lanes 5–8). However, the hyperphosphorylation of RB did not correlate with S phase entry. Two other RB-related proteins, p107 and p130, also became phosphorylated in the v-Abi tyrosine kinase. We also examined the level of Cyclin D1 and its associated kinase activity and found little difference between the two temperatures (not shown). Since many agents that block cells at G1 act through the Cdk inhibitors, we examined the level of p27Kip1 and p21Cip1. Activation of v-Abi did not have a significant effect on p27Kip1 or p21Cip1 (Fig. 1D). Thus, v-Abi-induced G1 arrest was correlated with the inhibition of CycE expression and the consequent reduction in Cdk2 activity. Because v-Abi did not inhibit the CycD- and CycE-dependent kinases, we tested whether it interfered with the phosphorylation of RB. With D4 cells at 39°C, RB was shifted to the hyperphosphorylated form by 12 h after serum stimulation, correlating with G1/S transition (Fig. 2A, lanes 1–4). The phosphorylation of RB also occurred at 32°C, beginning at 6 h and reaching completion by 18 h (lanes 5–8). However, the hyperphosphorylation of RB did not correlate with S phase entry. Two other RB-related proteins, p107 and p130, also became phosphorylated in D4 cells at either temperature (not shown).

Phosphorylation of RB and RB-related proteins is correlated with a functional inactivation (15, 16, 28). In particular, phosphorylation of RB, p107, and p130 leads to the release and the consequent activation of the transcription factor, E2F (15). RB

Fig. 1. Activation of v-Abl tyrosine kinase blocks CycA expression. A, the level of Cdk2, CycE, or CycA was determined by immunoblotting in synchronized D4 cells at 39°C (v-Abl-inactive) or 32°C (v-Abl-active). Samples were collected at the indicated hour after serum addition. B, the kinase activity of Cdk2, CycE, and CycA was assayed using GST-RB as a substrate. The relative activity of each kinase complex was quantitated and plotted (○, 39°C; ●, 32°C) as compared with the highest activity (lane 4) set to 100%. C, Cdk2 kinase is activated at 32°C in the uninfected N3T3 cells. D, the level of p21Cip1 or p27Kip1 was determined by immunoblotting in synchronized D4 cells. The protein level was quantitated and plotted (○, 39°C; ●, 32°C) with the level at 0 h set to 1. E, the steady state mRNA level of CycA was determined by Northern blot with total RNA prepared from synchronized D4 cells at two different temperatures.

Fig. 2. Phosphorylation and inactivation of RB occurs in v-Abl-arrested cell. A, phosphorylation of RB was determined by immunoprecipitation and immunoblotting with an an anti-RB antibody in the synchronized D4 cells (prB, hypophosphorylated form of RB; ppRB, hyperphosphorylated form). Results of FACS analysis (percentage of cells in each cell cycle phase) are shown for each sample. B and C, the protein binding activity of RB present in the synchronized D4 cells was determined by assaying for binding to either immobilized GST-E2F-1 (B) or immobilized T-Ag (C). Equal amounts of GST-E2F-1 or T-Ag were used in the binding reactions as shown in the lower panels.

RESULTS AND DISCUSSION

v-Abl Tyrosine Kinase Inhibits Cyclin A Expression—The temperature-sensitive A-MuLV-infected N3T3 cells, D4, were mad quiescent by serum starvation and then induced to re-enter the cell cycle with serum. At the restrictive temperature (39°C), D4 cells progressed through G1 into S phase with normal kinetics, and this was accompanied by the accumulation of CycE and CycA (Fig. 1A, 39°C). At the permissive temperature (32°C), v-Abl tyrosine kinase did not affect CycE expression but prevented the accumulation of CycA protein (Fig. 1A, 32°C), as well as CycA mRNA (Fig. 1E). Consistent with the protein level, CycE-associated kinase was activated by serum at both temperatures (Fig. 1B, CycE kinase), but the RB kinase was CycA-dependent in these cells. The reduction of Cdk2 activity was not observed in control N3T3 cells at 32°C (Fig. 1C), thus the observed inhibition was dependent on the v-Abl tyrosine kinase. We also examined the level of Cyclin D1 and its associated kinase activity and found little difference between the two temperatures (not shown). Since many agents that block cells at G1 act through the Cdk inhibitors, we examined the level of p27Kip1 and p21Cip1. Activation of v-Abl did not have a significant effect on p27Kip1 or p21Cip1 (Fig. 1D). Thus, v-Abl-induced G1 arrest was correlated with the inhibition of CycE expression and the consequent reduction in Cdk2 activity.

RB Protein Binding Assays—Cells were lysed in NET-N (100 mM NaCl, 1 mM EDTA, 100 mM Tris-Cl 8.0, 0.5% Nonidet P-40) supplemented with protease and phosphatase inhibitors and clarified by centrifugation (28). Clarified lysates of 400 μg of total protein were incubated with either 200 ng of T-Ag immobilized by immunoprecipitation or 500 ng of GST-E2F-1 immobilized on glutathione-agarose (28). Binding reactions were allowed to proceed for 2 h at 4°C with rotation, then washed four times with NET-N. Proteins bound to the immobilized T-Ag or GST-E2F-1 were solubilized by boiling in 1 × SDS sample loading buffer and resolved by 6.5% SDS-polyacrylamide gel electrophoresis. Immunoblotting for RB bound to T-Ag or GST-E2F-1 was carried out as described above.

E. S. Knudsen, unpublished data.
is phosphorylated on multiple Cdk sites (15, 16), and we have shown that different Cdk sites regulate the interaction of RB with different partner proteins (28). To confirm that the phosphorylated RB in the v-Abl-arrested cells had indeed lost its activity of these cyclins. We also tested two E2F family members, E2F-1 and E2F-4, for their effect on the v-Abl-induced G1 block (28, 30). Neither E2Fs could overcome the v-Abl-induced G1 block (Fig. 3, E2F-1, E2F-4). All the cyclin plasmids produce active proteins capable of driving RB phosphorylation3 and advancing S phase entry.4 Likewise, the E2F plasmids could stimulate transcription from E2F responsive reporters.5

The Role of v-Abl Tyrosine Kinase in G1/S—Data presented here are consistent with the model that v-Abl arrests N3T3 cells in G1 by blocking the activation of CycA expression. The activation of CycD1- and CycE-associated kinase by mitogenic stimuli was not inhibited by v-Abl (Fig. 1). These kinases drive the phosphorylation of RB and lead to the disruption of RB protein binding activity (Fig. 2), which allows for the release of E2F transcription factors. Although these G1 cyclins are active and RB is phosphorylated, CycA expression cannot be activated (Fig. 1). The induction of CycA expression at G1/S is at the level of transcription (29). Analysis of the CycA promoter has identified E2F-dependent and independent mechanisms for the cell cycle-regulated expression of CycA (29, 30–33). A variant E2F site in the CycA promoter has been shown to be required for the cell cycle-dependent regulation, and mutations of this site can have both positive and negative effects on the promoter activity (30, 32). If v-Abl blocks CycA expression by regulating E2F, it must act downstream of the E2F-binding proteins such as RB, p107, and p130, since these proteins are phosphorylated and inactivated in the arrested cells (Fig. 4). Phosphorylation of DP1, a subunit of E2F, has been shown to inhibit E2F DNA binding activity (34). Thus, E2F can be inhibited after it is released from RB. E2F sites are found in a number of promoters, including that of CycE (35). Because CycE expression is not affected by v-Abl, there must be some active E2F in the v-Abl-arrested cells. Thus, if v-Abl acts through the variant E2F site of CycA, it is acting through a CycA promoter-specific mechanism, but not a general inhibition of E2F function.

By mutational analyses, at least two other transcription factors binding sites, ATF and CCAAT, have been shown to be required for the activation of the CycA promoter (31, 33, 36). Therefore, the effect of v-Abl could be mediated through these or other yet unidentified factors that regulate the CycA promoter (Fig. 4). The ATF site mediates the cell-cell contact-induced repression of CycA expression (33). Conceivably, v-Abl tyrosine kinase could mimic contact signals to inhibit CycA expression. It will be of interest to identify the signaling pathway linking the v-Abl tyrosine kinase to the regulation of the CycA promoter.

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