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Abrogation of Retinoblastoma Protein Function by c-Abl through Tyrosine Kinase-Dependent and -Independent Mechanisms

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The decision to enter the cell division cycle is governed by the interplay between growth activators and growth inhibitors. The retinoblastoma protein (RB) is an example of a growth inhibitor whose main function appears to be the binding and inactivation of key cell cycle activators. One target of RB is a proto-oncoprotein, the c-Abl tyrosine kinase. RB binds to the ATP-binding lobe in the kinase domain and inhibits the nuclear pool of c-Abl in quiescent and G_1 cells. Phosphorylation of RB at G_1/S releases c-Abl, leading to the activation of this nuclear tyrosine kinase. In this report, we describe the construction of a mutant Abl, replacing the ATP-binding lobe of c-Abl with that of c-Src. The mutant protein AS2 is active as a tyrosine kinase and can phosphorylate Abl substrates, such as the C-terminal repeated domain of RNA polymerase II. AS2, however, does not bind to RB, and its activity is not inhibited by RB. As a result, the nuclear pool of AS2 is no longer cell cycle regulated. Excess AS2, but not its kinase-defective counterpart, can overcome RB-induced growth arrest in Saos-2 cells. Interestingly, wild-type c-Abl, in both its kinase-active and -inactive forms, can also overcome RB. Furthermore, overexpression of a kinase-defective c-Abl in rodent fibroblasts accelerates the transition from quiescence to S phase and cooperates with c-Myc to induce transformation. These effects, however, do not occur with the kinase-defective form of AS2. Thus, the growth-stimulating function of the kinase-defective c-Abl is dependent on the binding and the abrogation of RB function. That RB function can be abolished by the overproduction of one of its binding proteins is consistent with the hypothesis that RB induces cell cycle arrest by acting as a "molecular matchmaker" to assemble protein complexes. Exclusive engagement of RB by one of its many targets is incompatible with the biological function of this growth suppressor protein.

Tumor development is driven by two types of mutations in cellular genes. Mutations in proto-oncogenes generally lead to the constitutive activation of growth-promoting functions. Bialleleic inactivation of tumor suppressor genes, on the other hand, eliminates growth-inhibitory functions. Many activating mutations have been identified in human cancer, including point mutations, amplification, and rearrangement of a large number of proto-oncogenes. On the other hand, a smaller number of tumor suppressor genes have been found, and several of these appear to be mutated frequently in a wide variety of human cancers (22). The retinoblastoma susceptibility gene, *Rb-1*, is the prototype of tumor suppressor genes. Homozygous loss of *Rb-1* is associated with the development of retinoblastoma in humans and pituitary tumors in mice (9, 38, 42). Sporadic mutations of the retinoblastoma protein (RB) are also found in several other human tumors, albeit with lower frequency. Reintroduction of functional RB into certain RB-deficient tumor cells can result in both reduced tumorigenicity in nude mice and growth arrest in culture (9, 38, 42). In addition to tumor suppression, the *Rb-1* gene plays an essential role in mouse development. Mouse embryos that are homozygous for mutated *Rb* appear to develop normally but do not survive past day 16 of gestation (4, 16, 21). The mechanism by which RB suppresses tumor growth has not been elucidated. Moreover, the relationship between tumor suppression and the developmental regulation function of RB is not well understood.

The pleiotropic biological functions of RB are based on its ability to interact with many other cellular proteins (38). The RB protein is composed of several distinct protein-binding domains. The first to be described was the A/B pocket, defined as the binding site for the oncoproteins of DNA tumor viruses (14, 15, 18). The A/B pocket also binds cellular transcription factors such as E2F and Elf-1, and binding is correlated with inhibition of the transactivation function of these proteins (11, 35, 38). C-terminal to the A/B pocket is a second protein-binding domain, the C pocket, defined as the binding site for the nuclear c-Abl tyrosine kinase (40). Binding of c-Abl to the C pocket leads to the inactivation of this tyrosine kinase activity (40). Together, the A/B and C pockets make up an RB capable of growth suppression. Interestingly, both the A/B and C pockets must be present on the same RB molecule for growth suppression to occur (41). Inhibition of E2F or c-Abl by separate A/B or C pocket domains is not sufficient for RB to suppress cell growth. The assembly of protein complexes on RB through the simultaneous occupation of the A/B and C pockets appears to be important for the establishment of a cell cycle block (41).

The finding that RB inhibits c-Abl, a proto-oncoprotein, is in keeping with its role as a suppressor of tumor formation. c-Abl is a ubiquitous protein, being both cytoplasmic and nuclear (36). In the cytoplasm, c-Abl binds to F-actin (25, 26) and can bind to and phosphorylate the adaptor protein Crk (8, 31). In the nucleus, c-Abl binds to DNA (19) and can bind to and phosphorylate the C-terminal repeated domain (CTD) of RNA polymerase II (2, 2a, 5, 7). The ability of c-Abl to phosphorylate the CTD can be correlated with a transcription-enhancing function of this nuclear tyrosine kinase (2a, 37). The tyrosine kinase activity of c-Abl is tightly regulated in vivo. Deregulation of the tyrosine kinase has been shown to release the transforming potential (36). Oncogenic versions of Abl,

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i.e., v-Abl of the Abelson murine leukemia virus and Bcr-Abl of Philadelphia chromosome-positive leukemia, are exclusively cytoplasmic. The transforming potential of the nuclear c-Abl has not been described. In normal cells, it is only the nuclear pool of c-Abl that is regulated by RB (40). The fact that RB targets c-Abl for inhibition indicates that this nuclear tyrosine kinase may play a positive role in cell cycle progression.

To determine if a deregulated nuclear c-Abl tyrosine kinase can drive cell cycle progression, we decided to construct a mutant Abl that was resistant to the inhibition imposed by RB. We have shown that RB interacts with the ATP-binding lobe within the tyrosine kinase domain of Abl (40). Analyses of the three-dimensional structures of cyclic AMP-dependent kinase (20), cdk2 (6), ERK2 (43), and cdc2 (23) suggest that the overall folding diagram of the protein kinase domain is highly conserved (33). The kinase domain contains two globular lobes: the N-terminal ATP-binding lobe and the C-terminal substrate-binding lobe (33). We have found that the interaction with RB is specific for the ATP-binding lobe of c-Abl and does not occur with the closely related tyrosine kinase c-Src (40). Abl and Src are 46% identical (and 55% similar) in their ATP-binding lobes (10). By replacing the ATP-binding lobe of c-Abl with the one from c-Src, we should be able to generate a functional tyrosine kinase that is no longer regulated by RB.

In this report, we describe the construction and characterization of an RB-resistant Abl tyrosine kinase, AS2. We show that this deregulated tyrosine kinase is able to overcome RB-induced growth arrest in the human osteosarcoma cells, Saos-2. In addition, we have found that overproduction of Abl can override RB function by simply binding to RB, regardless of the kinase activity. Thus, overproduction of nuclear c-Abl can abrogate the suppression function of RB by two distinct mechanisms.

MATERIALS AND METHODS

Cell culture and synchronization. Saos-2, a human osteosarcoma cell line (American Type Culture Collection), Rat-1, and Rat-1/myc fibroblasts were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone). COS and NIH 3T3 cells were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% supplemented calf serum (Intergen). Fibroblasts were synchronized in quiescence and S phase as previously described (40). [^{3}H]thymidine labeling and analysis were performed as previously described (39).

Transfections and infections. COS and Saos-2 cells were transfected by the calcium phosphate method, as previously described (3). Vectors used were pBK-CMV (Stratagene) containing the Abl constructs, pCMV-Neo-Bam (1) containing full-length RB, and pBABE-puro expressing the puromycin resistance gene (27). The Abl-TAG construct was described previously (40). AS2 was constructed by using PCR to generate the ATP-binding lobe of murine c-Src (amino acids 263 to 343). This was cloned into pBK-Abl-TAG, replacing the corresponding ATP-binding lobe of c-Abl (amino acids 211 to 291). An identical strategy was used to clone the kinase-defective AS2 (AS2$^-$) with, as the PCR template, the kinase-defective c-Src whose critical lysine (amino acid 303) had been replaced with methionine (a gift from T. Hunter). For selection, puromycin (Sigma) was used at 1 μg/ml and G418 (Gibco BRL) was used at 500 μg/ml. To fix and stain cells, a 1% crystal violet solution in 20% ethanol was used. Supertransactivation experiments were performed as previously described (40).

NIH 3T3 cells stably expressing Abl-TAG and AS2-TAG were obtained by calcium phosphate transfection (3) of pBK-Abl-TAG and pBK-AS2-TAG, followed by selection in G418 (Gibco BRL) for 2 weeks. Single clones were picked and analyzed for expression of the exogenous protein. One clone of each was chosen for further experimentation; these clones expressed similar levels of either Abl-TAG or AS2-TAG.

Retroviral stocks of all Abl constructs were prepared by transient calcium phosphate transfection of BOSC-23 cells (28) with pSLX-CMV-based vectors containing the Abl cDNAs. Infections were performed for 2 h with Polybrene at a final concentration of 8 μg/ml. Pooled populations of G418-resistant cells were derived by selection in G418 for 2 weeks, beginning 48 h after retroviral infection.

Flow cytometry. Cells were harvested by trypsinization, washed once with phosphate-buffered saline (PBS), and fixed by the slow addition of 100% cold ethanol to a final concentration of 70%. Fixed cells were then resuspended in PBS containing 20 μg of propidium iodide (Sigma) per ml and 40 μg of RNase A per ml and subjected to analysis on a FACScan (Becton Dickinson) with CellFit software.

Biochemical analyses. Glutathione *S*-transferase (GST) purification, in vitro translation, and in vitro binding were performed as previously described (40). Fractionation, immunoprecipitation, and immunoblotting were performed as previously described (40). Abl autophosphorylation and substrate phosphorylation activity were measured as previously described (40).

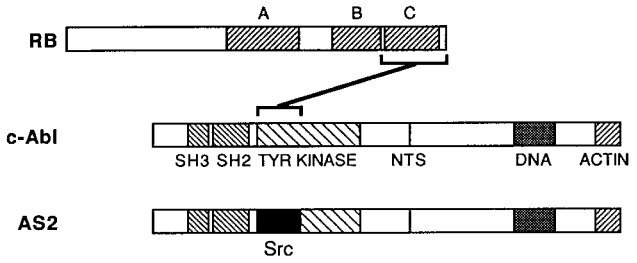

FIG. 1. Construction of AS2, an RB-resistant Abl tyrosine kinase. The C pocket of RB interacts with the ATP-binding lobe in the N-terminal half of the c-Abl tyrosine kinase domain. A mutant form of c-Abl, called AS2, is designed to have a functional tyrosine kinase domain that is no longer regulated by RB. To construct such a mutant, we took advantage of the observation that the closely related tyrosine kinase c-Src could not bind RB (40). In AS2, the ATP-binding lobe of c-Abl (common amino acids 211 to 291) is replaced with the identical region from murine c-Src (amino acids 263 to 343).

RESULTS

Construction of a mutant c-Abl that does not bind RB. We have shown that RB interacts with the ATP-binding lobe of c-Abl but not c-Src (40). The two proteins are 46% identical and 55% similar within this region (10). We surmised that by replacing the ATP-binding lobe of Abl with that of c-Src, we should create a protein that would no longer bind RB but would still have tyrosine kinase activity. In fact, exchange of the N-terminal region of Abl and Src kinase domains has previously been shown to maintain kinase activity (24). By a PCR-based strategy, we replaced the murine Abl common amino acids 211 to 291 with murine Src amino acids 263 to 343 (see Materials and Methods). The resulting mutant is called AS2, short for Abl-Src 2 (Fig. 1).

To establish that AS2 had lost RB-binding activity, we compared the ability of in vitro translated wild-type Abl and AS2 to bind RB in GST pull-down assays (Fig. 2A). As expected, wild-type Abl could bind GST-RB (lane 3) while AS2 could not (lane 6). To determine if this was also true in vivo, we expressed the hemagglutinin (HA)-tagged wild-type Abl (Abl-TAG) and AS2 (AS2-TAG) in COS cells and assayed their ability to coimmunoprecipitate with the endogenous RB (Fig. 2B). As previously shown (40), wild-type c-Abl is brought down in the anti-RB immunoprecipitation (Fig. 2B, lane 3, upper panel) and RB is precipitated by the anti-TAG antibody, 12CA5 (lane 2, lower panel). In contrast, AS2 is not brought down by anti-RB (lane 6, upper panel) and RB is not brought down by anti-TAG in AS2-TAG-transfected cells (lane 5, lower panel). Together, these results demonstrated that the AS2 mutant had lost the ability to bind RB both in vitro and in vivo.

AS2 kinase activity is not inhibited by RB. To determine if AS2 kinase activity was inhibited by RB, we tested whether a purified RB C pocket fragment could inhibit AS2 in immune complex kinase assays (Fig. 3A). Consistent with previous results (40), the activity of wild-type Abl decreased 8- to 10-fold when the C pocket of RB (RB-SE) was included in the kinase reaction (compare lanes 2 and 3), while a nonfunctional fragment of the C terminus of RB had no effect (RB-ME, lane 4). By contrast, AS2, which had a somewhat higher basal kinase

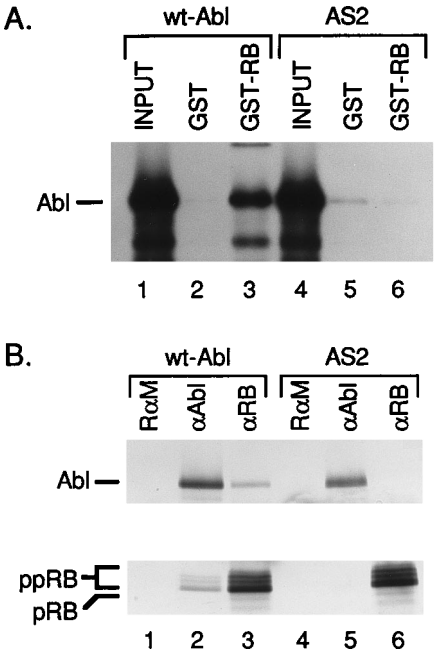

FIG. 2. AS2 does not bind RB. (A) In vitro translated c-Abl (lanes 1 to 3) or AS2 (lanes 4 to 6) (50 μl per binding reaction) was incubated with glutathione-agarose beads containing 1 μg of either GST (lanes 2 and 5) or GST-RB-SE, with RB amino acids 768 to 928 (lanes 3 and 6). Bound proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6.5% polyacrylamide) and visualized by fluorography. The input lanes (lanes 1 and 4) contain 1 μl of the translation mix. The position of the full-length Abl is marked. (B) COS cells were transfected with either pBK-Abl-TAG (lanes 1 to 3) or pBK-AS2-TAG (lanes 4 to 6). At 48 h posttransfection, lysates were immunoprecipitated with rabbit anti-mouse (lanes 1 and 4), 12CA5 anti-TAG (αAbl; lanes 2 and 5), or anti-RB (lanes 3 and 6). Precipitated proteins were separated by SDS-PAGE (6.5% polyacrylamide) and analyzed by anti-Abl (top panel) or anti-RB (bottom panel) immunoblotting. Positions of the phosphorylated RB (ppRB) and unphosphorylated RB (pRB) are marked.

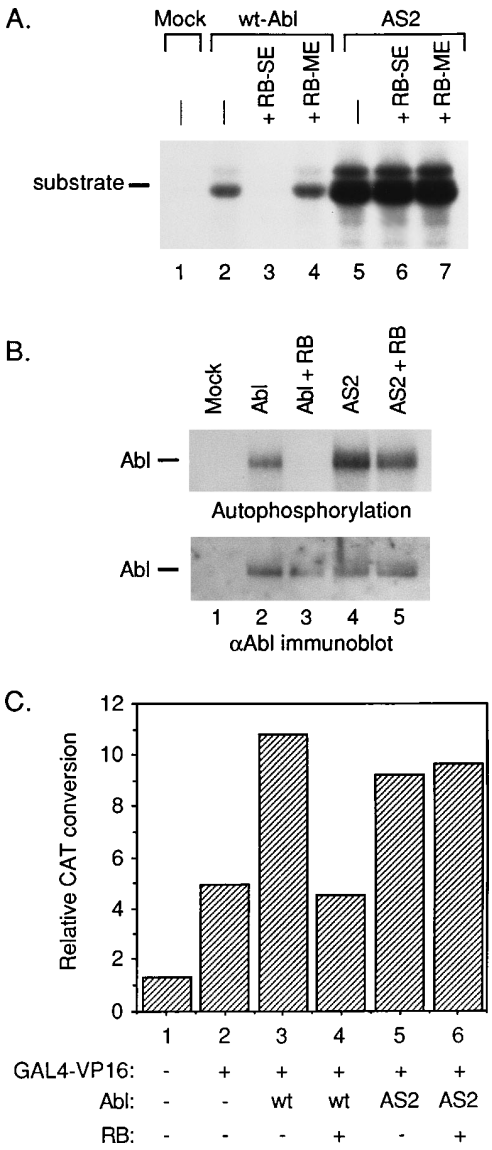

FIG. 3. AS2 tyrosine kinase is not inhibited by RB. (A) AS2 is not inhibited by RB-SE in vitro. COS cells were transfected with empty vector (Mock, lane 1), pBK-Abl-TAG (lanes 2 to 4), or pBK-AS2-TAG (lanes 5 to 7). At 48 h post-transfection, lysates were immunoprecipitated by anti-TAG (12CA5) antibodies and preincubated with 5 μg of either GST-RB-SE (amino acids 768 to 928 [lanes 3 and 6]) or GST-RB-ME (amino acids 835 to 928 [lanes 4 and 7]) for 30 min and then incubated with [γ-^{32}P]ATP and the NRD protein substrate (see Materials and Methods). The NRD substrate was separated from other proteins by SDS-PAGE (8% polyacrylamide) and visualized by autoradiography. (B) AS2 is not inhibited by RB in vivo. Saos-2 cells were transfected with pBK-Abl-TAG (lanes 2 and 3) or pBK-AS2-TAG (lanes 4 and 5) alone or in combination with pCMV-RB (lanes 3 and 5). At 48 h posttransfection, cells were fractionated and nuclear lysates were immunoprecipitated with anti-TAG antibody. The immunoprecipitate was incubated with [γ-^{32}P]ATP to assay for Abl autophosphorylation (upper panel) and then immunoblotted to reveal the Abl proteins (bottom panel). (C) AS2 has supertransactivation activity which is not inhibited by RB. Saos-2 cells were transfected with the 5× GAL4-CAT reporter, the GAL4-VP16 effector, pBK-Abl, pBK-AS2, and pCMV-RB as indicated, and CAT assays were performed 48 h later. Values represent average percent acetylation of [^{14}C]chloramphenicol, normalized to β-galactosidase activity (see Materials and Methods).

activity than Abl (lane 5), was not inhibited by the C pocket of RB (lane 6). To determine if AS2 could escape RB inhibition in vivo, we cotransfected AS2 with RB into Saos-2 cells and then analyzed the activity of AS2 immunoprecipitated from the nuclear extracts (Fig. 3B). When RB was cotransfected with wild-type Abl, the kinase activity of nuclear c-Abl was not detectable because of the formation of RB-Abl complexes (compare lanes 2 and 3). Nuclear AS2, however, was active in both the presence and in the absence of RB (compare lanes 4 and 5), showing that this tyrosine kinase is no longer regulated by RB.

We have previously shown that the wild-type Abl tyrosine kinase can enhance the transcriptional activity of GAL4-VP16 in transient-transfection assays (40). This supertransactivation is dependent on a functional tyrosine kinase that is localized to the nucleus and capable of binding DNA (40). Furthermore, this supertransactivation is inhibited upon cotransfection with either full-length RB or just the C pocket of RB (40), consistent with the requirement of the tyrosine kinase in supertransactivation. As shown in Fig. 3C, when wild-type Abl was coexpressed with GAL4-VP16 and a GAL4-chloramphenicol acetyltransferase (CAT) reporter plasmid, the CAT activity was increased two- to threefold above that seen with GAL4-VP16 alone (compare lanes 2 and 3) and this increase was completely abolished by RB (lane 4). When AS2 was coexpressed with GAL4-VP16 and the GAL4-CAT reporter, a similar supertransactivation activity was observed (compare lanes 3 and 5). Unlike wild-type Abl, however, AS2 supertransactivation was not affected by coexpression of RB (lane 6). The supertransactivation activity of Abl is tightly correlated with the ability of Abl to phosphorylate the CTD of RNA poly-

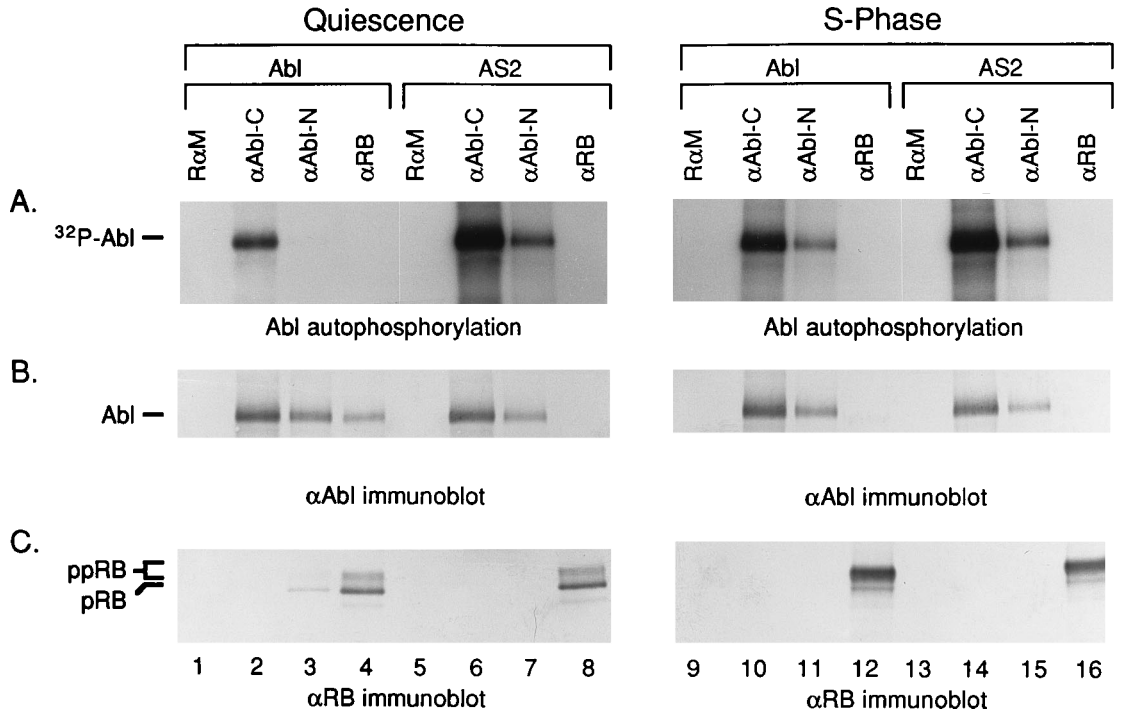

FIG. 4. Nuclear AS2 tyrosine kinase is not cell cycle regulated. NIH 3T3 cells, stably expressing either Abl-TAG (lanes 1 to 4 and 9 to 12) or AS2-TAG (lanes 5 to 8 and 13 to 16), were synchronized in quiescence (lanes 1 to 8) or S phase (lanes 9 to 16), as described in Materials and Methods. For the Abl immunoprecipitations, cytoplasmic (lanes 2, 6, 10, and 14) and nuclear (lanes 3, 7, 11, and 15) fractions were obtained from 10^7 cells. Following fractionation, lysates were immunoprecipitated with anti-TAG (αAbl) antibodies, to precipitate only the exogenously expressed Abl proteins. Lysates from 10^7 whole cells were immunoprecipitated with rabbit anti-mouse (lanes 1, 5, 9, and 13) or anti-RB 245 (lanes 4, 8, 12, and 16) antibodies. Immunoprecipitates were incubated with [γ-^{32}P]ATP to measure Abl autokinase activity (see Materials and Methods). Proteins were resolved by SDS-PAGE (6.5% polyacrylamide) and visualized by autoradiography (A) or by immunoblotting with anti-Abl (B) or anti-RB (C) antibodies. The Abl protein, phosphorylated RB (ppRB), and unphosphorylated RB (pRB) are indicated.

merase II (2a). c-Abl, but not c-Src, can processively phosphorylate the CTD (2), and it has been shown that AS2 is capable of processive CTD phosphorylation (7). This indicates that the presence of Src amino acids in the ATP-binding lobe of c-Abl does not affect substrate specificity. Together, these results indicated that the AS2 tyrosine kinase functions in vivo similarly to wild-type Abl but is no longer inhibited by RB.

Nuclear AS2 kinase is not cell cycle regulated. We have shown that the nuclear pool of Abl is bound to the hypophosphorylated forms of RB during quiescence and G_1 (40). During this time, the Abl tyrosine kinase is inactive. When cells enter S phase, RB becomes phosphorylated and c-Abl is released. This release is correlated with an activation of the nuclear Abl tyrosine kinase (40). Since we have demonstrated that AS2 is a functional kinase that is no longer regulated by RB, we would predict that AS2 would be activated during quiescence and G_1.

To address this question, NIH 3T3 cell clones that stably expressed either wild-type Abl-TAG or AS2-TAG were isolated (see Materials and Methods). These cells were made quiescent by serum starvation and then stimulated to enter S phase synchronously by serum addition. Both quiescent and S-phase cells were collected and fractionated. To measure the kinase activity of the exogenously introduced Abl, anti-TAG immunoprecipitates from either the cytoplasmic (Fig. 4A, lanes 2, 6, 10, and 14) or nuclear (lanes 3, 7, 11, and 15) fraction were incubated with [γ-^{32}P]ATP to detect Abl autophosphorylation activity. As expected, wild-type Abl kinase was not active in quiescent nuclei but became activated in S-phase nuclei (compare lanes 3 and 11). Note that the activity of the cytoplasmic pool of Abl was unchanged (compare lanes 2 and 10). In contrast, nuclear AS2 kinase was clearly active in quiescent cells (lane 7) and no further activation was observed in S phase (lane 15).

To verify that AS2 does not interact with RB in these cells, we examined the coimmunoprecipitation of RB and Abl (Fig. 4B and C). In the nuclei of quiescent cells expressing Abl-TAG, RB was found in the anti-TAG immunoprecipitates (Fig. 4C, lane 3). However, no RB protein was brought down by anti-TAG in cells expressing AS2-TAG (lane 7). In the reverse coimmunoprecipitations, wild-type Abl, but not AS2, was brought down with RB (Fig. 4B, compare lanes 4 and 8). Thus, deregulation of the nuclear AS2 kinase in quiescent cells is correlated with the lack of RB binding. In S-phase cells, RB becomes predominantly hyperphosphorylated (Fig. 4C, lanes 12 and 16). Under these conditions, no RB protein was in complex with either Abl or AS2 (lanes 11 and 15), nor was any wild-type Abl or AS2 protein brought down with anti-RB (Fig. 4B, lanes 12 and 16). Together, these results showed that the ATP-binding lobe of c-Abl is required for the cell cycle regulation of its kinase activity and that the G_1 inhibition of c-Abl is correlated with binding to the RB protein.

Both Abl and AS2 can overcome RB-induced growth suppression. Reintroduction of RB into human osteosarcoma Saos-2 cells induces an easily discernible flat-cell morphology (30, 34). These flat cells do not proliferate and are arrested in the G_1 phase of the cell cycle. To test if either c-Abl or AS2 could override the cell cycle arrest function of RB, each was cotransfected with RB into Saos-2 cells. When wild-type Abl was cotransfected with RB at a 1:1 DNA molar ratio, the number of flat cells was reduced by 10-fold (Fig. 5A, lane 3). However, the 1:1 cotransfection of AS2 with RB had little effect on the number of flat cells (lane 5). Since the difference

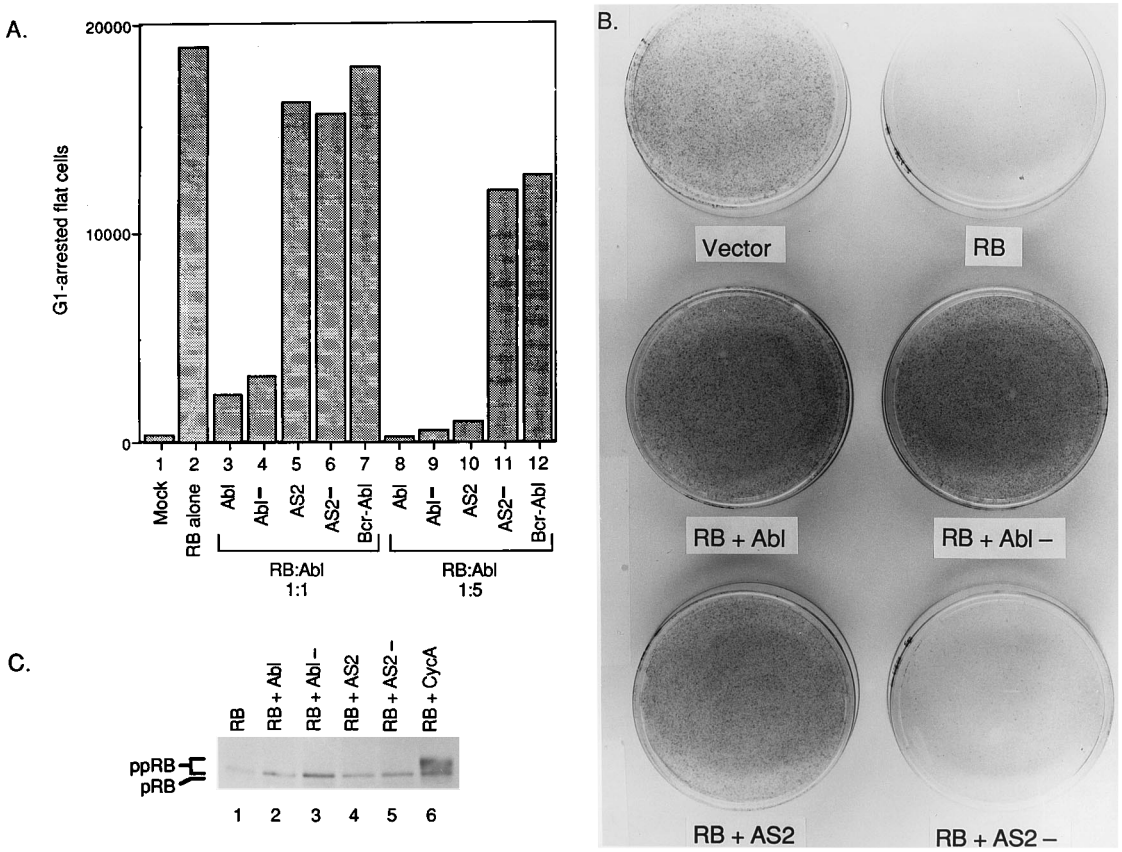

FIG. 5. Both Abl and AS2 can overcome RB-mediated growth arrest in Saos-2 cells. (A) Saos-2 cells (2×10^6) were cotransfected with either empty RB vector pCMV (lane 1) or pCMV-RB (lanes 2 to 12) and either empty Abl vector pBK (lanes 1 and 2), Abl (lanes 3 and 8), kinase-defective Abl (Abl^-; lanes 4 and 9), AS2 (lanes 5 and 10), kinase-defective AS2 ($AS2^-$; lanes 6 and 11), or Bcr-Abl (lanes 7 and 12). Plasmids were cotransfected with an RB/Abl ratio of either 1:1 (lanes 3 to 7) or 1:5 (lanes 8 to 12). Each transfection also included 0.5 μg of puromycin resistance plasmid. Cells were selected in puromycin for 7 days, and the resulting G_1-arrested flat cells were counted. (B) Abl, Abl^-, and AS2 prevent RB from suppressing colony formation. Saos-2 cells (2×10^6) were transfected with either empty RB vector pCMV (Vector) or pCMV-RB, as indicated. The pCMV vector also carries the neomycin resistance gene. Plates were cotransfected with either empty Abl vector pBK (Vector), Abl, kinase-defective Abl (Abl^-), AS2, or kinase-defective AS2 ($AS2^-$), as indicated, at an RB/Abl ratio of 1:5. Cells were selected in G418 (500 μg/ml) for 14 days and then stained with crystal violet. (C) Neither Abl nor AS2 induced RB phosphorylation. Saos-2 cells were cotransfected with RB and either Abl, kinase-defective Abl (Abl^-), AS2, kinase-defective AS2 ($AS2^-$) or pRC-CMV-cyclin A, as indicated, at an RB/Abl or RB/cyclin A ratio of 1:5. At 48 h posttransfection, lysates were immunoprecipitated with anti-RB antibodies and bound proteins were analyzed by anti-RB immunoblotting. Phosphorylated RB (ppRB) and unphosphorylated RB (pRB) are indicated.

between Abl and AS2 is in their ability to bind RB, these data suggested that Abl might be blocking RB not by its kinase function but by binding to RB. To test whether the overriding of RB required Abl kinase function, we cotransfected kinase-defective forms of Abl and AS2 with RB. Indeed, at a 1:1 ratio, the kinase-defective Abl also reduced the number of flat cells (Abl^-; lane 4), similar to the effect seen with wild-type Abl. The kinase-defective AS2, like its kinase-positive counterpart, had no effect on flat-cell formation ($AS2^-$; lane 6). Consistent with this observation, we found that the ATP-binding lobe of c-Abl was also capable of reducing the number of flat cells induced by RB (results not shown). Together, these data suggested that the binding of Abl to RB could abolish RB function in Saos-2 cells.

At a higher ratio of cotransfection (RB/Abl ratio, 1:5), AS2 also reduced the number of flat cells induced by RB (Fig. 5A, lane 10). The kinase-defective form of AS2, however, had only a minor effect on the number of flat cells ($AS2^-$; lane 11), showing the requirement of a functional tyrosine kinase in this effect. The wild-type and kinase-defective Abl reduced flat-cell number to the background level at this cotransfection ratio (lanes 8 and 9). We also tested Bcr-Abl, an activated kinase that is exclusively associated with F-actin (26). Despite the oncogenic potential of Bcr-Abl, it did not cause a significant reduction in the number of RB-induced flat Saos-2 cells at either cotransfection ratio (lanes 7 and 12), suggesting that only nuclear Abl could abrogate RB function in Saos-2 cells. Together, these results indicate that RB-induced growth arrest in Saos-2 cells can be abolished by nuclear Abl, either through the binding of Abl to RB or by the overexpression of an RB-resistant Abl tyrosine kinase.

The reduction in flat-cell number alone is not sufficient to prove that Abl and AS2 can reverse the RB-mediated growth arrest. Therefore, we performed a second assay in which the formation of colonies was scored. Expression of RB in Saos-2 cells reduces the number of stably transfected colonies when selected for resistance to neomycin (12, 29, 30, 34, 44). In our hands, a 10- to 20-fold reduction in Neo^r colonies was observed with RB-transfected Saos-2 cells relative to the vector control (Fig. 5B, top plates). Coexpression of RB with wild-type or kinase-defective Abl (middle plates) or AS2 (lower left plate), at an RB/Abl ratio of 1:5, brought the number of Neo^r colonies back to levels similar to that obtained without RB. The kinase-defective AS2 could not reverse the effect of RB on Neo^r

colony formation (lower right plate). These data confirmed the results of the flat-cell assay and showed that Abl and AS2 could overcome the growth suppression activity of RB in Saos-2 cells.

In normal cells, entry into the cell cycle is accompanied by RB phosphorylation (38). To determine if Abl or AS2 promotes cell cycle progression by inducing RB phosphorylation, Saos-2 cells were cotransfected with RB and each of the various Abl constructs, and the phosphorylation status of RB was examined (Fig. 5C). As a positive control, cyclin A was used to drive RB phosphorylation (lane 6) (13). Neither wild-type nor kinase-defective Abl induced RB phosphorylation (lanes 2 and 3). Similarly, AS2 also did not alter the phosphorylation status of RB (lane 4). We have previously shown that Saos-2 cells can proliferate in the presence of unphosphorylated RB when the RB function is disrupted by the coexpression of its C pocket fragment (41). The Abl proteins appeared to also overcome RB without activating RB phosphorylation.

Kinase-defective Abl but not AS2 can accelerate S-phase entry and promote transformation. In contrast to our results with Saos-2 cells indicating a positive function of Abl tyrosine kinase in cell cycle progression, Sawyers et al. (32) have proposed that nuclear Abl can cause G_1 arrest in rodent fibroblasts. This suggestion was based mainly on the observation that a kinase-defective Abl could shorten the transition from quiescence to S phase and could collaborate with Myc to transform Rat-1 cells (32). These authors concluded that the kinase-defective Abl was functioning as a dominant negative mutant, preventing normal c-Abl from restraining growth. We show here that the kinase-defective Abl can inactivate RB function. This raises an interesting alternative interpretation, i.e., that kinase-defective Abl could be accelerating S-phase entry or collaborating with Myc by overriding the growth-inhibitory activity of RB. If so, we would predict that the kinase-defective AS2 should not have these effects on the Rat-1 cells.

To test this hypothesis, we isolated polyclonal populations of Rat-1 fibroblasts that were stably transfected with the kinase-defective forms of Abl or AS2. Both proteins were overexpressed about 10- to 20-fold more than the endogenous c-Abl (Fig. 6C, compare lanes 3 and 5 with lane 1). In contrast to the previous report by Sawyers et al. (32), we found that the kinase-positive forms of Abl and AS2 could also be overproduced in Rat-1 cells; however, only fragments of these proteins were accumulated in the stably transfected clones (lanes 2 and 4). The degradation of kinase-positive Abl and AS2 indicated that only the full-length form of Abl was intolerable when overproduced. Since full-length proteins were not maintained, we were unable to study the biological effects of kinase-positive Abl or AS2 in these Rat-1 cells.

To examine the effect of the kinase-defective Abl and AS2 on cell cycle progression, we first analyzed cycling cells. There was no difference in the doubling time, indicating that Abl^- and $AS2^-$ did not accelerate the cell cycle. Furthermore, flow cytometry analysis showed that these cells had the same cell cycle distribution as mock-transfected cells. Thus, overexpression of Abl^- or $AS2^-$ did not affect the length of G_1, S or G_2/M in cycling cells. We next examined the kinetics of exit from quiescence. Rat-1 clones were arrested by serum starvation and then stimulated to reenter the cell cycle by the addition of serum. At various times after serum stimulation, cells were assayed for DNA content by flow cytometry (Fig. 6A). In the control Rat-1 fibroblasts, over 75% of the cells entered S phase 15 h after serum stimulation (left panels). Consistent with the observation of Sawyers et al., cells overexpressing kinase-defective Abl entered S phase approximately 5 h earlier, with 78% of the cells being in S phase after 10 h (middle panels). However, cells overexpressing kinase-defective AS2 did not show such an acceleration, since nearly 60% of the cells were still in G_1 after 10 h (right panels). By 15 h, a majority of the $AS2^-$ cells had entered or proceeded through S phase, only slightly ahead of the control cells.

We also determined the timing of DNA synthesis by measuring the incorporation of [^{3}H]thymidine (Fig. 6B). Again, cells overexpressing kinase-defective Abl entered S phase between 4 and 5 h earlier than the control cells did. Cells overexpressing the kinase-defective AS2 began synthesizing DNA just slightly ahead of the control cells and were about 3 to 4 h behind the cells overexpressing Abl^-. These data showed that only Abl^-, but not $AS2^-$, could significantly advance the transition from quiescence to S phase. It is interesting that Abl^- affected the kinetics of exit from quiescence but did not affect the length of G_1 in cycling cells. This suggested that Abl^- might interfere with the establishment of the quiescent state; in other words, cells expressing Abl^- might not be arrested at the same G_0/G_1 point as the control cells were. Since $AS2^-$ did not have the same effect, the ability to bind to RB was a major factor in the action of Abl^-.

Overexpression of kinase-defective Abl has also been shown to cooperate with c-Myc to cause transformation in Rat-1 cells (32). Again, if this effect is dependent on the ability of Abl^- to bind and inactivate RB, one would predict that $AS2^-$ would be unable to cooperate with c-Myc. To test this, we infected Rat-1/Myc cells with retrovirus expressing either Abl^- or $AS2^-$ and assayed growth in soft agar (Table 1). In vector-virus-infected culture, small soft agar colonies were observed, and they occurred at a low frequency (321 to 355 colonies per 10^6 cells seeded). Infection with the kinase-defective Abl virus caused a 20- to 42-fold increase in the number of soft agar colonies, and these colonies were also much larger (Table 1). Infection with the kinase-defective $AS2^-$ virus had only a three- to fivefold effect on the number of soft agar colonies (Table 1). Most of these colonies were also smaller than those generated by the expression of Abl^-. These results indicated that the ability to bind RB was critical for the kinase-defective Abl to cooperate with Myc to transform Rat-1 cells.

DISCUSSION

By replacing the ATP-binding lobe of c-Abl with the corresponding amino acids of c-Src (Fig. 1), we have created a functional tyrosine kinase AS2 which can neither bind (Fig. 2) nor be regulated by (Fig. 3) RB. As a result, the nuclear pool of AS2 is no longer cell cycle regulated (Fig. 4). Both c-Abl and AS2 can abrogate the RB-mediated growth suppression in human osteosarcoma Saos-2 cells. While the abolition of RB function requires the kinase activity of AS2, the wild-type c-Abl can overcome RB even in its kinase-defective form (Fig. 5). When overproduced in rodent fibroblasts, the kinase-defective Abl, but not the kinase-defective AS2, can accelerate the transition from quiescence to S phase (Fig. 6) and cooperate with c-Myc to induce growth in soft agar (Table 1).

RB binding regulates nuclear Abl activity in the cell cycle. We have previously shown a strong correlation between the disruption of RB/c-Abl complex and the activation of nuclear Abl tyrosine kinase as cells enter S phase (40). We have proposed that the G_1 inhibition of nuclear c-Abl is due to RB binding and that the G_1/S activation is due to the release of c-Abl from RB. As shown here, the AS2 mutant that is deficient in RB binding is equally active in quiescent and S-phase nuclei. This result supports our hypothesis that the inhibition of nuclear c-Abl tyrosine kinase during G_1 is mediated by binding to RB. However, we cannot rule out the possibility that other regulatory mechanisms also act on the ATP-binding lobe

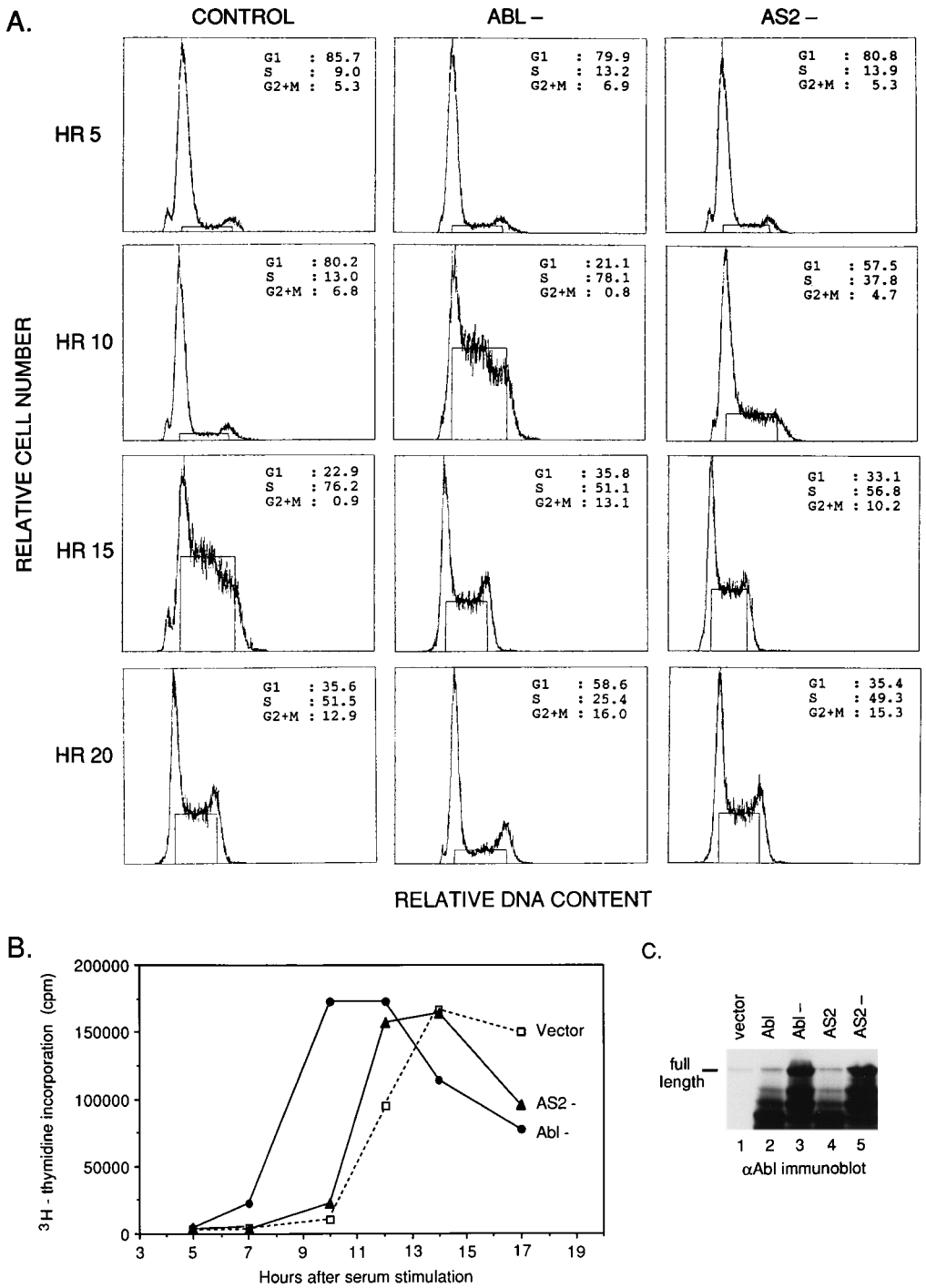

FIG. 6. Kinase-defective Abl, but not kinase-defective AS2, accelerates the transition from quiescence to S phase in Rat-1 cells. (A) Polyclonal populations of Rat-1 fibroblasts expressing either the retroviral vector pSLX alone (CONTROL), kinase-defective Abl (ABL^-), or kinase-defective AS2 ($AS2^-$) were derived by retroviral infection followed by G418 selection for 2 weeks. Cells were made quiescent by culturing in 0.1% serum for 48 h and then stimulated to synchronously reenter the cell cycle by the addition of fresh medium containing 5% serum. At 5, 10, 15, and 20 h after serum stimulation, the cell cycle profile of each population was determined by analysis of DNA content by flow cytometry (see Materials and Methods). The percentages of cells in G_1, S and G_2/M for each time point are shown in the upper right of each panel. (B) Identical cell populations were synchronized in quiescence and then subjected to serum stimulation, as described for panel A. At various time points after serum stimulation, the cells were incubated with [^{3}H]thymidine (see Materials and Methods) to measure DNA synthesis and entry into S phase. Values represent average [^{3}H]thymidine incorporation from duplicate samples. (C) Expression of kinase-positive and kinase-defective Abl and AS2 in polyclonal populations of Rat-1 cells. Rat-1 cells were infected with retroviruses containing each of the indicated Abl constructs and selected in G418 for 2 weeks. Whole-cell lysates were immunoprecipitated with anti-Abl 8E9, and the precipitated proteins were separated by SDS-PAGE (6.5% polyacrylamide) and analyzed by anti-Abl immunoblotting. Abl^- and $AS2^-$ were overproduced to similar levels. Abl and AS2 were also overproduced, but only degraded forms were accumulated in these Rat-1 cells.

TABLE 1. Soft-agar colony formation of Rat-1/Myc cells is enhanced by kinase-defective Abl but not kinase-defective AS2

Retrovirus sequence[a]	No. of soft agar colonies[b]	
	Expt 1	Expt 2
Vector	321	335
Abl^-	14,208	8,307
$AS2^-$	963	1,846

[a] Helper-free retroviruses containing the indicated sequences were generated and used to infect Rat-1/Myc cells as described in Materials and Methods.

[b] At 48 h postinfection, 10^6 cells were seeded into soft agar and cultured for 3 weeks with regular feeding. The number of soft agar colonies per 10^6 cells is shown.

of Abl to inhibit its activity during the G_1 phase of the cell cycle.

Abl tyrosine kinase can abrogate RB-induced growth arrest. Since RB targets the nuclear c-Abl and this tyrosine kinase is activated at the G_1/S transition, it is reasonable to assume that c-Abl may be able to antagonize the suppression function of RB. By using the AS2 mutant, we have found that RB-induced growth suppression can be overcome by Abl through two different mechanisms, one requiring the Abl tyrosine kinase and the other involving the binding of Abl to RB.

As shown by the ability of AS2 to overcome RB in Saos-2 cells, the Abl tyrosine kinase can indeed abolish RB function. Since AS2 does not induce RB phosphorylation, its effect must be mediated by the phosphorylation of critical substrates to bypass the RB block. AS2 retains the ability to phosphorylate the CTD of RNA polymerase II (7) and to enhance transcription (Fig. 3). However, AS2 has to be expressed to a relatively high level, as indicated by the requirement of a higher level of DNA input, to override RB. A possible explanation for this is as follows. Since the A/B and C pockets of RB can be simultaneously occupied, RB could potentially bring substrates to Abl (38). When RB becomes phosphorylated, Abl will be activated, and it can phosphorylate the proteins bound to the A/B pocket of RB. We have shown that the A/B and C pockets of RB are regulated by different cyclin-dependent kinase (cdk) phosphorylation sites (20a). A partial phosphorylation of RB on the cdk sites that regulate Abl binding can conceivably lead to the activation of c-Abl but not the complete disruption of the RB-assembled complexes, and this can facilitate the phosphorylation of Abl substrates (38). The inefficiency of AS2 to overcome RB could therefore be due to its inability to join RB-assembled complexes, and thus it may not effectively phosphorylate substrates that are normally brought to c-Abl by RB (38, 41). Alternatively, the growth promotion function of AS2 may be the result of nonspecific phosphorylation events due to the overexpression of this deregulated kinase.

At physiological levels of expression in NIH 3T3 cells, AS2 did not have any obvious effect on the cell cycle. These AS2-expressing cells could be made quiescent (Fig. 4), and they entered S phase with normal kinetics (results not shown). It is possible that AS2 abolishes certain aspects of G_1/S regulation for which there is currently no assay. It is interesting that the full-length Abl or AS2 could not be stably overproduced in rodent fibroblasts, consistent with previously published results (17, 32). In NIH 3T3 cells, stable transfectants of AS2 expressed the protein only at levels similar to or below that of the endogenous Abl (Fig. 4). In Rat-1 cells, kinase-positive Abl or AS2 could be expressed to high levels but only degraded forms were accumulated (Fig. 6C). At present, it is not clear why rodent cells cannot tolerate a long-term overproduction of Abl tyrosine kinase. The fact that AS2 is also not tolerated shows that exchanging the ATP binding with Src does not affect the apparent growth-inhibitory activity of Abl in rodent fibroblasts.

Abl protein with an RB-binding domain can also override RB. The second mechanism for Abl to override RB requires its RB-binding domain and is independent of the kinase activity. The kinase-defective Abl rescued Saos-2 cells, while the kinase-defective AS2, lacking the RB-binding domain, did not override RB. Moreover, overexpression of the kinase-defective Abl, but not the kinase-defective AS2, could accelerate the transition from quiescence to S phase and enhance Myc transformation in Rat-1 cells.

The c-Abl tyrosine kinase binds the C pocket of RB (40). The C pocket by itself is capable of inhibiting c-Abl tyrosine kinase (40) but does not possess growth suppression function (41). Thus, the inhibition of c-Abl alone is not enough for RB to suppress cell proliferation. This is in keeping with the observation that AS2 does not activate S-phase entry when it is expressed at the physiological level. Although it alone is not sufficient to suppress cell growth, the C pocket is necessary for RB to induce G_1/S arrest. We have shown that growth suppression requires the assembly of protein complexes through simultaneous binding to the A/B and C pockets of RB (41). We have also shown that the A/B and C pockets of RB cannot function in *trans* but have to be in one molecule to suppress cell growth. Moreover, overexpression of the C pocket of RB can disrupt RB function. This has led us to propose that RB suppresses cell growth by acting as a molecular matchmaker, bringing together proteins that would otherwise not interact with one another (38, 41). The RB-assembled protein complexes can either silence the expression of growth activators or induce the expression of growth inhibitors to cause cell cycle arrest.

The fact that Abl can override RB through a binding-dependent mechanism is consistent with the hypothesis that RB suppresses growth by assembling protein complexes. Binding of the kinase-defective Abl to the C pocket of RB can have two consequences. First, Abl^- could occupy the C pocket, thus leading to the release and activation of the endogenous c-Abl tyrosine kinase. However, because the C pocket alone does not suppress cell growth and AS2 does not stimulate S-phase entry at normal levels of expression, activation of the endogenous c-Abl is not likely to account for the abrogation of RB function by Abl^-. We favor a second possibility in which occupation of the RB C pocket by the excess Abl^- precludes the binding of other proteins that also join the RB-assembled complexes through the C pocket. Given the fact that RB binds many different proteins (38), the growth suppression function of RB may be dependent on the proper assembly of more than one type of protein complexes. If so, the exclusive engagement of the RB C pocket with Abl^- would be expected to disrupt the RB function.

Is c-Abl a cell cycle activator or inhibitor? The inability of rodent fibroblasts to accommodate an overabundance of Abl tyrosine kinase has previously been taken as evidence that normal c-Abl has growth-inhibitory activity (32). As discussed above, that conclusion was based mainly on the observation that kinase-defective Abl could advance the onset of S phase and enhance the transforming function of Myc. Since overproduction of both AS2 and Abl is intolerable, the dominant negative model would have predicted that both Abl^- and $AS2^-$ should promote G_1 progression and Myc transformation in Rat-1 cells. This is clearly not the case. The small effect of $AS2^-$ on the formation of soft-agar colonies of Rat-1/Myc cells may be due to the titration of other yet unidentified growth-inhibitory proteins through binding to the other domains of Abl.

In Saos-2 cells, overexpression of AS2 can overcome RB-induced growth suppression. Thus, overexpression of Abl kinase can have a positive effect on the cell cycle. The inability to demonstrate this positive effect in rodent fibroblasts is probably related to the difficulty in maintaining high levels of expression of this tyrosine kinase in the nucleus. The "growth-inhibitory" effect associated with the overexpression of kinase-positive Abl may not reflect the physiological function of this protein. It is conceivable that the inappropriate activation of an S-phase function in G_1 may have a deleterious effect on G_1 progression. The currently available data indicate that overproduction of the nuclear c-Abl tyrosine kinase can have either an inhibitory or a stimulatory effect on the cell cycle, and this appears to be determined by the cell context. Perhaps the osteosarcoma cells Saos-2 lack a certain function which is present in rodent fibroblasts to make them intolerable of high levels of Abl tyrosine kinase. The fact that Abl tyrosine kinase can overcome RB under specific conditions indicates that this nuclear tyrosine kinase can function as a cell cycle activator.

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