Induction of *c-myc* transcription by the v-Abl tyrosine kinase requires Ras, Raf1, and cyclin-dependent kinases

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v-Abl is an oncogenic form of the c-Abl nonreceptor tyrosine kinase. v-Abl induces transcription of *c-myc*, and c-Myc function is a necessary but not sufficient component of the v-Abl transformation program. Previously we showed that the E2F site in the *c-myc* promoter is a v-Abl response element and that v-Abl appears to induce *c-myc* by initiating a phosphorylation cascade that ultimately activates E2F-binding proteins. In this work we have investigated the signaling pathway between the v-Abl tyrosine kinase and activated E2F proteins. We show that the Ras GTPase and Raf1 serine/threonine kinase are required in this pathway. However, in contrast to other aspects of v-Abl signaling, induction of *c-myc* transcription is independent of the Rac GTPase. Our results also establish a requirement for activated cyclin-dependent kinases (cdks), as v-Abl-dependent induction of *c-myc* transcription is blocked by cdk inhibitor p21 and induction of *c-myc* is accompanied by activation of cdk2 and cdk4. Finally, we show that v-Abl-dependent induction of *c-myc* is accompanied by hyperphosphorylation of pRb, p107, and p130. On the basis of these data, we propose a model for the signaling path from v-Abl to *c-myc*.

[Key Words: v-Abl; *c-myc*; Ras/Raf; Rac; cdk; signal transduction]

Received October 17, 1996; revised version accepted January 21, 1997.

Abelson murine leukemia virus (A-MuLV) encodes an oncogenic form of the Abelson kinase, v-Abl (Risser and Green 1988). v-Abl is a fusion protein in which portions of the retroviral Gag protein replace the amino-terminal SH3 domain of c-Abl, rendering the tyrosine kinase activity constitutive. Until recently, little was known about functionally important substrates of the Abelson kinases or about possible signal transduction paths which their action might initiate. Development of temperature-sensitive forms of v-Abl led to the identification of the *c-myc* gene as an important target of v-Abl activity. In fibroblasts, myeloid cells, and pre-B cells, v-Abl induces transcription of *c-myc* which is independent of new protein synthesis, suggesting that v-Abl acts by post-translational modification of existing *c-myc* regulatory proteins (Cleveland et al. 1989; Chen and Rosenberg 1992; Renshaw et al. 1992). Subsequently, dominant-negative forms of c-Myc were used to show that c-Myc was required for transformation by either *v-abl* or *bcr/abl*, establishing the importance of the *c-myc* gene as an ultimate target for the activity of v-Abl and BCR/ABL (Sawyers et al. 1992).

We have been studying v-Abl-dependent induction of *c-myc* transcription as a way of gaining further under-

standing of this critical aspect of v-Abl signaling. Our previous work showed that v-Abl can induce *c-myc* transcription through the E2F site located upstream of the P2 transcription initiation site in the *c-myc* promoter. We showed further that E2F sites alone are sufficient to confer v-Abl responsiveness on a heterologous promoter (Wong et al. 1995). This induction requires the SH1 (kinase) and SH2 domains of v-Abl but does not require the carboxy-terminal nuclear localization signal, SH3 binding, DNA-binding, or actin-binding domains (Wong et al. 1995). Thus, v-Abl appears to induce *c-myc* transcription by initiating a phosphorylation cascade that ultimately increases the transcriptional activating ability of E2F site binding proteins. Because we knew that the initial signal for *c-myc* transcriptional induction came from v-Abl tyrosine kinase and the penultimate event was activation of E2F-dependent transcription, we wanted to determine the intermediate biochemical steps in this signal transduction pathway.

In this study we have determined the components of the signaling pathway that links v-Abl and induction of *c-myc* transcription. The roles of the Ras GTPase, Raf1 serine/threonine kinase, and Rac GTPase were assessed by cotransfecting a series of dominant-negative and constitutively active mutants. v-Abl induces *c-myc* transcription via a pathway that requires Ras and Raf1, but not Rac. Furthermore, we show that overexpression of

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p21, an inhibitor of cyclin-dependent kinases (cdks), blocks v-Abl-dependent induction of *c-myc* transcription. Consistent with this finding, v-Abl causes activation of cdk2 and cdk4 and hyperphosphorylation of the retinoblastoma (Rb) protein, p107, and p130. We propose that v-Abl-dependent activation of Raf1 is linked to activation of cdk2/cdk4 through the recently identified cdc25 pathway. Thus, these studies have resulted in the identification of the major components of a signaling pathway between v-Abl and one of its functionally important target genes, *c-myc*.

Results

Ras GTPase and Raf1 Ser/Thr kinase are required for the induction of c-myc transcription by v-Abl

Our previous work suggested that induction of *c-myc* transcription by v-Abl depended on initiation of a phosphorylation cascade (Wong et al. 1995). We wondered if this pathway involved activation of Ras which, like induction of *c-myc* (Sawyers et al. 1992), is required for Abl-dependent transformation (Goga et al. 1995; Sawyers et al. 1995). To test this hypothesis, we utilized our previously developed cotransfection assay (Wong et al. 1995). A v-Abl expression plasmid (pAB160-19) and a lu-

ciferase reporter (Luc) dependent on the murine *c-myc* promoter (extending from nucleotide -140 to nucleotide +340) were cotransfected into 4A2 fibroblasts (Renshaw et al. 1992) with or without a plasmid encoding a dominant-interfering mutant of Ras, Ras N17 (Stacey et al. 1991). v-Abl activated the *c-myc* promoter reporter approximately six- to sevenfold compared to the appropriate control lacking v-*abl* cDNA (Fig. 1A, lanes 1,2). However, in the presence of dominant-negative Ras N17, v-Abl-dependent activation was ablated completely (Fig. 1A, lanes 3,4). We then tested the effect of cotransfecting a plasmid encoding a constitutively active Ras mutant, Ras V12 (Conrad and Gutierrez-Hartmann 1992). Ras V12 activated *c-myc* transcription, and v-Abl was unable to activate it further in the presence of Ras V12 (Fig. 1A, lanes 5,6). These results show that Ras GTPase activity is required downstream of v-Abl for v-Abl to activate *c-myc* transcription.

The Ser/Thr kinase Raf1 is an immediate downstream effector for many Ras-dependent events (Marshall 1995). Therefore, we used the same approach to ask whether Raf1 was also required for v-Abl-dependent activation of *c-myc* transcription. Expression of RafC4, a dominant-negative form of Raf1 (Bruder et al. 1992), efficiently inhibited activation of the *c-myc* promoter by v-Abl (Fig. 1B lanes 3,4). In addition, a constitutively active mutant

Figure 1. Ras and Raf are required for *trans*-activation of *c-myc* by v-Abl. (A) 4A2 cells were transfected with 5 μ g of a luciferase reporter dependent on regions of the murine *c-myc* promoter from -140 to +340 bp and expression plasmids for v-Abl and dominant-negative (N17) or constitutively active (V12) Ras. The hatched bars were transfected with 5 μ g of pLTR, the promoter control plasmid. The solid bars were transfected with 5 μ g of pAB160-19wt, expression plasmid encoding wild-type v-Abl. (Lanes 1,2) Control plasmid; (lanes 3,4) 0.5 μ g of Ras N17, expression plasmid of dominant-negative Ras; (lanes 5,6) 5 μ g of Ras V12, expression plasmid of constitutively active Ras. The average luciferase activity of unstimulated cells transfected with vector plasmid was set as 1. The fold induction shown is the mean and standard deviation determined from three independent transfections of one representative experiment. (B) 4A2 cells were transfected with dominant-negative or constitutively active Raf1 expression constructs and the

same reporter and v-Abl expression plasmids as in A. (Lanes 1,2) Vector for Raf1 mutants; (lanes 3,4) 5 μ g of RafC4, expression plasmid of dominant-negative Raf1; (lanes 5,6) 5 μ g of RafBXB, expression plasmid of constitutively active Raf1. (C) 4A2 cells were transfected with 5 μ g of a reporter dependent on +70 to +160 bp of the murine *c-myc* promoter and the combination of v-Abl and dominant-negative Ras or Raf expression plasmids. (Lanes 1,2) Vector control; (lanes 3,4) 0.5 μ g of Ras N17; (lanes 5,6) 5 μ g of RafC4. (D) 4A2 cells were transfected with 5 μ g of a luciferase reporter dependent on regions of the murine *c-myc* promoter from -1100 to +580 bp and the combination of p65 and dominant-negative Ras or Raf. The hatched bars were transfected with 5 μ g of pCMV, the control expression vector (cytomegalovirus). The solid bars were transfected with 5 μ g of CMVp65, expression plasmid encoding the large subunit of NF- κ B. (Lanes 1,2) Vector; (lanes 3,4) 0.5 μ g of Ras N17; (lanes 5,6) RafC4. In each panel, a diagram of the reporter, not drawn to scale, is shown.

of Raf1, RafBXB (Bruder et al. 1992) activates the *c-myc* promoter alone and there is no further activation with v-Abl (Fig. 1B, lanes 5,6). We also found that dominant-negative forms of Ras and Raf1 blocked v-Abl-dependent activation of a truncated *c-myc* promoter reporter construct, +70/+160Luc (Fig. 1C), and a minimal TATA box dependent on four E2F-binding sites (data not shown). In these templates the response to v-Abl depends solely on the E2F sites (Wong et al. 1995).

We wished to be sure that expression of dominant-negative forms of Ras and Raf1 did not inhibit activation of *c-myc* transcription nonspecifically. Therefore, we tested the ability of the p65 subunit of NF- κ B to activate the *c-myc* promoter in the absence or presence of dominant-negative Ras or Raf1, as NF- κ B activation does not involve Ras or Raf and we have shown previously that NF- κ B sites are not involved in activation of *c-myc* by v-Abl (Wong et al. 1995). We cotransfected a p65 expression plasmid with -1100/+580Luc, a larger *c-myc* promoter reporter construct which contains the two NF- κ B responsive sites (Duyao et al. 1990 a,b), into 4A2 cells. p65 activated this reporter approximately fivefold (Fig. 1D, lanes 1,2). In the presence of dominant-negative Ras N17 (Fig. 1D, lanes 3,4) or dominant-negative RafC4 (Fig. 1D, lanes 5,6), p65 still fully activated *c-myc* transcription. Thus, we conclude that inhibition of v-Abl-dependent activation of *c-myc* transcription by Ras N17 and RafC4 is specific. Taken together, these data demonstrate that activation of Ras and Raf1 Ser/Thr kinase is required as a downstream effector for v-Abl-dependent *trans*-activation of *c-myc* transcription.

Activity of the Rac GTPase is not required for v-Abl to activate *c-myc* transcription

The v-Abl tyrosine kinase apparently uses more than one independent signaling pathway to transform cells. It has been shown that Rac and Ras are both important for v-Abl to activate mitogenesis, and a dominant-negative Rac mutant can inhibit v-Abl-induced activation of the TPA response element (TRE) and the serum response element (SRE) (Renshaw et al. 1996). Therefore, we tested whether Rac activity is required for v-Abl to activate *c-myc* transcription. In the cotransfection assay shown in Figure 2, v-Abl activated the *c-myc* promoter reporter approximately sixfold (Fig. 2A, lanes 1,2); how-

ever, in contrast to the results using dominant-negative forms of Ras and Raf, this activation was fully retained in the presence of an expression plasmid encoding dominant-negative Rac, V12N17rac1 (Ridley et al. 1992) (Fig. 2A, lanes 3,4). The activity of cotransfected V12N17rac1 in our system was confirmed by its ability to inhibit v-Abl-dependent activation of an SRE-dependent promoter (Fig. 2B, cf. lanes 3 and 4 with lanes 1 and 2) as reported previously (Renshaw et al. 1996). Thus, we conclude that Rac activity is not required for v-Abl to *trans*-activate *c-myc* transcription in our system.

v-Abl activates *cdks*

Because v-Abl activation of *c-myc* transcription requires the E2F site, we assume that E2F site binding proteins are activated in response to v-Abl. The activity of E2F-DP heterodimers is modulated by association with Rb family proteins and the ability of Rb family proteins to associate is regulated by phosphorylation (Adams and Kaelin 1995; Chen et al. 1995; Weinberg 1995; Cobrinik 1996). Rb family proteins are phosphorylated by cdk: cdk4-cyclin D is active early in G₁, and cdk2-cyclin E is active later in G₁ (Weinberg 1995). Therefore, we asked whether cdk activity changed in response to v-Abl signaling.

We took advantage of the D5 cell line, a 3T3 subclone transformed with a temperature-sensitive form of v-Abl (Renshaw et al. 1992). At the permissive temperature (32°C), D5 cells are transformed, displaying serum- and anchorage-independent growth and increased transcription of their endogenous *c-myc* gene. At the nonpermissive temperature (39°C), the cells revert to serum- and anchorage-dependent growth typical of 3T3 cells. D5 cells were made quiescent by serum starvation at 39°C for 48 hr. Subsequently the temperature was shifted to 32°C to activate v-Abl; under these conditions, growth is dependent solely on v-Abl, as the cells are still serum starved. We also compared cells growing in serum at the permissive and nonpermissive temperatures; at the permissive temperature these cells are receiving signals from both v-Abl and serum. Immunoprecipitated v-Abl was immunoblotted with antibody to phosphotyrosine to confirm that v-Abl kinase was temperature sensitive in our system (Fig. 3A, panel a).

Figure 2. Rac is not required for *trans*-activation of *c-myc* by v-Abl. (A) 4A2 cells were transfected with 5 μ g of a luciferase reporter dependent on regions of the murine *c-myc* promoter from -140 to +340 bp and the combination of v-Abl and dominant-negative Rac expression constructs. (Lanes 1,2) Control plasmid; (lanes 3,4) 5 μ g of V12N17rac1, expression plasmid for dominant-negative Rac. (B) 4A2 cells were transfected as in A, except with 5 μ g of SRE-Fluc, a luciferase reporter dependent on *c-fos* SRE.

Figure 3. v-Abl activates cdk2/cdk4 and cdk activity is necessary for v-Abl-dependent *trans*-activation of *c-myc* transcription. (A, a) v-Abl autophosphorylation in D5 cells. D5 cells were made quiescent by serum starvation at 39°C for 48 hr, shifted to 32°C and harvested 0, 2, 4, and 8 hr after temperature shift. D5 cells exponentially growing at both 39°C and 32°C with 10% serum were also analyzed. Lysates (50 µg of protein) were immunoprecipitated with v-Abl antiserum, run on a 6% gel, and probed with phosphotyrosine antibody. (b) Cyclin D1-associated kinase activity in D5 cells. The same D5 cell lysates (200 µg of protein) as in a were immunoprecipitated with an anti-cyclin D1 antibody or preimmune serum. Immune complexes were assayed for kinase activity using GST-Rb as substrates. ³²P-Labeled GST-Rb was shown. The relative amounts are given below the lanes. (c) The same protein lysates (25 µg of protein) were immunoblotted with anti-cdk4 antibody. (d) cdk2 kinase activity in D5 cells. D5 cells lysates were made the same way as above. Lysates (50 µg of protein) were immunoprecipitated with an anti-cdk2 antibody or preimmune serum. Immune complexes were assayed for kinase activity using histone H1 as substrates. ³²P-Labeled histone H1 was shown. The bands were quantitated and the amounts relative to 0 hr are shown below the lanes. (e) The same protein lysates (25 µg of protein) as in d were immunoblotted with anti-cdk2 antibody. (B) 4A2 cells were transfected with 5 µg of a luciferase reporter dependent on regions of the murine *c-myc* promoter from -140 to +340 bp and the combination of expression plasmids for v-Abl and p21. (Lanes 1,2) Control plasmid; (lanes 3,4) 5 µg of CMVp21. (C) 4A2 cells were transfected with 5 µg of the SRE-Fluc, a luciferase reporter dependent on *c-fos* SRE and the combination of expression plasmids for v-Abl and p21. (Lanes 1,2) Control plasmid; (lanes 3,4) 5 µg of CMVp21.

D-type cyclin-associated kinase activity was assayed *in vitro* by immunoprecipitating with cyclin D1 antibodies at various time following temperature shift, and the associated kinase activity was assayed by using glutathione *S*-transferase (GST)-Rb protein substrates. This activity primarily belongs to cdk4, as cdk4 is the major partner of D-type cyclin complexes in fibroblasts (Matsushime et al. 1994). A small but reproducible increase (twofold) in kinase activity was observed following activation of v-Abl in the absence of serum (Fig. 3A, panel b). D-type cyclin activity was increased at both temperatures in the presence of serum, although it was slightly higher at the permissive temperature. In all cases, the amount of cdk4 protein, determined by immunoblot (Fig. 3A, panel c), did not change significantly.

Cdk2 activity was similarly assayed *in vitro* using histone H1 as substrate. An increase in cdk2 activity was observed at 2 hr and reached 5.7-fold by 8 hr (Fig. 3A, panel d). Cdk2 activity was elevated at both temperatures in the presence of serum, although activity was slightly higher in the presence of v-Abl. The amount of cdk2 protein, determined by immunoblot (Fig. 3A, panel

e), did not change significantly in any sample. Thus, we observe a v-Abl-dependent increase in cdk activity, especially for cdk2, which is consistent with the possibility of v-Abl-dependent phosphorylation of Rb family proteins and subsequent activation of E2F proteins.

Cdk activity is required for v-Abl-dependent induction of c-myc transcription

To test directly whether cdk activity is required for v-Abl-dependent induction of *c-myc* transcription, we expressed p21 in the cotransfection assay along with v-Abl and the reporter dependent on the *c-myc* promoter. p21 is a universal inhibitor of cdk activity (Xiong et al. 1993). v-Abl activated the -140/+340Luc *c-myc* promoter reporter approximately sixfold (Fig. 3B, lane 1,2); however, in the presence of p21, this activation was ablated completely (Fig. 3B, lane 3,4). As a control, we examined the effect of p21 on v-Abl-dependent activation of a reporter dependent on an SRE. Because v-Abl-dependent activation of a SRE requires Rac (Fig. 2; Renshaw et al. 1996), it appears to proceed by a different signal transduction

path. v-Abl activated SRE-Fluc (a luciferase reporter dependent on *c-fos* SRE) more than sixfold (Fig. 3C, lanes 1,2), and this activation was not affected by cotransfection of the p21 expression plasmid (Fig. 3C, lanes 3,4). Thus, inhibition of v-Abl-dependent induction by p21 expression is specific for *c-myc* transcription and is not required for SRE-dependent transcription. We conclude that cdk activity is required for *trans*-activation of *c-myc* transcription by v-Abl.

Rb family proteins are hyperphosphorylated in response to v-Abl signaling

Because Rb family proteins are primary substrates of the cdks, as well as regulators of E2F proteins, we examined the phosphorylation states of pRb, p107, and p130 in D5 cells. Hypophosphorylated forms of pRb, p107, or p130 migrate faster in SDS-polyacrylamide gels than hyperphosphorylated forms and can be discerned using immunoblots. Cells were grown at both temperatures in the absence of serum as described above. Whole-cell extracts were prepared from these cells, and immunoblots were analyzed with antisera against c-Myc, pRb, p107, and p130. Activation of the v-Abl tyrosine kinase (Fig. 4A, panel a) led to an increase in c-Myc (Fig. 4A, panel b), consistent with previous reports (Renshaw et al. 1992) and thereby demonstrating that v-Abl was signaling correctly. Before temperature shift, approximately half of the pRb was in the high-mobility hypophosphorylated form; after temperature shift, nearly all was found in the low-mobility hyperphosphorylated form (Fig. 4A, panel c). Similarly, p107 became increasingly hyperphosphorylated, as evidenced by lower mobility, following v-Abl activation by temperature shift (Fig. 4A, panel d). Of the three family members, the phosphorylation state of p130 changed most dramatically. At the nonpermissive tem-

perature, p130 was in two higher mobility hypophosphorylated forms; 8 hr after temperature shift, p130 was entirely in a lower mobility hyperphosphorylated form (Fig. 4A, panel e). In addition, less p130 was observed 8 hr after temperature shift, although we do not know whether the amount actually decreased or whether the hyperphosphorylated form reacts less well with antiserum. We conclude that pRb, p107, and p130 are hyperphosphorylated in response to v-Abl signaling and that the amount of p130 may decrease in response to v-Abl.

Overexpression of pRb blocks v-Abl-dependent induction of *c-myc* transcription

Association of E2F-DP heterodimers with pRb, p107, and/or p130 inhibits their ability to act as transcriptional activators and can create transcriptional repressors (Cobrinik 1996). Thus, our results are consistent with a model in which v-Abl signaling results in activation of cdks, phosphorylation of Rb family proteins, release of Rb proteins from association with E2F-DP and, finally, activation of *c-myc* transcription via the E2F site. To examine this model further, we used the cotransfection assay to test whether overexpression of pRb could block v-Abl-dependent *trans*-activation of *c-myc* transcription. Our results show that ectopic pRb does block *c-myc* transcription in this assay (Fig. 4B, cf. lanes 3 and 4 with lanes 1 and 2). As a control, we examined the effect of pRb on v-Abl-dependent activation of a reporter dependent on a SRE; this activation was not affected by cotransfection of the Rb expression plasmid (Fig. 4C, cf. lanes 3 and 4 with lanes 1 and 2). These results are consistent with the idea that *c-myc* transcription is sensitive to the levels of hypophosphorylated pRb and lend support to the suggestion that v-Abl activation via E2F sites depends on phosphorylation of Rb family proteins.

Figure 4. Rb family proteins are hyperphosphorylated following v-Abl activation, and overexpression of pRb blocks v-Abl-dependent *trans*-activation of *c-myc* transcription. (A) D5 cells were made quiescent by serum starvation at 39°C for 48 hr, and shifted to 32°C. Whole-cell extracts were prepared from these cells at indicated time points and subjected to Western blot analysis using antibodies against c-Myc, pRb, p107, and p130. For a, the same D5 cell lysates (50 µg) were immunoprecipitated with an antiserum against v-Abl and probed with phosphotyrosine antibody. (B) 4A2 cells were transfected with 5 µg of a luciferase reporter dependent on regions of the murine *c-myc* promoter from -140 to +340 bp and the combination of v-Abl and Rb expression plasmids. (Lanes 1,2) Control plasmid; (lanes 3,4) 5 µg of pSV-Rb, expression plasmid for Rb. (C) 4A2 cells were transfected with 5 µg of SRE-Fluc and the combination of expression plasmid for v-Abl and Rb. (Lanes 1,2) Control plasmid; (lanes 3,4) 5 µg of pSV-Rb.

Discussion

v-Abl induces *c-myc* transcription by initiating a phosphorylation cascade that ultimately activates E2F transcription factors (Wong et al. 1995). In this study we have identified key components of the signaling pathway between v-Abl and activated E2F site binding proteins. We propose that v-Abl induces *c-myc* transcription by the pathway illustrated in Figure 5. This path is not the only signaling pathway that v-Abl utilizes to carry out its full program of cell transformation and may not be the only path used to induce *c-myc*. However, this represents the most complete understanding to date of a signaling pathway from v-Abl to a functionally important target gene.

Ras and Raf1 are required for v-Abl-dependent induction of c-myc

A requirement for the Ras GTPase in v-Abl-dependent induction of *c-myc* is consistent with previous data showing that both Ras and c-Myc are required for v-Abl- and BCR/ABL-dependent transformation (Smith et al. 1986; Sawyers et al. 1992, 1995). Although v-Abl and BCR/ABL activate Ras via adaptor proteins that associate with BCR sequences (Pendergast et al. 1993) or the proline-rich SH3-binding domain (SBD) in the carboxyl terminus (Feller et al. 1994; Nichols et al. 1994; Ren et al. 1994; Goga et al. 1995), we believe that association of

v-Abl with Shc, which requires the v-Abl SH2 domain (Raffel et al. 1996), is critical for activation of E2F proteins. The SH2 domain, but not the SBD of v-Abl, is required for v-Abl-dependent *trans*-activation of *c-myc* transcription (Wong et al. 1995), and c-Myc complements mutations in the SH2 domain of BCR/ABL in a transformation assay (Afar et al. 1994). However, our data do not rule out the possibility (broken line in Fig. 5) that activation of Ras by an adaptor such as Crkl may be an alternate path by which v-Abl induces *c-myc* transcription via Ras.

E2F activation by v-Abl does not require Rac

While c-Myc is required for v-Abl-dependent transformation, its induction is not sufficient for transformation (Sawyers et al. 1992). The Ras-related GTPase Rac has been shown to be necessary for activation of a mitogenic program by v-Abl (Renshaw et al. 1996). Rac is required for induction of TRE-dependent transcription by v-Abl, whereas Rac, Ras, and Raf1 are required for v-Abl to induce SRE-dependent transcription (Renshaw et al. 1996). We show that Ras and Raf1, but not Rac, are required for v-Abl to induce the *c-myc* promoter. This emphasizes that v-Abl-dependent induction of promoters containing SREs and TREs (presumably corresponding to *c-fos* and *c-jun*, respectively *in vivo*) proceeds by pathways distinct from that for *c-myc* and is consistent with the facts that neither the *c-fos* nor the *c-jun* promoter is regulated by E2F sites and that v-Abl increases expression of c-Myc but not Fos or JunB in a myeloid line (Cleveland et al. 1989).

Cdc25 may link v-Abl-dependent activation of Raf1 to activation of cdk

Our work and that of others suggests that mitogen-activated protein kinase (MAPK) may not be involved in E2F site-dependent activation by v-Abl via Raf1. Dominant-negative Rac inhibits v-Abl-dependent activation of MAPK (Renshaw et al. 1996) but fails to block v-Abl-dependent induction of *c-myc* transcription (Fig. 2). In addition, a synthetic inhibitor of the MAPK pathway, PD 098059 (Dudley et al. 1995) failed to inhibit v-Abl *trans*-activation of *c-myc* in our standard assay (data not shown). Finally, in the murine mast cell line ICDP, v-Abl induces Shc phosphorylation but not MAPK activation (Owen-Lynch et al. 1995).

We suggest, but have not yet demonstrated, that v-Abl is linked to activation of E2F proteins via a second path from Raf1 that involves the cdc25A phosphatase. cdc25 activates cdc2 and cdk2 by dephosphorylating a critical tyrosine (Y15) in their nucleotide-binding domains (Jinno et al. 1994; Galaktionov et al. 1995). cdk4 and cdk6 may also be activated by cdc25 because this tyrosine in their nucleotide-binding domains is conserved (Terada et al. 1995).

cdks are required for v-Abl-dependent induction of c-myc transcription

cdk inhibitor p21 specifically blocked the v-Abl-depen-

Figure 5. Model for signal transduction from v-Abl to activation of *c-myc* transcription. Steps that are consistent with current data, but not definitely established, are indicated by broken lines. Because the role of cdc25A is implied but not proven, it is shown in parenthesis.

dent induction of *c-myc* transcription but not induction of an SRE-dependent promoter (Fig. 3B,C), demonstrating that cdk activity is required for v-Abl-dependent *trans*-activation of *c-myc* transcription. This is corroborated by increased activity of cdk2 and cdk4 when D5 cells are signaled by v-Abl to proliferate (Fig. 3A). cdk2 can form complexes with both E2F-p107 and E2F-p130. These higher-order complexes may be intermediates whose subsequent disruption yields free, active E2F-DP heterodimers (Zhu et al. 1995; Shiyanov et al. 1996). This possibility is consistent with the fact that cdk2-containing E2F complexes are seen during S phase in normal cells but are observed throughout the cell cycle in v-Abl-transformed D5 cells (Wong et al. 1995). Alternatively, activated cdk2 may directly phosphorylate pocket proteins associated with E2F-DP heterodimers and release free E2F-DP heterodimers.

Hyperphosphorylation of Rb family proteins appears to be important for v-Abl-dependent induction of c-myc transcription

The penultimate step in v-Abl-dependent induction of *c-myc* via the E2F site is a functional change in proteins that bind the E2F site. Upon association with Rb family "pocket proteins" the ability of E2F-DP heterodimers to activate transcription is ablated or changed to repression. Hyperphosphorylation releases pocket proteins and changes the activity of E2F-DP heterodimers (Chellapan 1994; Cobrinik 1996; Slansky and Farnham 1996). Our demonstration (Fig. 4A) that v-Abl signaling leads to hyperphosphorylation of pRb, p107, and p130 is consistent with the idea that release of Rb family proteins from E2F-DP heterodimers is a critical step for induction of *c-myc* via the E2F site (Fig. 5). However, we cannot distinguish whether the actual mechanism of this induction is activation (by E2F-DP heterodimers), relief of repression (removal of E2F-DP-Rb complexes), or both.

pRb, p107, and p130 each bind to particular members of the E2F family at distinct stages of the cell cycle and act in concert to limit the activation of E2F and the expression of E2F-responsive genes (Vairo et al. 1995; Weinberg 1995; Shiyanov et al. 1996). Our data do not distinguish specific roles for individual pocket proteins in the v-Abl-dependent induction of *c-myc* transcription. Although pRb repressed *c-myc* promoter activity in the cotransfection assay (Fig. 4B), this does not establish a key role for pRb *in vivo*, as overexpression of any pocket protein would be expected to alter the balance between active and inactivate E2F-binding proteins.

Autoregulatory loops

c-Myc/Max was recently shown to activate transcription of *cdc25A* at a time in G₁ when c-Myc induction peaks (Galaktionov et al. 1996). This is consistent with previous work showing that c-Myc stimulates the activity of cyclin E- and D1-dependent kinases, possibly via *cdc25*, and leads to hyperphosphorylation of pRb (Steiner et al. 1995). c-Myc has also been reported to activate transcrip-

tion of cyclin A and D1, and there are binding sites for c-Myc/Max in the E2F-1 promoter (Jansen-Durr et al. 1993; Johnson et al. 1994). If v-Abl activated E2F proteins by another Ras/Raf-dependent path, elevated c-Myc could induce transcription of *cdc25*, cyclin D1, cyclin A, and/or E2F-1. In this model c-Myc induction would cause and not result from increased cdk activity, hyperphosphorylation of pocket proteins, and altered E2F-binding proteins. However, this model is not consistent with our observation that p21 blocks the ability of v-Abl to *trans*-activate *c-myc*, which establishes a requirement for cdk activity in the path from v-Abl to *c-myc*. We favor the view that v-Abl leads to a dynamic situation with autoregulatory loops in which c-Myc, *cdc25*, cdk, and E2F-1 can be both regulators and subject to regulation.

Our data provide the most complete understanding to date of a signaling pathway initiated by v-Abl and raise important questions for further investigation. For example, a role for *cdc25A* and the prediction that other E2F-dependent genes may be activated by v-Abl or that other *cdc25*- or p21-regulated proteins may be sensitive to v-Abl signaling can be tested. Experiments can be designed to ask whether signaling by v-Abl mimics a normal proliferative signal or whether there are qualitative or quantitative differences in activation of specific proteins in the path. Finally, we can ask how v-Abl signaling may be related to signaling by BCR/ABL or c-Abl.

Materials and methods

Cells and transfections

Monolayer culture of 4A2 (P-3T3) and D5 fibroblasts were grown in Iscove's modified Dulbecco's medium containing 10% newborn calf serum. DNA transfection was performed by the calcium phosphate precipitation method described by Chen and Ohayama (1988). Cells (5×10^5) were seeded 1 day prior to transfection and fed with fresh medium 3 hr before transfection. The cells were usually transfected with 5 μ g of reporter plasmid and 5 μ g of each expression plasmid unless indicated otherwise, and the total amount of transfected DNA was balanced by pUC19 plasmid. After an overnight incubation, the cells were washed, transferred to medium containing 0.5% serum, and harvested 96 hr later, or transferred to medium containing 0.2% serum and harvested 24 hr later. Luciferase activity for each transfection was measured as described previously (Riggs et al. 1993).

Plasmids

The *c-myc* promoter reporters -1100/+580Luc, -140/+340Luc, and +70/+160Luc have been described previously (Wong et al. 1995). The plasmid expressing v-Abl, pAB160-19, and pLTR promoter control plasmid have also been described previously (Wong et al. 1995). Plasmid Ras N17, expressing the dominant-negative Ras, was kindly provided by A. M. Pendergast and has been described previously (Pendergast et al. 1993). Plasmid Ras V12, expressing the constitutive active Ras, was kindly provided by A. Gutierrez-Hartmann and has been described previously (Conrad and Gutierrez-Hartmann 1992). The plasmids expressing dominant-negative and constitutive active mutants of Raf, RafC4, and RafBXB were kindly provided by U.R. Rapp and have been described previously (Bruder et al. 1992). Plasmid

V12N17rac1, expressing dominant-negative Rac, was a gift from A. Hall and has been described previously (Ridley et al. 1992). Plasmid CMVp21 was kindly provided by S.J. Elledge and described previously (Harper et al. 1993). Plasmid p65 was kindly provided by D. Baltimore (Massachusetts Institute of Technology, Cambridge). Plasmid pSV-Rb, an expression construct of Rb, was kindly provided by S.P. Goff (Columbia University). Plasmid SRE-Fluc was kindly provided by R. Prywes and described previously (Johansen and Prywes 1994).

Immunoprecipitation and kinase assays

For immunoprecipitation, cells were washed twice with ice-cold PBS, harvested in lysis buffer containing 50 mM HEPES (pH 7.5), 10% glycerol, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, and 0.1% Tween 20, supplemented with the phosphatase and protease inhibitors 1.0 mM NaF, 0.1 mM sodium orthovanadate, 5 µg of leupeptin per ml, 10 µg of aprotinin per ml, 5 µg of pepstatin A per ml, and 1.0 mM PMSF and lysed by repeated passage through a 25-gauge needle. Cellular debris was removed from soluble extracts by centrifugation at 16,000g for 10 min at 4°C. Lysates were precleared by incubation with protein A(G)-agarose (Santa Cruz Biotechnology) for 30 min at 4°C. Endogenous cdk2-containing complexes were immunoprecipitated for 2 hr at 4°C using 1 µg cdk2 antibody and then incubated with 20 µl of protein A-agarose beads (50% vol/vol) for 2 hr at 4°C. Immunoprecipitates were washed four times with lysis buffer and twice with the kinase reaction buffer (50 mM HEPES at pH 7.5, 10 mM MgCl₂, 1 mM DTT, 2.5 mM EGTA). The kinase activity associated with anti-cdk2 immunocomplexes was assayed in 30 µl of kinase reaction buffer containing 5 µg of histone H1 (Boehringer Mannheim), 5 µCi of [γ -³²P]ATP. Reactions were carried out for 15 min at 30°C and stopped by the addition of Laemmli sample buffer, and the reaction products were electrophoresed in a 10% SDS-polyacrylamide gel, whereupon the gels were dried, visualized by autoradiography, and quantitated with a PhosphorImager (Molecular Dynamics). Endogenous cdk4-containing complexes were immunoprecipitated using 20 µl of protein G-agarose precoated with 2 µg of cyclin D1 antibody. The kinase assay was performed by using 1 µg of GST-Rb (769-921) fusion proteins (Santa Cruz Biotechnology) as the substrates. Reactions were carried out for 30 min at 30°C, the products were electrophoresed in a 10% SDS-polyacrylamide gel, and visualize and quantitated as above. v-Abl was immunoprecipitated using 5 µl of antiserum according to standard procedure.

Western blot (immunoblot) analysis

The D5 whole-cell extracts were made the same way as the lysates for immunoprecipitation (see above). Equal amounts of cellular proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blotted with 5% nonfat milk in Tris buffer at pH 7.5 (50 mM Tris-HCl, 125 mM NaCl, 0.2% Tween 20) for 1 hr at room temperature and then immunoblotted with the indicated primary antibodies in 2% nonfat milk in the same Tris buffer for 2 hr at room temperature or overnight at 4°C. Antibodies immunoblotted to the membrane were detected by using horseradish peroxidase-linked secondary antibodies and an enhanced chemiluminescence detection system from Pierce (Rockford, IL).

Antibodies

To characterize proteins by immunoblot and to immunoprecipitate cdk complexes the following antibodies were used: The

pRb monoclonal antibody (Q3-245) was obtained from Pharmingen (San Diego, CA). The p107 (SD9), p130 (C-20), cdk2 (M2), and cdk4 (C-22) polyclonal antibodies and phosphotyrosine monoclonal antibody (PY20) were from Santa Cruz Biotechnology (Santa Cruz, CA). The cyclin D1 monoclonal antibody (DCS-11) was from NeoMarkers (Fremont, CA). Murine c-Myc antiserum was described previously (Shrivastava et al. 1996). Antiserum against v-Abl was a gift from Dr. S. Goff.

Acknowledgments

We are grateful to Drs. S. Goff, N. Rosenberg, O. Witte, D. Cobrinik, S. Chellapan, and members of our laboratories for helpful discussions and to Drs. S. Goff, S. Chellapan, D. Cobrinik, and A. Henderson for critically reading the manuscript. We appreciate receiving cell lines and plasmids from Drs. J. Wang, A.M. Pendergast, A. Gutierrez-Hartmann, U.R. Rapp, D. Baltimore, A. Hall, S.J. Elledge, and R. Prywes. This work was supported by grants from the American Cancer Society (VM130) and the U.S. Army Medical Research and Material Command to K.C.

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Genes Dev. 1997, 11:

Access the most recent version at doi:[10.1101/gad.11.5.654](https://doi.org/10.1101/gad.11.5.654)

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