Genetic Requirement for Ras in the Transformation of Fibroblasts and Hematopoietic Cells by the *Bcr-Abl* Oncogene

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Summary

To determine the functional importance of *Ras* in transformation by *Abl* oncogenes, we used a genetic approach to measure the effect of impaired Ras activity on the ability of Bcr-Abl or v-Abl to transform cells. Expression of the catalytic domain of the GTPase activating protein for Ras (Gap C terminus) impaired soft agar colony formation by fibroblasts expressing v-Abl or Bcr-Abl by 70–80%. To test Ras function in a model that more closely resembles clinical diseases involving *Bcr-Abl*, double gene retroviruses expressing *Bcr-Abl* paired with the Gap C terminus or dominant negative *Ras* were introduced into naive mouse bone marrow cells. Transformation by Bcr-Abl was completely blocked in both situations. Coexpression of normal c-H-Ras accelerated the transforming activity of Bcr-Abl. These findings show that Ras activation is essential for the leukemogenic activity of *Abl* oncogenes in two distinct model systems. The results genetically define a connection between the Bcr-Abl cytoplasmic tyrosine kinase and Ras and add to the accumulating evidence that deregulation of Ras is a central event in the genesis of a number of molecularly distinct forms of human myeloid leukemia.

The *Ras* family of G-proteins plays a central role in relaying growth stimulatory signals from outside the cell to the nucleus. Mutations in *Ras* which disrupt this normal signaling pathway lead to deregulated growth and cancer. Surveys of oncogene abnormalities in human leukemia have found a high frequency of activating *Ras* mutations in acute myeloid leukemias (AML) and myelodysplastic syndromes (MDS), particularly those of the chronic myelomonocytic leukemia (CMML) subtype (1). *Ras* mutations are also common in patients with chronic myelogenous leukemia (CML[1]) who lack the *Bcr-Abl* fusion gene (2). These findings suggest that *Ras* mutations are a common feature of myeloid leukemias. A notable exception to this trend is the absence of *Ras* mutations in *Bcr-Abl*–positive CML (2, 3).

One explanation for this apparent paradox is that Ras is regulated in an abnormal fashion in CML cells. Ras is activated by stimulatory GDP/GTP exchange molecules such as Sos (4, 5) which convert Ras from its inactive GDP-bound state to its active GTP-bound state. Active Ras can bind and target the cytoplasmic Raf kinase to the cell membrane, where a kinase cascade involving Mek and mitogen-activated protein (MAP) kinase is propagated (6, 7). Negative control of Ras is mediated by inhibitory GTPase-activating molecules such as RasGap and neurofibromin (NF-1), which convert Ras back to its inactive state.

Philadelphia chromosome–positive CML is characterized by the tumor-specific t(9,22) translocation which creates the cytoplasmic *Bcr-Abl* tyrosine kinase (TK) oncogene. Bcr-Abl transforms fibroblasts and hematopoietic cells in culture and produces leukemias in mice (recently reviewed in 8). Mutational analysis has defined several requirements within Bcr-Abl for fibroblast transformation. These include on oligomerization domain within Bcr (9), a Grb-2 binding site in Bcr (10), the SH2 domain (11), a catalytically active kinase domain (12), the major site of tyrosine autophosphorylation (12), and an actin binding domain in the Abl COOH terminus (13). Dominant negative experiments have shown that the c-Myc transcription factor is required for Bcr-Abl transformation (14). A connection between Myc and SH2 domain of Bcr-Abl is suggested by the observation that overexpression of c-Myc specifically restores transforming activity to the inactive SH2 mutant (11). The results from complementation experiments such as these define at least two independent pathways required for Bcr-Abl transformation.

Is Ras part of one of these pathways? Studies of the Ras protein in cells expressing Bcr-Abl suggest abnormal levels of activation because a greater than normal proportion of Ras is bound to GTP (15). Several molecules which can affect Ras activity, such as RasGap (16), Grb-2 (10, 17), Shc (18), and Crk-L (19), can bind Bcr-Abl. Mutagenesis experiments show

[1] *Abbreviations used in this paper:* CML, chronic myelogenous leukemia; TK, thymidine kinase.

a correlation between loss of Grb-2 binding and loss of transforming activity (10). In this report we have used a genetic approach to evaluate directly if Ras is essential for this transformation signal. Interruption of Ras function by overexpression of the catalytic domain of RasGap or of dominant negative c-H-Ras impairs the transforming activity of Bcr-Abl and v-Abl in fibroblasts and bone marrow cells. The results show that Ras is required for the oncogenic activity of *Abl* oncogenes and suggest that Ras activity is essential for human CML. Together with the high frequency of activating *Ras* mutations in AML and MDS, abnormal Ras activity appears to be a common theme in multiple types of human myeloid malignancies.

Materials and Methods

Retroviral Plasmids and Preparation of Virus Stocks. cDNAs encoding the *Gap* C terminus (provided by Jeff DeClue and Doug Lowy, National Institutes of Health, Bethesda, MD), c-H-*Ras* and the dominant negative Asn 17 c-H-*Ras* mutant (provided by Simon Cook, Onyx Phamaceuticals, Richmond, CA) were subcloned into the pSRalphaMSVtkNeo retrovirus vector (20) after addition of EcoRI linkers. For bone marrow experiments, the pSRαMSVtkNeo vector was modified to allow simultaneous expression of two genes from a single retrovirus. First, the tkNeo cassette was removed by digestion with ClaI. Secondary HindIII and ClaI sites in the backbone of the plasmid were destroyed, creating the vector pSRαMSV with unique cloning sites for EcoRI, HindIII, and ClaI within the LTRs. To provide an internal promoter, the HindIII site was changed to NotI and sequences encoding the TK promoter were subcloned into the NotI site. *Gap* C terminus, c-H-*Ras*, Asn 17 *Ras*, or *Neo* were subcloned into the upstream EcoRI site, and $p185^{Bcr\text{-}Abl}$ was subcloned into the downstream ClaI site (see Fig. 4).

With the exception of *v-Mos*, all retrovirus stocks were prepared by transient transfection of Cos-7 cell using $CaCl_2$ as described previously (20). V-mos retrovirus was obtained from a rat-1 fibroblast producer cell line (14). For retroviruses expressing *Bcr-Abl* or *v-Abl*, titers were determined indirectly by measuring Bcr-Abl or v-Abl protein expression 48 h after infection of NIH3T3 cells by immunoblot or immunohistochemistry.

Transformation Assays. The rat-1/Gap C terminus cell line was generated by infection of rat-1 fibroblasts with helper-free retrovirus followed by selection in G418 (0.5 mg/ml) for 2–3 wk. Cells were maintained continuously in G418 to prevent the outgrowth of cells that might delete the retrovirus construct. Transformation by v-Abl and Bcr-Abl was measured using a soft agar colony assay as described (14). Hematopoietic cell transformation assays were performed using fresh bone marrow from the tibias and femurs of 4-wk-old BALB/c mice as described previously (21).

Protein Analysis. Expression of the Gap C terminus protein was measured by two-cycle immunoprecipitation of rat-1/Gap C terminus cells labeled with [^{35}S]methionine with polyclonal rabbit antisera raised against the full-length Gap protein (kindly provided by Bonnee Rubinfeld, Onyx Pharmaceuticals). Bcr-Abl and v-Abl protein expression was measured by immunoblot or immunohistochemistry using the pex-5 anti-Abl monoclonal antibody (11).

Results

Overexpression of the Catalytic Domain of RasGap in rat-1 Fibroblasts. Transformation by *Abl* oncogenes can be quantitatively measured in a single-step soft agar colony assay using rat-1 fibroblasts acutely infected with retroviruses expressing either *v-Abl* or *Bcr-Abl* (22). To determine the role of Ras in this transformation process, we inhibited Ras function by overexpression of the GTPase activating protein for Ras (RasGap), thereby maintaining a greater pool of Ras in the inactive GDP bound state. This approach has been previously used to inhibit the transforming activity of c-H-Ras (23), v-Src (24, 25), and c-Fms (26).

cDNAs for the full-length bovine RasGap protein and a truncated form of *RasGap* (*Gap* C terminus) containing the catalytic GTPase activating domain but not the NH_2-terminal SH2 and SH3 domains, were subcloned into the retrovirus vector pSRαMSVtkNeo (20). This vector contains a *cis*-linked G418 resistance gene and allows the generation of high titer retrovirus stocks by transient transfection of Cos-7 cells. To ensure that all cells at risk for transformation by v-Abl or Bcr-Abl overexpressed a Gap protein, populations of rat-1 fibroblasts infected with *RasGap* or *Gap* C terminus retrovirus were derived by G418 selection.

To verify that the appropriate RasGap protein was expressed in the G418 resistant populations, immunoprecipitations were performed on lysates from cells labeled with [^{35}S]methionine. The results showed stable expression of the Gap C terminus protein at a level comparable with that of endogenous RasGap (Fig. 1). We were unable to derive similar populations overexpressing the full-length RasGap protein despite screening three different G418 populations and 10 independent clones. One explanation for this result might be a toxic effect of constitutive overexpression of full-length RasGap in rat-1 cells, although overexpression of full-length RasGap has been described in NIH3T3 cells (25). Importantly, overexpression of the Gap C terminus showed no toxic effects on cell growth. The number of G418 resistant clones recovered after infection with *Gap* C terminus retrovirus ($\sim 10^5$/ml of supernatant) was comparable with control Neo virus of similar titer, and the growth rates of rat-1/Gap C terminus cells and rat-1/Neo were comparable (data not shown).

Overexpression of Gap C Terminus Impairs the Transforming Activity of Bcr-Abl and v-Abl. To measure the effect of Gap C terminus overexpression on transformation by *Abl* oncogenes, rat-1/Neo and rat-1/Gap C terminus cells were infected with retroviruses expressing either *v-Abl* or *Bcr-Abl*. Retroviruses expressing *c-H-Ras*, *v-Mos*, or an empty vector containing only the *Neo* gene were used as controls. 2 d after infection, cells were plated in soft agar, and transformation was measured by comparing the number of colonies after 2–3 wk.

In three independent experiments, overexpression of the Gap C terminus consistently reduced colony formation by Bcr-Abl and v-Abl by 70–80% (Fig. 2 *A*). Photographs of agar plates from a representative assay are shown in Fig. 3. rat-1/Neo cells (Fig. 3, *top left*) and rat-1/Gap C terminus cells (*bottom left*) failed to form colonies in agar after infection with control Neo retrovirus. After infection with *v-Abl* retrovirus, rat-1/Neo cells formed numerous large colonies (Fig. 3, *top right*) whereas rat-1/Gap C terminus cells did not

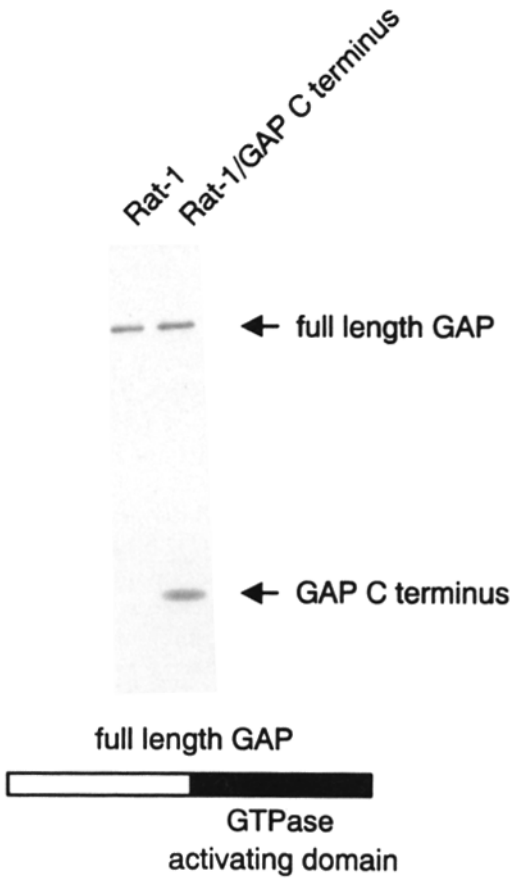

Figure 1. Expression of the Gap C terminus in rat-1 fibroblasts. Rat-1 fibroblasts were infected with retrovirus expressing the Gap C terminus and selected in G418 for 3 wk. Lysates from Rat-1 cells and rat-1/Gap terminal cells labeled for 3 h with [^{35}S]methionine were subjected to two cycles of immunoprecipitation with Gap antisera and visualized by autoradiography after SDS-PAGE. A cartoon of the RasGap protein is shown below.

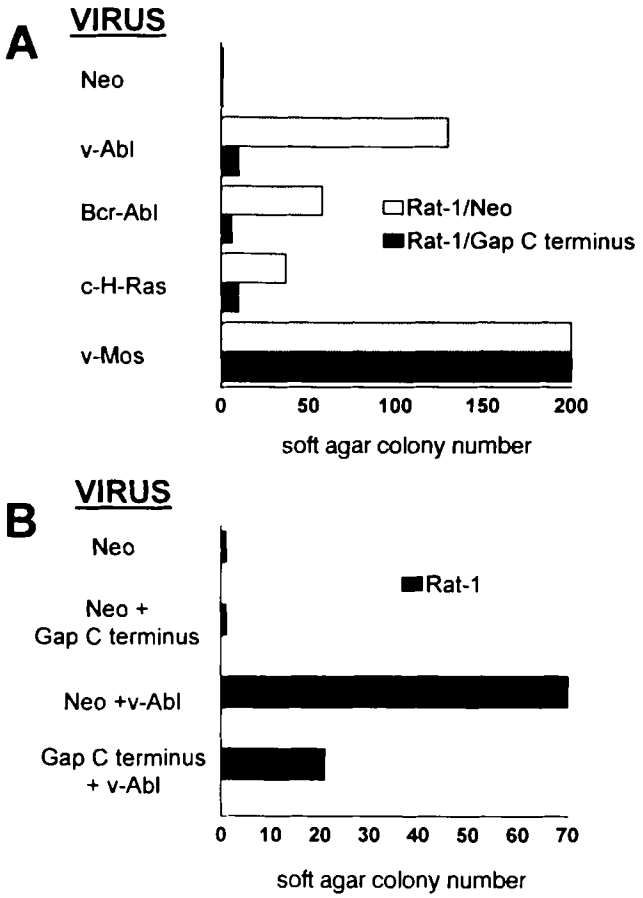

Figure 2. Expression of the Gap C terminus impairs the transforming activity of v-Abl, Bcr-Abl, and c-H-Ras, but not v-Mos. (*A*) rat-1/Neo and rat-1/Gap C terminus cells were infected with retroviruses expressing v-Abl, Bcr-Abl, c-H-Ras, or v-Mos and plated in soft agar. Colonies were counted after 2 wk. The average colony counts from three experiments are shown. (*B*) Rat-1 cells were infected with the retrovirus stock combinations listed along the left and plated into soft agar. The average colony numbers from two experiments are shown.

(*bottom right*). To be sure that both populations were successfully infected with *v-Abl* retrovirus, anti-Abl immunoblots were performed at the time of the cells were plated in soft agar. The results confirmed equivalent levels of v-Abl protein expression in both populations (data not shown).

We have postulated that the transformation suppression activity of the Gap C terminus is due to downregulation of Ras. If this is the case, then overexpression of the Gap C terminus should impair the transforming activity of normal c-H-Ras. In contrast, the Gap C terminus should not affect the transforming activity of oncogenes known to signal independently of Ras. To test this hypothesis, we infected rat-1/Neo and rat-1/Gap C terminus cells with retroviruses expressing either the normal *c-H-Ras* gene or the *v-Mos* serine kinase oncogene, which has been previously shown to transform NIH3T3 cells in a Ras-independent fashion (23, 27). Similar to the results with v-Abl and Bcr-Abl, fewer colonies were seen in rat-1/Gap C terminus cells after infection with *c-H-Ras* retrovirus compared with the rat-1/Neo control. However, there was no difference in colony number in rat-1/Neo or in rat-1/Gap C terminal cells after *v-Mos* infection (Fig. 2 *A*). These results indicate that rat-1/Gap C terminus cells suppress transformation in a Ras-specific manner and support the hypothesis that the mechanism for this effect is through downregulation of Ras.

To be sure that the transformation suppression activity in the rat-1/Gap C terminus cells was due to the Gap C terminus protein and not some other mutation acquired during G418 selection, we repeated the experiment in a fashion that did not require derivation of any cell lines. rat-1 cells were infected simultaneously with two different retrovirus stocks expressing either the Gap C terminus or v-Abl and plated directly into soft agar after 48 h. As previously observed using the rat-1/Gap C terminus cell lines, fewer colonies were seen after simultaneous infection of rat-1 cells with *v-Abl* and *Gap* C terminus retroviruses as compared with *v-Abl* and *Neo* control retroviruses (Fig. 2 *B*). The magnitude of the suppressive effect of the Gap C terminus on v-Abl transformation was lower than with the rat-1/Gap C terminus population

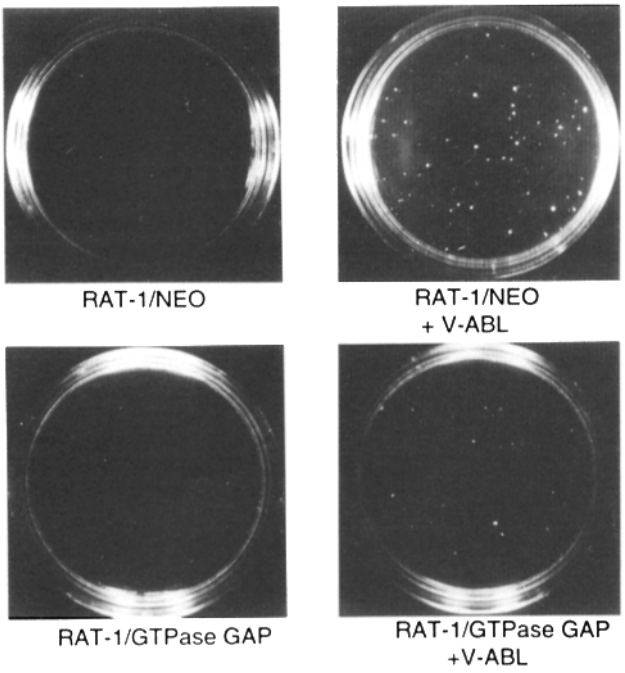

Figure 3. Expression of the Gap C terminus impairs soft agar colony formation by v-Abl. Photographs are shown of representative soft agar plates from v-Abl experiment scored in Fig. 2. Plates seeded with rat-1/Neo cells and rat-1/Gap C terminus cells are shown on the top left and bottom left, respectively. Plates seeded with the same cells after infection with *v-Abl* retrovirus are shown on the top right and bottom right.

because we were unable to introduce the *Gap* C terminus gene into every cell at risk for v-Abl transformation.

Overexpression of Gap C Impairs the Transforming Activity of Bcr-Abl in Hematopoietic Cells. The fibroblast assay is a valuable model system for the transformation by *Abl* oncogenes, but hematopoietic cells are the true target tissue for *Bcr-Abl* in clinical disease. To test for genetic dependence of Bcr-Abl on Ras in this tissue, we used an in vitro bone marrow transformation assay (21). The culture conditions for this assay select for the growth of immature progenitor cells which develop to the pre-B cell stage over a 5–6-wk latent period from an initial inoculum of fresh murine bone marrow. If the bone marrow is exposed to *Bcr-Abl* retrovirus before plating, the culture is overtaken by dense outgrowths of pre-B cells within 2–4 wk. These Bcr-Abl–expressing pre-B cells form tumors at high frequency in syngeneic mice.

To measure the effect of the Gap C terminus on transformation by Bcr-Abl in this assay, it was critical to ensure that every cell at risk for Bcr-Abl transformation also received a *Gap* C terminus gene. This experiment required simultaneous introduction of *Bcr-Abl* and the *Gap* C terminus gene into the same cell using a single retrovirus system. To accomplish this, the *Bcr-Abl* and *Gap* C terminus cDNAs were cloned into a vector previously developed by us for studies of *Bcr-Abl* and dominant negative *Myc* (14). The plasmid was modified by incorporating sequences from the pSRαMSV-tkNeo vector (20) which give higher titers after transient transfection in Cos-7 cells. To control for possible effects of the upstream gene (*Gap* C terminus) on expression of the downstream gene (*Bcr-Abl*), the *Gap* C terminus plus *Bcr-Abl* retrovirus was compared to a symmetrically balanced construct in which the *Neo cDNA* was subcloned into the upstream position (*Neo plus Bcr-Abl*).

After infection with the retroviruses listed in Fig. 4, the nonadherent cells from each plate were counted at 10 and 21 d. Plates with greater than 5×10^5 nonadherent cells per ml were scored positive. The number of positive plates over the total number plated at the 10- and 21-d time points are shown. 87% (13/15) of the plates infected with *Neo plus Bcr-Abl* retrovirus or retrovirus expressing Bcr-Abl alone were transformed within 21 d compared with 0/20 infected with *Gap* C terminus plus *Bcr-Abl*. This result shows that the Gap C terminus blocks the transforming activity of Bcr-Abl in bone marrow cells.

Dominant Negative Ras Inhibits, Whereas Wild-type Ras Stimulates Bone Marrow Transformation by Bcr-Abl. In addition to its role as a negative regulator of Ras, RasGap may also act as a downstream effector of Ras (28). Therefore, it is possible that the suppressive effect of the Gap C terminus on Bcr-Abl transformation might result from interference with a Ras effector pathway. To address this issue, we inhibited Ras function by a different strategy, using a dominant inhibitory Ras mutant containing a serine to asparagine mutation at position 17 (Asn 17 Ras) (27). This mutant shows preferential affinity for GDP over GTP due to improper Mg^{2+} binding and presumably acts as a competitive inhibitor of normal Ras by binding to guanine nucleotide exchange factors (29).

The murine bone experiments described above were repeated using either normal *c-H-Ras* or the dominant negative Asn 17 *Ras* paired with *Bcr-Abl* in the two gene retrovirus system.

VIRUS	TRANSFORMED CULTURES (#plates with >5x10^{5}cells/total # of plates) 10 DAYS	21 DAYS
TK NEO	0/20	0/20
TK c-H-RAS	0/4	0/4
TK BCR-ABL	3/12	9/11
NEO — TK BCR-ABL	0/4	4/4
c-H-RAS — TK BCR-ABL	9/12	10/10
Asn17 RAS — TK BCR-ABL	0/20	0/20
GAP-C — TK BCR-ABL	0/20	0/20

Figure 4. Expression of either the Gap C terminus or dominant negative Ras impairs bone marrow transformation by Bcr-Abl. Mouse bone marrow cells obtained from the femurs and tibias of BALB/c mice were infected with retrovirus expressing the gene combinations shown on the right and seeded into multiple dishes. After 10 and 21 d, the nonadherent cells were counted. Plates with a density >5 × 10^5 cells/ml were scored positive for transformation. The transformation frequency of each retrovirus at 10 d (column *1*) and at 21 d (column *2*) is shown as the number of positive plates over the total number plated.

As with the Gap C terminus experiments, Asn 17 *Ras* blocked the transforming activity of Bcr-Abl. None of the plates (0/20) from bone marrow infected with Asn 17 *Ras* plus *Bcr-Abl* retrovirus scored positive. The titers of all retroviruses were tested indirectly by measuring Bcr-Abl protein expression after infection of NIH3T3 cells. The Asn 17 *Ras plus Bcr-Abl* and *Gap* C terminus plus *Bcr-Abl* retroviruses were of comparable titer to the *Neo plus Bcr-Abl* retrovirus (data not shown).

Interestingly, coexpression of normal c-H-Ras with Bcr-Abl significantly shortened the latency for Bcr-Abl transformation. 75% (9/12) of plates infected with c-H-*Ras plus Bcr-Abl* retrovirus scored positive at 10 d compared with 18% (3/16) of plates from cells infected with *Neo plus Bcr-Abl* retrovirus (0/4) or *Bcr-Abl* retrovirus alone (3/12). This result was not due to an independent effect of c-H-*Ras* because none of the plates from bone marrow infected with c-H-*Ras* retrovirus alone scored positive (0/4), even at 3–4 wk. These experiments demonstrate that inhibition of Ras with a dominant negative mutant blocks Bcr-Abl transformation. The fact that additional normal c-H-Ras accelerates Bcr-Abl transforming activity implies that Ras is limiting for the Bcr-Abl transformation signal.

Discussion

Studies of a number of different receptor and cytoplasmic TKs show a correlation between Ras activation and mitogenic activity (30). However, there have been relatively few direct tests of the requirement for Ras for biological activity (31). For the Bcr-Abl TK, biochemical studies show an elevated ratio of active GTP-Ras versus GDP-Ras in cells expressing Bcr-Abl (15). A Bcr-Abl mutant which fails to activate transcription from a Ras-responsive reporter construct in NIH3T3 cells cannot transform rat-1 fibroblasts (10). Although data such as these support a correlation between Ras activation and transforming activity by Bcr-Abl, they fail to directly test the requirement for Ras in transformation.

In this work we have used a genetic approach to demonstrate directly that Ras is required for the transforming activity of Bcr-Abl. We have impaired Ras function in fibroblasts and in hematopoietic cells using two strategies that affect different sides of the Ras regulatory cycle. Overexpression of the COOH terminal GTPase activating domain of RasGap impairs Ras activity by converting active GTP-bound Ras to inactive GDP-bound Ras. The result is a higher than normal percentage of Ras in the GDP-bound state. The dominant inhibitory Asn 17 Ras mutant acts as a competitive inhibitor of normal Ras for binding to GDP/GTP exchange molecules, thereby preventing normal Ras activation. In both cases, transformation by Bcr-Abl is suppressed.

Our findings are consistent with a recent report (32) showing that the growth of a CML cell line expressing Bcr-Abl is impaired by antisense oligonucleotides directed against *Ras*. One interpretation of these results is that inhibition of Ras leads to generalized growth suppression rather than a specific effect on the Bcr-Abl transformation signal. In fact the Asn 17 Ras mutant, which is widely used by a number of groups to impair Ras activity, is known to suppress normal growth in fibroblasts (27). Our studies of the Gap C terminus mutant in nontransformed cells show that this is not the case. High level expression of Gap C terminus showed no toxic effects on cell growth in fibroblasts or hematopoietic cells (our unpublished observations). The suppressive activity of the Gap C terminus on transformation was specific because it did not impair the transforming activity of a Ras-independent gene, *v-Mos*.

Our findings suggest that Bcr-Abl activates an essential signaling pathway that flow through Ras. Recent biochemical experiments showing Bcr-Ab1 binds to Grb-2 (10, 17) provide a potential mechanism for a Bcr-Abl connection to Ras. Whether Grb-2 is the critical connection to Ras is not clear. Although a Bcr-Abl molecule with a mutation of the Grb-2 binding site (Y177) is no longer able to transform rat-1 fibroblasts, it does have growth stimulatory activity in hematopoietic cells (Goga, A., D. Afar, J. McLaughlin, C. Sawyers, and O. Witte, unpublished observations). These findings indicate there may be additional pathways from Bcr-Abl to Ras, perhaps through other adaptor molecules known to bind Bcr-Abl such as Shc (18) or Crk-L (19). The recent identification of interactions between c-Abl and the Crk SH3 domain provides a potential mechanism for such a pathway (33, 34).

In addition to Ras, Myc is another protein essential for transformation by Bcr-Abl (14). How do Ras and Myc fit together in the Bcr-Abl transformation pathway? Complementation experiments show that overexpression of c-Myc specifically restores transforming activity to a *Bcr-Abl* gene rendered inactive due to a mutation in the phosphotyrosine binding region of its SH2 domain (11). These experiments define a Myc pathway and suggest that the SH2 domain is responsible for a signal to *Myc*, perhaps through its promoter. If the Grb-2 binding site in Bcr-Abl is indeed the primary connection to Ras, the fact that Myc does not complement a Grb-2 binding mutant of Bcr-Abl may indicate that Ras and Myc act independently. Further complementation analysis is required to sort out these issues.

Our findings support accumulating evidence that Ras deregulation is a common theme in many types of human myeloid leukemia. In AML, MDS, and *Bcr-Abl* negative CML, point mutations in *Ras* are common and create a constitutively active protein. Molecular studies of myeloid leukemias which are not associated with *Ras* mutations, are beginning to uncover abnormalities in Ras regulation. Leukemia cells from children with juvenile CML, a disease which phenotypically resembles adult CML but is not associated with a *Bcr-Abl* translocation or with *Ras* mutations, contain mutations in both alleles of *NF-1* (35). Similar to RasGap, NF-1 is a negative regulator of Ras. Loss of function should lead to abnormal Ras activation. Our results suggest that Ras activation is also abnormal in adult CML because of constitutive activation by Bcr-Abl. Through a similar mechanism, it appears likely that the fusion protein in patients with t(5,12) associated CMML may also activate Ras. This translocation fuses the platelet-derived growth factor receptor (*PDGFR-β*) to a novel gene called *Tel* (36). Structural characteristics predict a receptor TK which is no longer regulated by binding to its ligand.

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