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Differential Complementation of Bcr-Abl Point Mutants with c-Myc

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A complementation strategy was developed to define the signaling pathways activated by the Bcr-Abl tyrosine kinase. Transformation inactive point mutants of Bcr-Abl were tested for complementation with c-Myc. Single point mutations in the Src–homology 2 (SH2) domain, the major tyrosine autophosphorylation site of the kinase domain, and the Grb-2 binding site in the Bcr region impaired the transformation of fibroblasts by Bcr-Abl. Hyperexpression of c-Myc efficiently restored transformation activity only to the Bcr-Abl SH2 mutant. These data support a model in which Bcr-Abl activates at least two independent pathways for transformation. This strategy may be useful for discerning signaling pathways activated by other oncogenes.

Cancer is a progressive disease caused by the accumulation of mutations, some of which result in abnormal signal transduction events (*1*). Because oncogenes are mutated forms of normal cellular counterparts, it is conceivable that oncogenesis results from the amplification or alteration of a normal signal, the generation of multiple signals, or a combination of these mechanisms. In this report, we examine signaling in mammalian cells by Bcr-Abl.

The *bcr-abl* chimeric oncogene is generated from a reciprocal translocation between chromosomes 9 and 22, which results in the fusion of *bcr* sequences upstream of the second exon of *c-abl*. Two Bcr-Abl proteins can be generated, P185 and P210, which are most commonly associated with acute lymphocytic and chronic myelogenous leukemias, respectively (*2*).

We developed a strategy to define genetically the minimum number of signals needed for oncogenesis. Point mutations were generated in domains of Bcr-Abl that are expected to participate in connecting its tyrosine kinase signal to downstream effector molecules. If the mutation impaired the transforming activity of Bcr-Abl by uncoupling a critical signaling pathway, then the overexpression of a downstream molecule would be expected to restore transforming activity. If all mutants were rescued by the downstream gene, then the same signaling pathway would likely have been inactivated by the different mutations. Complementation of one point mutant, but not another, would signify that each mutation blocks a different pathway.

Point mutations in the major tyrosine autophosphorylation site of the kinase domain (*3*) and in the tyrosine phosphorylation site at position 177 in the Bcr region (*4*) impair transformation activity of Bcr-Abl. A mutation at position 177 in P185 prevents association with Grb-2 (*4*), an adapter molecule involved in Ras activation (*5*).

We created a point mutation in the phosphotyrosine binding site of the Src–homology 2 (SH2) region of Bcr-Abl. The most highly conserved sequence in the SH2 domain is the FLVRES motif (*6*), where the Arg residue makes direct contact with the phosphate group of a phosphotyrosine moiety (*7*). Mutations in the FLVRES motif of transforming mutants of mouse type IV c-Abl and Src decrease their transformation potential in mammalian cells (*8*). In the FLVRES motif of P185 Bcr-Abl, Arg^{552} was substituted with Leu to create P185 R552L (*9*).

Wild-type (WT) P185, P185 R552L (FLVRES mutant), P185 Y813F (autophosphorylation mutant), and P185 Y177F (Grb-2 binding mutant) were transfected into 293T cells and analyzed by immune-complex kinase assays to assess autophosphorylation activity (Fig. 1). Although the mutation at Tyr^{177} had no effect on in vitro autophosphorylation, the FLVRES mutant and the autophosphorylation mutant had activities one-third and one-half that of WT P185, respectively.

Cells of the rat-1 line expressing transforming forms of the Abl kinase exhibit tyrosine phosphorylation of a protein doublet in the 62- to 65-kD range (*10*). To assess intracellular tyrosine phosphorylation, WT P185 and point mutants were introduced into rat-1 cells by retroviral infection (*10*). The expression of Bcr-Abl in cells was determined 48 hours after infection by protein immunoblotting. Viral titers for different Bcr-Abl variants were equivalent. No change in protein stability was detected in the point mutants as compared to WT P185. Although mutant and WT P185 proteins were expressed in approximately equal amounts, immunoblot analysis with antibodies to phosphotyrosine revealed that cells expressing the FLVRES mutant had severely reduced amounts of intracellular phosphotyrosine (Fig. 1). Although mutant P185 proteins exhibited ty-

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Table 1. Differential rescue of Bcr-Abl point mutants with Myc (*22*).

Retrovirus	Neo colonies			Myc colonies		
	Number	Size	Acidification	Number	Size	Acidification
Neo	0		–	2	S	–
WT P185	92	L	+	135	L	+
P185 R552L*	8	S	–	93	L	+
P185 Y813F†	3	S	–	12	S	–
P185 L552-F813‡	0		–	1	S	–
P185 Y177F§	5	S	–	7	S	–

*FLVRES mutant. †Autophosphorylation mutant. ‡Double mutant. §Grb-2 binding mutant.

rosine phosphorylation, phosphorylation of the p62 and p65 doublet was not detected in cells expressing the FLVRES mutant. In contrast, tyrosine phosphorylation of p62 and p65 in cells expressing the autophosphorylation and Grb-2 binding mutants was indistinguishable from that in cells expressing WT P185. These results suggest that one function of the SH2 region in Bcr-Abl is to regulate substrate recognition and phosphorylation.

The binding of Grb-2 by Bcr-Abl correlates with the transformation of fibroblast and lymphoid cells (*4*). To test whether point mutations distal to the Grb-2 binding site (Tyr^{177}) influenced the ability of Bcr-Abl to interact with Grb-2, we overexpressed different P185 proteins in 293T cells and immunoprecipitated them with antibodies to Abl. The samples were then analyzed by immunoblotting with antibodies to Abl or Grb-2. All P185 variants, with the exception of P185 Y177F, bound to Grb-2 (Fig. 2) (*12*).

The transforming potential of WT P185 was compared to that of mutant P185 molecules in rat-1 fibroblasts, in a soft agar growth assay. Cells of the rat-1 line were infected with P185 retroviruses that contained a cis-linked *neo* gene as a selectable marker. After the selection of infected cells in G418 for 14 days, overexpression of Bcr-Abl was verified by protein immunoblotting. The cells were plated in soft agar, which represents a quantitative measurement of transformation after infection with retrovirus (*10, 13*). The samples were visually inspected for colony formation 2 to 3 weeks after plating.

Wild-type P185 induced both the growth of large colonies (0.5 to 3 mm in diameter) and the acidification of the culture medium (Table 1). Cells expressing the FLVRES mutant failed to show signs of transformation. A small number of colonies (<0.5 mm in diameter) formed, and acidification of the culture medium was not detected. Point mutations in the tyrosine autophosphorylation site (*3*) or in the Grb-2 binding site (*4*) also blocked transformation. Cells expressing WT Bcr-Abl formed 20 times as many colonies as cells expressing the point mutants.

The hyperexpression of the nuclear proto-oncogene *c-myc* synergizes with Bcr-Abl to cause transformation (*13*). This gene is also believed to function downstream of a Bcr-Abl signal, because dominant negative forms of Myc block Bcr-Abl–mediated transformation (*14*). To determine if c-Myc can restore transformation activity to point mutants of Bcr-Abl, rat-1 cells expressing P185 variants were infected with a retrovirus encoding *c-myc* and tested for growth in soft agar assays.

In five independent experiments (Table 1), the hyperexpression of c-Myc increased P185-mediated transformation of rat-1 cells by 50%. Large (>1 mm in diameter) colonies were formed, and the culture medium was acidified. The hyperexpression of Myc did not significantly increase the number of colonies formed in soft agar by cells expressing the autophosphorylation and Grb-2 binding mutants. The colonies that did form were small (<0.5 mm in diameter), and no acidification of the culture medium was observed.

The overexpression of c-Myc restored the transformation phenotype of the FLVRES mutant of Bcr-Abl. The overexpression of Myc induced a 10-fold increase in the number of colonies formed in soft agar by the FLVRES mutant, which ranged in size from 0.5 to 3 mm in diameter. The recovered colonies did not represent a reversion of the FLVRES mutant back to WT P185 (*15*).

The FLVRES mutation appears to couple Bcr-Abl to an alternative pathway that is complemented by Myc hyperexpression. We therefore generated P185 L552-F813, which contains the FLVRES mutation and the autophosphorylation site mutation in the kinase domain. Although active as a kinase (Fig. 1), the double mutant did not transform cells, even in the presence of increased amounts of Myc (Table 1). This mutation at the autophosphorylation site uncoupled the ability of Myc to rescue the transforming activity of the FLVRES mutant.

Our results support the concept that at least two signaling pathways are required for transformation. Complementation by Myc of the FLVRES mutant, but not of other mutants, segregates these pathways. The differential ability of point mutants to bind Grb-2 may separate a signal dependent on autophosphorylation from a signal dependent on Grb-2 binding. However, with our data we cannot rule out the possibility that the signals generated by Grb-2 binding and autophosphorylation of the kinase domain ultimately feed into the same pathway.

An autophosphorylation mutant of the CSF-1 (colony-stimulating factor–1) receptor is rescued for mitogenesis by the overexpression of c-Myc (*16*). It is possible that Myc hyperexpression replaces a similar pathway that is deficient in both the autophosphorylation mutant of the CSF-1 receptor and the FLVRES mutant of Bcr-Abl.

The activity of Ras is an essential component of signaling by many tyrosine kinases (*5, 17*), including Bcr-Abl (*18*). Bcr-Abl interacts directly with Grb-2 (*4, 19*),

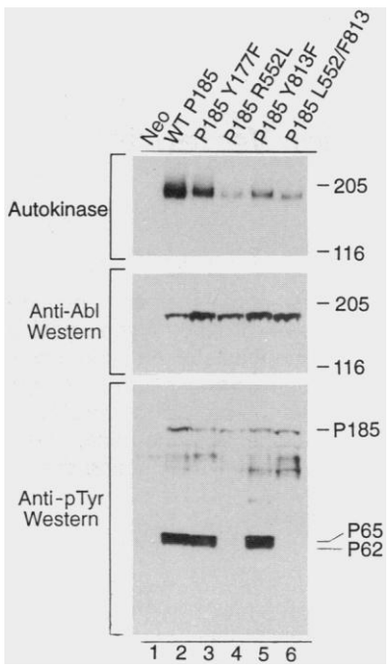

Fig. 1. Protein analysis of Bcr-Abl point mutants. Bcr-Abl proteins were overexpressed in 293T cells (*20*) and analyzed by immune-complex tyrosine kinase assays (*21*). Rat-1 cells were harvested 48 hours after infection and analyzed by immunoblotting with monoclonal antibody to Abl (anti-Abl; pex5) and monoclonal antibody to phosphotyrosine (Anti-pTyr) (PY20 from ICN) (*1*). Molecular size markers are indicated in kilodaltons. The position of P185 Bcr-Abl and the p62 and p65 proteins are indicated. Samples are Neo (lane 1), WT P185 (lane 2), P185 Y177F (Grb-2 binding mutant, lane 3), P185 R552L (FLVRES mutant, lane 4), P185 Y813F (autophosphorylation mutant, lane 5), P185 L552/F813 (double mutant, lane 6).

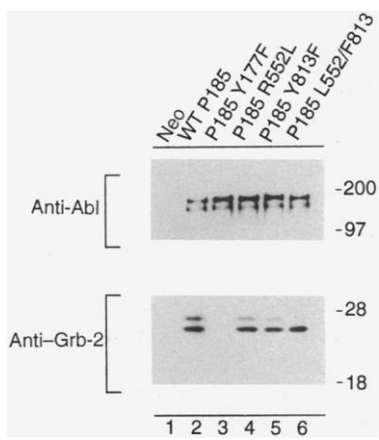

Fig. 2. Grb-2 binding by point mutants of Bcr-Abl. The human cell line 293T was transfected with Bcr-Abl variants by calcium phosphate precipitation. At 48 hours after transfection, the samples were processed and immunoprecipitated with protein A–Sepharose coupled to polyclonal antibodies to Abl (pex5) (*4*). The samples were analyzed by immunoblotting with monoclonal antibodies to Abl (pex5) and monoclonal antibodies to Grb-2 (anti–Grb-2) (Transduction Laboratories, Lexington, Kentucky). Molecular size markers are indicated in kilodaltons. Samples are as in Fig. 1.

and this interaction correlates with transformation (*4*). Point mutants of Bcr-Abl that are defective in transformation still bound Grb-2, indicating that they can activate Ras (*4*, *5*). This activity demonstrates that, whereas Grb-2 binding is necessary for transformation, it is not sufficient. Transformation by the Grb-2 binding mutant was not enhanced by the overexpression of Myc, suggesting that Myc and Ras may operate in different pathways.

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9. The mutation in the FLVRES motif of P185 Bcr-Abl was generated by polymerase chain reaction (PCR)–based mutagenesis. The mutagenic oligonucleotide 5′ primer used to replace Arg^{552} in the SH2 domain of P185 with Leu was 5′-TATCCGCTGAGCAGCGGGATCAATGGCAGCTTCTTGGTGCTTGAGAGTGAGAGCAGTCC-3′, where the underlined sequence replaced the WT codon of CGT. The downstream primer used was 3′-CGTAAACCTCATAACGAAACCTTCGAACGC-5′. Sequences of *c-abl* were used as a template to PCR-amplify the mutant SH2 domain. The PCR product was subcloned into *c-abl* pBluescript (Stratagene) and analyzed by dideoxy DNA sequencing (Sequenase, United States Biochemical) to verify the presence of the point mutation. The FLVRES mutation was then reconstructed into Bcr-Abl by ligation of a Kpn I and Hind III digest of *c-abl* FLVRES mutant to an Eco RI and Kpn I digest of P185 *bcr-abl*. The P185 FLVRES mutant was then subcloned into the pSRαMSVtkneo retroviral vector (*10*) as an Eco RI and Hind III fragment. The autophosphorylation mutant P185 Y813F was generated by the recombination of the 5′ end of P185 *bcr-abl* with a Kpn I and Hind III fragment of P210 *bcr-abl* 1294F (*3*). To generate the double mutant P185 L552 and F813, P185 Y813F was digested with Bsr GI and Hind III and recombined with the 5′ end of P185 R552L.
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22. Soft agar transformation by Bcr-Abl forms was determined as described in (*10*, *13*). Infected cells were grown for 2 days in culture before selection in G418 for 14 days. Cell populations were then superinfected with *neo* retrovirus or human *c-myc* retrovirus (*14*). 48 hours after infection, cells were plated in agar at a density of 5×10^4 cells per 6-cm dish. The samples were plated in duplicate in medium containing fetal calf serum (20%). Colonies equal to and larger than 0.5 mm in size were counted for 2 to 3 weeks after growth in soft agar. The numbers of colonies grown in soft agar represent averages from five independent experiments for cells expressing WT P185. Colonies expressing mutant P185 proteins were averaged from two to three independent experiments. Large colonies (L) represent sizes ranging from 0.5 to 3.0 mm in diameter. Small colonies (S) represent visually detectable colonies ≤0.5 mm in diameter. G418-resistant populations expressing P185 proteins were superinfected with retrovirus containing the *neo* gene or retrovirus containing *c-myc*.

23. We thank A. M. Pendergast for providing the Grb-2 binding mutant of Bcr-Abl (P185 Y177F); S. Smale, D. Black, A. Berk, L. Zipursky, D. Saffran, and L. Cohen for critical review of the manuscript; S. Quan and J. C. White for photography; and J. Shimaoka for assistance in preparation of the manuscript. D.E.H.A. is a fellow of the Medical Research Council of Canada. A.G. is a fellow of the UCLA medical scientist training program. O.N.W. is an Investigator of the Howard Hughes Medical Institute. Supported by the Physician Scientist Award CA 01551 from the National Cancer Institute (C.L.S.) and by grant CA 53867 from the National Institutes of Health (O.N.W.).

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A Dual Embryonic Origin for Vertebrate Mechanoreceptors

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Neuromasts, the mechanoreceptors of the lateral line system of fishes and aquatic amphibians, have previously been thought to develop exclusively from embryonic epidermal placodes. Use of fate mapping techniques shows that neuromasts of the head and body of zebrafish, Siamese fighting fish, and *Xenopus* are also derived from neural crest. Neural crest migrates away from the neural tube in developing vertebrates to form much of the peripheral nervous system, pigment cells, and skeletal elements of the head. The data presented here demonstrate that neuromasts are derived from both neural crest and epidermal placodes.

Neuromasts are sensory structures of fishes and aquatic amphibians that function to detect vibrations in the nearby water, and thereby facilitate schooling, prey capture, and predator avoidance (*1*). They are predominantly arranged in lines on the body surface and head, and together comprise the lateral line system. Neuromasts display a highly conserved structure, with a core of sensory hair cells that are surrounded and underlain by support cells and are covered with a gelatinous cupula (*1*, *2*). Neuromasts evolved in the common ancestor of vertebrates and are retained in lampreys, sharks and rays, fishes, and aquatic amphibians (Fig. 1). Vertebrates have hair cells in the inner ear that are considered homologous to the hair cells of neuromasts by virtue of their similarity in structure and function.

In amphibians, a variety of classical experimental techniques (*3*–*7*) have demonstrated that neuromasts of the head and body develop from epidermal placodes that all originate on the head. Placodes caudal to the otic capsule migrate from head to tail tip, dropping off clusters of cells that differentiate as neuromasts. Although it is assumed that neuromasts develop in the same manner in teleosts and although postotic placode migration has been documented

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