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Complementation by *BCL2* and C-HA-*RAS* Oncogenes in Malignant Transformation of Rat Embryo Fibroblasts

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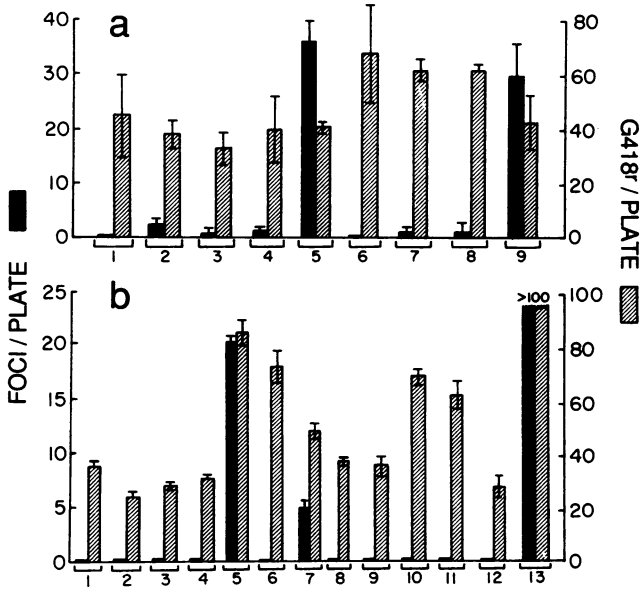
The *BCL2* (B cell lymphoma/leukemia-2) and C-HA-*RAS* oncogenes encode membrane-associated proteins of 26 and 21 kilodaltons, respectively. Although RAS proteins have long been known for their ability to bind and hydrolyze GTP, recent investigations suggest that *BCL2* encodes a novel GTP-binding protein (S. Haldar, C. Beatty, Y. Tsujimoto, and C. M. Croce, Nature [London] 342:195–198, 1989). Cotransfection of *BCL2* and HA-*RAS* oncogenes resulted in morphological transformation of early-passage rodent fibroblasts, rendering these cells tumorigenic in animals and enabling them to grow in semisolid medium. In contrast, cotransfection of *BCL2* with oncogenes that encode nuclear proteins (*E1A* and C-*MYC*) did not produce malignant transformation, whereas HA-*RAS* did complement with these genes. These findings suggest that proteins encoded by oncogenes such as *BCL2* and HA-*RAS*, although having similar subcellular locations and perhaps similar biochemical properties, can regulate distinct complementary pathways involved in cellular transformation.

The *BCL2* gene is commonly rearranged and overexpressed in human B-cell lymphomas (11, 17, 21). This gene encodes two proteins through alternative splicing mechanisms; these proteins have been termed p26-BCL2-alpha and p22-BCL2-beta (18). Although little is known about the BCL2-beta protein, the more abundant 26-kilodalton BCL2-alpha protein has been found primarily in association with intracellular membranes, presumably because of its hydrophobic carboxyl terminus (3, 19). Recently, evidence has been obtained suggesting that the 26-kilodalton BCL2 protein specifically binds GTP (5). If this is confirmed by others, BCL2 would appear to represent a novel low-molecular-weight GTP-binding protein, since it shares little sequence homology with RAS and related proteins.

We have been interested in the biological actions of the BCL2 gene and have previously used gene transfer approaches to demonstrate its growth-promoting effects in human B lymphocytes (14). In this work we have explored the ability of *BCL2* to complement with other oncogenes in the malignant transformation of early-passage rodent fibroblasts isolated from rat embryos (REF cells). Malignant transformation in REF cells can be induced in vitro by transfection of two complementary oncogenes, whereas usually each gene individually is insufficient for transformation (6, 7, 15). We therefore transfected monolayers of REF cells with *BCL2* expression plasmid DNAs in combination with various other oncogenes. To control for variations in transfection efficiencies, oncogene-containing plasmids were always cotransfected with a plasmid conferring G418 resistance (pcD-NEO) (2), and a portion of the transfected cells were cultured with this antibiotic, fixed, and stained to enumerate colonies of G418-resistant cells.

Cotransfection of *BCL2* (pSV2-BCL2-alpha) (13) and *RAS* (pEJ6.6) (7) plasmids resulted in the development of numerous foci within monolayers of REF cells (Fig. 1a). Like many malignant cells in culture, these REF cells transfected with *BCL2* plus *RAS* had a spindle shape and increased refractility and grew in a disorganized manner (Fig. 2c). However, unlike cells transfected with the classical complementary pair of *MYC* and *RAS* oncogenes (data not shown), cells transfected with *BCL2* plus *RAS* did not pile up appreciably in cultures and did not round up and grow loosely attached to the culture dish. In contrast to foci resulting from cotransfection of C-*MYC* and C-HA-*RAS* plasmids, the *BCL2*-plus-*RAS* foci took about 1 week longer to form and remained relatively small in diameter throughout the culture period. When recovered from monolayers with the use of cloning cylinders and expanded in vitro to provide enough cells for injections into irradiated athymic mice (5×10^6 cells per injection), however, morphologically altered cells trans-



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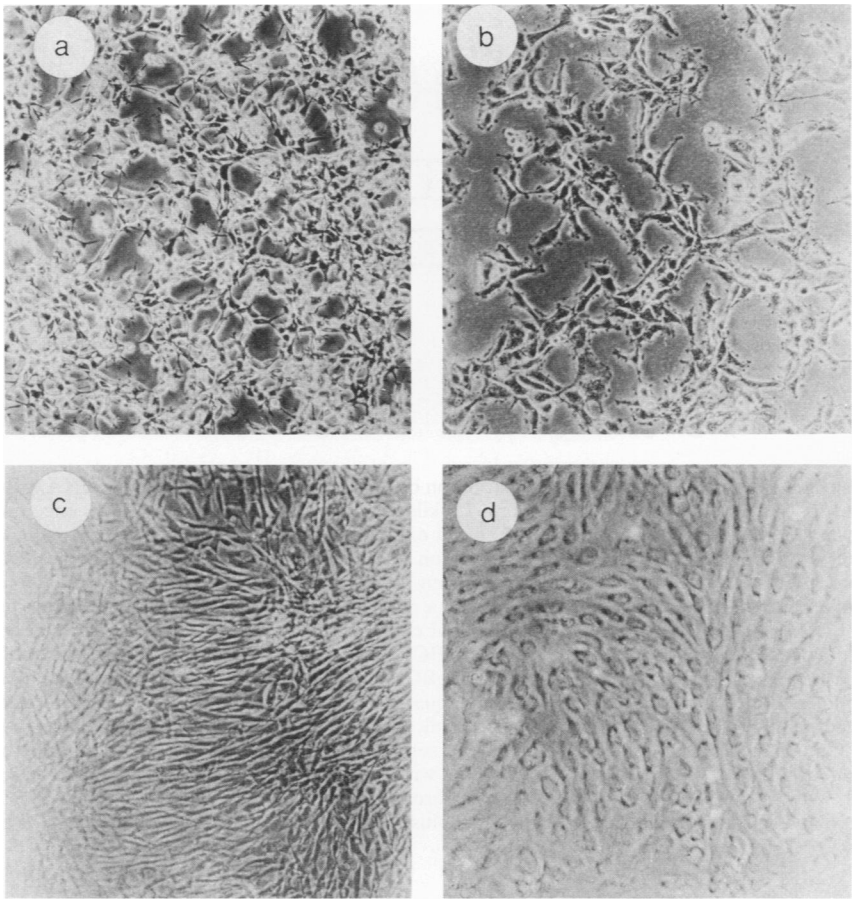

FIG. 2. Morphology of transfected REF cells. REF cells were photographed by using a phase microscope equipped with a 35-mm camera. Shown are photographs of hygromycin-resistant, morphologically transformed cells from cotransfections of pBCL2-alpha/HYG and pT24/NEO (Fig. 1b) at higher cell density and ×40 magnification (a) and at lower cellular density and ×100 magnification (b); abnormal cells (focus) resulting from cotransfections of pSV2-BCL2-alpha and pEJ6.6 (Fig. 1a) at ×40 magnification (c); and normal untransfected REF cells at ×100 magnification (d).

fected with *BCL2* plus *RAS* gave rise to tumors within 2 weeks (five of five injections), like their counterparts transfected with C-*MYC* plus *RAS* (data not shown). By comparison, injections of normal REF cells or of cells recovered from cultures transfected with *BCL2* or *RAS* alone did not produce tumors (three injections each) (data not shown).

These initial findings suggesting complementation of *BCL2* and C-HA-*RAS* were unexpected, since (at least for cellular oncogenes) pairs of complementary oncogenes have usually consisted of a nuclear protein with DNA-binding capacity (such as C-*MYC*) and a nonnuclear protein usually having biochemical properties suggestive of a role in transmembrane signal transduction (such as *RAS*) (6, 7, 15). To more readily assess the role of *BCL2* in complementation with C-HA-*RAS*, therefore, we prepared *BCL2*-alpha and *BCL2*-beta expression constructs in a plasmid, p290, that contains a resistance gene for hygromycin (14) (Fig. 3a) and thus allows rapid determination of the presence of *BCL2* plasmids in transfected REF cells by using antibiotic selection. As a negative control, the pB-Trun/HYG plasmid was also prepared, in which a portion of the *BCL2* open reading frame was interrupted. This control plasmid failed to produce stable transcripts based on RNase protection assays (data not shown).

When these *BCL2*/*HYG* plasmids were cotransfected into REF cells with plasmids containing a G418 resistance gene and either the C-HA-*RAS* oncogene from the T24 human bladder carcinoma (pT24/NEO) or an *E1A* gene (15) from adenovirus (pE1A/NEO), and the cells were subsequently

FIG. 1. Focus formation induced by cotransfection of *BCL2* and *RAS* oncogenes. REF cells were transfected as described previously (13) with various plasmid DNAs (2, 4, 7, 10, 13), individually or in combination. Transfected cells were grown with or without G418 for 3 weeks, and then G418-resistant colonies and foci of morphologically transformed cells were enumerated (mean ± standard deviation of two determinations). (a) Transfections were performed with pcDNEO together with the following plasmids: (1) pSV2-gpt; (2) pSV2-BCL2-alpha; (3) pSV2-C-MYC-1; (4) pEJ6.6; (5) pSV2-BCL2-alpha plus pEJ6.6; (6) pSV2-BCL2-alpha-AS plus pEJ6.6; (7) pSV2-gpt plus pEJ6.6; (8) pSV2-BCL2-alpha plus pSV2-C-MYC-1; and (9) pSV2-C-MYC-1 plus pEJ6.6. (b) In these experiments, the foci are G418-resistant colonies with transformed morphology (see text). Transfections were performed with the following plasmids: (1) pBCL2-alpha/HYG plus pcD-NEO; (2) pB-Trun/HYG plus pcD-NEO; (3) pE1A/NEO; (4) pT24/NEO; (5) pBCL2-alpha/HYG plus pT24/NEO; (6) pB-Trun/HYG plus pT24/NEO; (7) pBCL2-beta/HYG plus pT24/NEO; (8) pBC-140 plus pT24/NEO; (9) p290 plus pT24/NEO; (10) pBCL2-alpha/HYG plus p290-C-MYC(2,3) plus pcD-NEO; (11) pBCL2-alpha/HYG plus pE1A/NEO; (12) pBCL2-beta/HYG plus pE1A/NEO; and (13) pE1A/NEO plus pT24/NEO.

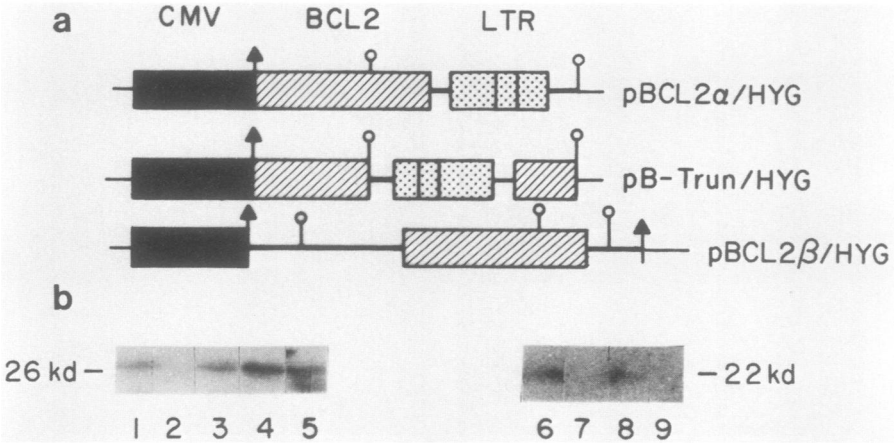

FIG. 3. Expression of pBCL2/HYG in REF cells. (a) Construction of *BCL2* expression plasmids. A cDNA (pB4) containing the complete open reading frame for the 26-kilodalton (kd) BCL2 protein or a 7.8-kilobase-pair genomic clone (p18-214) encoding the 22-kilodalton BCL2-beta protein (▨) (18) was inserted into the *Hin*dIII site (▲) of an expression plasmid, p290 (a kind gift of B. Sugden). The p290 vector contains a strong promoter-enhancer from the immediate-early region of cytomegalovirus (CMV) for driving *BCL2* expression (■), as well as a hygromycin resistance gene under the control of sequences from the thymidine kinase gene of herpes simplex virus (data not shown). For termination-polyadenylation, a *Bam*HI fragment (♀) containing a portion of the pB4 cDNA and the 3′ long terminal repeat (LTR) from Moloney leukemia virus (▩) was excised from a *BCL2* retroviral construct (Reed et al., in press) and cloned in both orientations into *Bam*HI-cleaved and phosphatased p290-pB4 to create the plasmids pBCL2-alpha/HYG and pB-Trun/HYG. (b) Immunoblot analysis. REF cell clones were analyzed by Western immunoblotting for expression of pBCL2/HYG plasmids by using an antiserum prepared against a synthetic peptide corresponding to amino acids 20 to 34 of BCL2 (5, 14). Equal amounts of total protein (150 μg) were size fractionated in sodium dodecyl sulfate–12% polyacrylamide gels and transferred to nitrocellulose. Autoradiograms represent a 1-day exposure of ^{125}I-labeled protein A-treated blots to X-ray film. Incubation of blots with preimmune serum produced no bands in the region of the BCL2 protein (data not shown). Lanes: 1, *E1A*-immortalized cells subsequently transfected with pBCL2-alpha/HYG and selected in 50 μg of hygromycin per ml; 2 and 9, cells transfected with pE1A/NEO plus pT24/NEO; 3 through 5 pBCL2-alpha/HYG-plus-pT24/NEO-transfected clones with transformed morphology; 6 and 8, transformed pBCL2-beta/HYG-plus-pT24/NEO clones; 7, pBCL2-beta/HYG-plus-pT24/NEO clone with normal morphology.

subjected to G418 selection, only co-transfections of *BCL2* plasmids with pT24/NEO produced morphologically transformed cells (Fig. 1b). The pE1A/NEO plasmid, which, like C-*MYC*, encodes a nuclear DNA-binding oncoprotein, failed to complement with *BCL2* (but, as expected, did complement with C-HA-*RAS*). Interestingly, the transformed G418-resistant colonies resulting from cotransfection of pBCL2/HYG plasmids with pT24/NEO displayed classical transformed morphology similar to that observed when cells were cotransfected with plasmids containing *RAS* and either C-*MYC* or *E1A* (Fig. 2a and b) (data not shown). Thus, contrasting these findings (Fig. 1b) with the results of the previous experiment (Fig. 1a) suggests that normal neighboring cells in culture maintained without G418 may have suppressed the transformed phenotype of the cells transfected with *BCL2* plus *RAS* but not that of REF cells cotransfected with *RAS* plus *E1A* or *RAS* plus *MYC* (6, 16).

Because the frequency of transformed G418-resistant colonies was relatively low for REF cells cotransfected with pT24/NEO and the pBCL2/HYG plasmids (20 to 25% for *BCL2*-alpha and 10 to 15% for *BCL2*-beta), we further characterized these cells with regard to hygromycin resistance to determine which G418-resistant clones contained pBCL2/HYG plasmids. Several G418-resistant colonies resulting from *BCL2* plus *RAS* transfections (both transformed and normal morphology) were isolated with the use of cloning cylinders and individually passaged in vitro (Table 1). REF cells transfected with *BCL2* plus *RAS* and displaying transformed morphology survived better in long-term cultures than did their normal-morphology counterparts (Table 1). These *BCL2*-plus-*RAS*-transfected clones with transformed morphology also displayed more rapid doubling times in culture and grew to densities 5 to 7 times higher than *BCL2*-plus-*RAS*-transfected cells with normal morphology (data not shown).

When these REF clones capable of long-term growth in culture were next subjected to selection in hygromycin, *BCL2*-plus-*RAS*-transfected clones having transformed morphology were, without exception, hygromycin resistant. Nontransformed clones isolated from the same culture dish were sensitive to hygromycin in all cases but one (Table 1). Examination of several of these hygromycin-resistant *BCL2*-plus-*RAS*-transfected clones for expression of their pBCL2/HYG plasmids by immunoblotting (Fig. 3) demonstrated a clear correlation between production of high levels of BCL2 proteins and malignant transformation through complementation with an activated *RAS* gene. The one hygromycin-resistant pBCL2-beta/HYG+RAS clone with normal morphology produced much less BCL2-beta protein (Fig. 3, lane 7) than did the other hygromycin-resistant clones with transformed morphology. Furthermore, examination of several additional nontransformed hygromycin-sensitive cells, including those transfected with *E1A*, *RAS*, *BCL2*, or various combinations of these plasmids, revealed no detectable BCL2 protein (data not shown).

Several REF cell clones that grew in long-term cultures were then functionally evaluated for the ability to form colonies in semisolid medium and tumors in syngenic rats, revealing complete correlation between morphological transformation and malignant biological behavior (Table 1). Because none of the five G418-resistant REF clones that we randomly picked from cultures cotransfected with *E1A* and *BCL2*-alpha plasmids were hygromycin resistant (presumably reflecting the absence of the pBCL2-alpha/HYG plas-

TABLE 1. Characterization of transfected REF cell clones

Plasmids	Morphology[a]	No. with long-term growth/total no.[b]	No. Hyg[r]/total long-term no.[c]	No. with anchorage-independent growth/ total no.[d]	No. of rats with tumors/total no. injected[e]
1. pBCL2-alpha/HYG + pcDNEO	N	4/31	4/4	0/2	0/4
2. pB-Trun/HYG + pcDNEO	N	0/22			
3. pT24/NEO	N	4/10	NA[f]	0/2	0/4
4. pE1A/NEO	N	9/10	NA	0/2	0/9
5. pBCL2-alpha/HYG + pT24/NEO	N	4/23	0/4	0/2	0/4
6. pBCL2-alpha/HYG + pT24/NEO	T	7/11	7/7	6/6	19/21
7. pBCL2-beta/HYG + pT24/NEO	N	2/7	1/2	0/2	0/2
8. pBCL2-beta/HYG + pT24/NEO	T	2/4	2/2	2/2	4/5
9. pBCL2-alpha/HYG + pE1A/NEO	N	15/17	0/5	ND[g]	ND
10. pE1A/NEO → pBCL2-alpha/HYG	N	NA		No	0/8
11. pB-Trun/HYG + pT24/NEO	N	3/11	2/3	0/2	0/2
12. pE1A/NEO + pT24/NEO	T	ND	NA	Yes	4/6

[a] REF cells transfected with various combinations of plasmids were grown in the presence of 400 μg of G418 per ml. N, Normal morphology; T, transformed morphology.

[b] Individual G418-resistant colonies (both normal and transformed morphology) were isolated and cultured for 3 months to assess their potential for long-term growth in vitro. Any cultures experiencing crisis were discarded and scored as negative for long-term growth. Note that all of the oncogenes examined were capable of immortalizing REF cells, albeit with markedly different efficiencies (*E1A* > *RAS* > *BCL2*).

[c] Clones that survived in long-term cultures were tested for resistance to 50 μg of hygromycin per ml.

[d] Shown is the fraction of clones exhibiting high-efficiency colony formation in 0.3% agar-containing medium. Growth in semisolid medium was also assessed for *E1A*-plus-*BCL2*-alpha- (row 10) and *E1A*-plus-*RAS* (row 12)-transfected cell lines. Positive cell lines and clones formed colonies at ≥30% efficiency; negative cell lines formed colonies at ≤5% efficiency.

[e] For tumorigenicity experiments, 10^6 cells were injected subcutaneously into 3-week-old rats. Several independent clones were injected. Three injections of each of the *BCL2*-alpha/*RAS*-transformed clones and two or three injections of the *BCL2*-beta/*RAS*-transformed cells were performed. Hygromycin-resistant cells were used for both injections in rows 7 and 11. Note that because rows 10 and 12 refer to cell lines, these represent several independent injections (10^6 cells) and not individual clones. All tumors formed within 2 weeks. In some cases, tumors were analyzed by Southern blotting, confirming the presence of appropriate plasmid DNAs (data not shown).

[f] NA, Not applicable.

[g] ND, Not done.

mid) (Table 1, row 9), we transfected pE1A/NEO-containing REF cells with pBCL2-alpha/HYG, selected stable transfectants with hygromycin, verified expression by immunoblotting (Fig. 3, lane 1), and used these *E1A*-plus-*BCL2*-alpha-transfected cells for comparisons with *RAS*-plus-*BCL2*-alpha-transfected clones (Table 1, row 10). The introduction of *BCL2* into these cells that had been previously immortalized by *E1A* had no effect on their morphology (data not shown) or on their ability to display anchorage-independent growth or tumorigenicity (Table 1). Thus, *BCL2* clearly does not cooperate with *E1A* in these cells. These findings, showing that *BCL2* belongs to a different complementation group of oncogenes (as well as our previous results with NIH 3T3 cells demonstrating morphological transformation of these cells by *RAS* but not by *BCL2* oncogenes [13]), indicate important qualitative differences in BCL2 and RAS oncoproteins, notwithstanding their possible biochemical similarities (5).

Previous studies have shown that either sequentially transfecting REF cells twice with the T24-*RAS* oncogene or overexpressing T24-*RAS* via a retroviral long terminal repeat can result in malignant transformation when neighboring normal cells are removed by antibiotic selection (6, 16). It is unlikely, however, that the observed complementation of *BCL2* and *RAS* was attributable to the strong promoter-enhancer associated with the *BCL2* expression plasmids, since cotransfections of C-HA-*RAS* plasmids (pEJ6.6 and pT24/NEO) with several control plasmids (pSV2-BCL2-alpha-AS [antisense *BCL2*], pB-Trun/HYG [Fig. 3], pSV2-gpt [guanine phosphoribosyltransferase], and pBC140 [has long terminal repeat and cytomegalovirus enhancers]) (4, 10, 13) that contain potent enhancers did not result in transformation (Fig. 1). Because recent findings have suggested that *BCL2* encodes a GTP-binding protein (5), however, the combined expression of *BCL2* and C-HA-*RAS* may in some ways be functionally equivalent to overexpressing *RAS* in this transformation assay. In this regard, it is intriguing to speculate that fibroblasts may normally express genes that encode GTP-binding proteins and that (like *BCL2*) can regulate transformation pathways complementary with *RAS*. If this were true, one could imagine an explanation for the *RAS* overexpression data (6, 16) wherein production of RAS oncopeptides at extremely high (nonphysiological) levels substitutes for these other GTP-binding proteins, thereby activating both the *RAS* and the complementary pathways.

In contrast to our findings with REF cells, we and others have previously observed synergistic actions of *BCL2* and C-*MYC* oncogenes in lymphoid cells with regard to in vitro growth and tumor formation (12, 20; J. Reed, M. Cuddy, S. Haldar, C. Croce, P. Nowell, D. Makover, and K. Bradley, Proc. Natl. Acad. Sci. USA, in press). Although the mechanisms for this tissue specificity of oncogene complementation are unknown, a similar phenomenon has also been observed previously for *ABL* oncogenes, for which complementation with *MYC* was found in fibroblasts (9) but not in lymphocytes (1). Further investigations will therefore focus on the molecular basis for the differential actions of *BCL2* in various cellular lineages.

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