Mechanisms of Oncogene Cooperation: Activation and Inactivation of a Growth Antagonist

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Gene transfer experiments have defined limitations with regard to the ability of individual oncogenes to transform cultured cells to a tumorigenic state. The stable transformation of REFS2 cells by either the \textit{ras} or \textit{sia} oncogenes requires the continuous expression of a second collaborating oncogene, such as adenovirus-5 E1A or SV40 large T-antigen. Our studies suggest that the function of the nuclear collaborators is to antagonize dominant growth controls which limit the ability of REFS2 cells to proliferate in response to mitogenic stimuli.

Introduction

Oncogenes are genes implicated in carcinogenesis by virtue of their associations with oncogenic viruses and tumor-specific chromosome abnormalities and by their ability to transform cultured cells to a tumorigenic state (1,2). Mutations and virus associations enable oncogenes to facilitate autonomous cell growth either by increasing the expression of activities that promote cell proliferation or by interfering with controls that normally serve to restrict cell growth. As carcinogenesis is generally believed to be a multistep process, malignant phenotypes may frequently require multiple alterations affecting several levels of growth control (3). This is supported by gene-transfer experiments in which two or more oncogenes are required to transform normal cells to a malignant state (4–6).

Collaborations between oncogenes indicate that regulatory mechanisms that preclude transformation by one oncogene can be circumvented by a second oncogene. This implies that certain oncogenes actively influence the way that cells respond to other oncogenes. Gene transfer experiments indicate that expression of an activated ras oncogene is sufficient to transform cells from a variety of established cell lines (7–9). In contrast, primary and early passage rodent cells are transformed by ras oncogenes only at very low frequencies unless cotransfected with collaborating genes such as E1A or \textit{myc} (9,10).

Several observations suggested that in \textit{vitro} establishment is a necessary prerequisite to transformation by ras oncogenes. First, the susceptibility of normal hamster cells to transformation by ras coincides with \textit{in vitro} establishment (9). Second, genes such as E1A, p53, SV40 large T-antigen, and \textit{myc}, which collaborate with ras to transform primary cells, also facilitate immortalization (11–15). Finally, mutational analysis of E1A revealed a tendency for mutations that affect \textit{in vitro} establishment functions to also influence the ability of E1A to collaborate with ras in the transformation of primary cells (16,17). However, some E1A genes are defective in immortalization but are nevertheless able to collaborate with ras in transformation (17–19).

Though informative, the use of primary cells in transformation studies has several limitations. First, a variety of factors including variations in cell type, levels of ras expression, inhibitory effects of normal cells, and other environmental factors can influence transformation by ras alone (20–24). Second, the fact that immortalization appears to be a necessary prerequisite for oncogenic transformation \textit{in vitro} may simply reflect difficulties imposing a transformed phenotype over a dominant commitment to senescence. Finally, problems working with nonestablished cell clones complicate any assessment of the phenotypic effects of ras alone. As a result, the mechanisms responsible for oncogene cooperation in nonestablished cells have been difficult to analyze.
We have examined oncogene interactions using an established line of Fisher rat embryo fibroblasts, REF52, which, unlike most established lines, is not stably transformed by activated ras oncogenes. Instead, transformation of REF52 cells by ras strictly requires the presence of a collaborator, such as adenovirus E1A or SV40 large T-antigen (25). The transformation properties of the REF52 line indicate that the role of the viral oncoproteins extends beyond that of promoting establishment and suggests that while immortality may be a necessary prerequisite for transformation by ras, it is not sufficient. While other established cell lines resist transformation by ras (14,26), the REF52 line provides the clearest evidence that oncogenes can complement in a genetic sense to elicit a malignant phenotype.

**Multistep Transformation of REF52 Cells**

In earlier studies, REF52 cells were transduced with T24-Ha-ras linked to the neomycin-resistance gene and clones were selected in G418. The vast majority of the resistant clones were morphologically indistinguishable from the parental line, unable to grow in soft agar, not tumorigenic in nude mice, and expressed low levels of p21T24Ha-ras (30% of the level of endogenous Ha-ras p21). While in 1000 clones was morphologically transformed, these rare ras-only transformants experienced morphological crisis and ceased growing. p21T24Ha-ras levels in these abortive transformants were extremely high (greater than 100-fold higher than the endogenous Ha-ras p21), indicating that even high levels of the activated ras oncoprotein are not sufficient to induce stable transformation in the absence of a collaborating oncogene (25).

In contrast, cells transduced with T24Ha-ras/neob and either adenovirus E1A or SV40 large T-antigen, gave rise to transformed neo-resistant colonies (25,27). While p21T24Ha-ras levels in these transformed clones varied considerably, ranging from levels 2- to 10-fold greater than the endogenous p21c-Ha-ras, all of the transformed clones tested formed colonies in soft agar and induced rapidly lethal tumors in nude mice. These studies indicate that relatively low levels of the oncogenic p21 are sufficient to transform in the presence of a collaborator and that the collaborating oncogene supplies activities that are not duplicated by increased ras expression.

Conditional transformation of REF52 cells has also been achieved by cotransferring a temperature-sensitive allele of SV40 large T-antigen (tsA58) and T24 Ha-ras (27). Thus, two-thirds of the clones transfected with T24 Ha-ras and tsA ceased growing, arresting predominantly in G2 or late S-phase, when transformed from a permissive to a nonpermissive temperature for T-antigen expression. p21T24 Ha-ras levels were unaffected by the temperature shift. This study indicates that transformation by ras requires continuous expression of a collaborating oncogene, and suggests that E1A and SV40 large T-antigen may enable ras to transform REF52 cells by circumventing cellular responses to ras which inhibit cell proliferation.

**Oncogenic p21 Induces Quiescent REF52 Cells to Proliferate**

To investigate early phenotypic effects of ras, activated and normal forms of p21 were introduced into serum-arrested REF52 cells using the bead-loading method (28). The method utilizes small (75–500 μm) glass beads to mechanically disrupt the plasma membrane to an extent that allows proteins to enter cells without significantly affecting cell viability. p21Ha-ras proteins were purified from insect cells infected with baculovirus vectors expressing high levels of posttranslationally modified T24 and c-Ha-ras p21s. As shown in Figure 1, T24Ha-ras was observed 12 to 24 hr after exposure to bead loaded p21T24Ha-ras. At concentrations greater...
than 1 mg/mL, p21T24Ha-ras also induced morphological transformation as well as stimulating DNA replication.

Similar results have been reported using microinjected p21 (29); thus, although resistant to transformation, REF52 cells are mitogenically stimulated by introduction of p21T24Ha-ras. In this regard, REF52 cells respond to oncogenic p21 in a manner not unlike cells that are susceptible to transformation (29,30). As ras is thought to transduce signals from growth factor receptors, controls that prevent ras from transforming REF52 cells would appear to act at a level subsequent to or independent of a mitogenic response to ras.

E1A Enables the sis Oncogene to Transform REF52 Cells

Similar growth controls may also regulate transformation of REF52 by c-sis, the B chain of the platelet-derived growth factor (PDGF) (21,22). Transformation by sis is thought to result from autocrine stimulation resulting from binding to the growth factor to PDGF receptors.

Quiescent REF52 cells were stimulated to enter S phase by recombinant p28c-sis, indicating that these cells have receptors for PDGF (Fig. 2). In addition, p28c-sis induced, with expected kinetics, the transcription of several growth factor-induced early response genes (i.e., myc, fos, and JE), suggesting that the signal transduction pathway from the cell surface to the nucleus is intact (data not shown). However, when REF52 cells are cotransfected with c-sis and the gene for neomycin resistance, all of the resistant colonies were distinguishable from REF52 cells transfected with the neomycin-resistance gene alone. In each case, similar numbers of neo-resistant colonies were obtained, suggesting that the sis gene was not toxic to REF52 cells. E1A, introduced together (Table 1) or in a secondary transfection (data not shown), enabled c-sis to transform REF52 cells to anchorage independence.

These results suggest that the ability of cells to be mitogenically stimulated by sis growth factor, while perhaps necessary, was not sufficient for stable transformation. As with ras, E1A appears to circumvent controls that prevents sustained exposure to a mitogenic stimulus from transforming.

Collaborating Oncogenes Have Complementary Effects on Cellular Responses to Growth Factors

Since oncogenic transformation of REF52 cells can require the activities of two oncogenes acting in concert, these cells provide an opportunity to study the biological effects of individual oncogenes in the absence of secondary phenotypic changes resulting from transformation. REF52 cells expressing low levels of activated Ha-ras p21 (T24REF) are morphologically normal and fail to form either colonies in soft agar or tumors in nude mice. However, these cells express functional ras oncoproteins, since E1A and SV40 large T-antigen each efficiently transform when transfected subsequently (clone N6) (25).

When transferred to serum-free media, T24REF (clone RN14) cells cease growing with kinetics indistinguishable from the neo-resistant REF52 control (clone N6) (Fig. 3). Nevertheless, a marked difference was evident between the cell lines upon restimulation by individual mitogens. As shown in Figure 2, the presence of even low levels of activated p21 interfered with the ability of REF52 cells to progress into S phase upon stimulation by phorbol 12-myristate 13-acetate, epidermal growth factor, PDGF or PDGF and epidermal growth factor together. The response to serum, however, was unaffected or even moderately enhanced by T24Ha-ras.

Cell lines individually expressing either E1A or SV40 large T antigen, although morphologically altered, were not transformed, as assessed by their failure to grow in soft agar or from tumors in nude mice. However, as shown in Figure 2, the presence of either SV40 large T antigen or E1A in REF52 cells allows for extended proliferation in the absence of serum growth factors. Thus, there is an apparent functional complementation: ras inhibits proliferative responses to mitogenic stimuli, while E1A and large T each enable REF52 cells to proliferate in the absence of mitogenic stimuli. In other words, REF52 cells appear to possess growth controls
which limit the ability of the cells to proliferate in response to mitogenic stimuli. These controls are activated by ras and are circumvented by E1A and SV40 large T-antigen.

**A Dominant Antagonist Regulates Transformation by ras and E1A**

A series of cell fusion experiments were performed to determine whether the growth controls that prevent ras from transforming REF52 cells are dominant or recessive. In principle, REF52 could differ from other established rodent lines, which are transformed by ras alone, in one of two ways: a) REF52 cells lack activities that are required for transformation by ras, and E1A supplies the missing cellular functions; b) REF52 cells possess an antagonist that prevents ras from transforming, and E1A inactivates of circumvents the antagonist. In order to distinguish between these two possibilities, cell lines, shown in Table 2, containing either hygromycin or neomycin resistance markers, were fused using polyethylene glycol and selected in the presence of both antibiotics (33). Hybrid colonies were pooled and plated in soft agar to assess whether any expressed transformed phenotypes (Table 3).

When NRK cells transformed by ras were fused with REF52 cells, 20-fold fewer colonies were obtained than when normal NRK cells were fused with REF52. Furthermore, the hybrid colonies obtained were morphologically normal and failed to produce any soft agar colonies, indicating that transformation of NRK cells by ras was suppressed by fusion with REF52 cells. In contrast, the fusion of ras-transformed NRK cells with E1A-expressing REF52 cells yielded transformed colonies, which formed colonies in soft agar with a frequency of 4%. Similar results were obtained with Rat2 cells were substituted for NRK cells (Table 3). Thus, the growth controls that prevent ras from transforming and act to inhibit proliferation in the presence of ras are dominant, trans-acting, and are either circumvented or negated by function(s) of E1A.

Further evidence that REF52 cells possess a dominant antagonist of transformation has come from the analysis of spontaneous transformants induced by activated ras oncogenes. We pooled 10^6 to 10^7 ras transformants and generated resistance colonies isolated after transfecting REF52 cells with T24Ha-ras, and 5 × 10^6 cells were injected into nude mice. Tumors arose within 4 to 6 weeks, and the cell lines derived from each tumor were
re-established in culture and used in fusion experiments (Table 4). Fusions between tumor cells and the parental REF52 cells failed to produce transformed hybrids, whereas fusions between tumor cells and NRK or Rat2 lines yielded transformed colonies which grew in soft agar. In addition, fusions between some of the tumor lines and REF52 cells expressing E1A produced transformed hybrids, indicating that the antagonist was neutralized by E1A. This series of fusions suggest that the ras-induced tumors arose as a result of the loss of antagonist functions which normally prevent transformation and not from the activation of cellular functions analogous to E1A.

While the biochemical mechanisms are yet unknown, this genetically defined antagonist resembles an oncogene, a gene whose functional loss leads to transformation. This is provocative, since the ability of E1A proteins to bind the retinoblastoma gene product, p105RB, is tightly linked to E1A activities that enable ras to transform primary baby rat kidney cells (34–36). This raises the possibility that p105RB is a component of the transformation antagonist of REF52 cells. If so, one would expect to observe differences in the levels of p105RB in normal REF52 cells, and ras-transformed REF52 tumor cells. However, the eight ras-induced tumor lines examined thus far maintain a normal number of intact Rb genes as well as unaltered levels of Rb message. These results do not exclude the possibility

### Table 2. Cell lines used as fusion partners.*

| Cell line         | Plasmid  | Transfected gene(s) | Selection  | Morphology | Soft agar plating efficiency |
|-------------------|----------|---------------------|------------|------------|-----------------------------|
| REF52.H1          | pY3      | hph                 | hyg<sup>B</sup> | Normal    | <0.01                        |
| REF52.E1aH1       | p1Ahgm   | E1A, hph            | hyg<sup>B</sup> | Normal    | <0.01                        |
| Rat2.H1           | pY3      | hph                 | hyg<sup>B</sup> | Normal    | <0.01                        |
| Rat2.G1           | Homer6   | neo                 | neo<sup>B</sup> | Normal    | <0.01                        |
| Rat2.T24G3        | pT24neo  | ras, neo            | ras, neo   | 34         |
| NRK49F.H1         | pY3      | hph                 | hyg<sup>B</sup> | Normal    | 0.2                          |
| NRK49F.T24G3      | pT24neo  | ras, neo            | neo<sup>B</sup> | Transformed | 10                          |
| NRK49F.T24G3      | pT24neo  | ras, neo            | neo<sup>B</sup> | Transformed | 3                           |
| NRK49F.T24G3      | pT24neo  | ras, neo            | neo<sup>B</sup> | Transformed | 0.6                         |
| N5 (REF52)        | pKOneo   | neo                 | neo<sup>B</sup> | Transformed | 5                           |
| RT1               | pT24neo  | ras, neo            | neo<sup>B</sup> | Transformed | 1                           |
| RC6T1             | H06T1    | ras, neo            | neo<sup>B</sup> | Transformed | 3                           |
| RC6T2             | H06T1    | ras, neo            | neo<sup>B</sup> | Transformed | 0.6                         |
| 15-26-4           | H06T1    | ras, neo            | neo<sup>B</sup> | Transformed | 5                           |

*Cells were fused with polyethylene glycol as described (33). After 9 to 12 days of selection in media containing 400 µg/ml G418 and 100 µg/ml hygromycin B, hybrid colonies were counted, trypsinized, and plated in 0.37% soft agarose. The soft agar plating efficiency refers to the percent of cells forming viable colonies after 3 weeks. NRK49F.T24G3 and Rat2.T24G1 are NRK49F and Rat2 cell lines transformed by T24 Ha-ras, respectively. Means and SEs for at least two experiments are presented.

### Table 3. Resistance of REF52 cells to transformation by ras is dominant over susceptibility to transformation.*

| Neo<sup>B</sup> parent × Hyg<sup>B</sup> parent | Hybrids/10<sup>6</sup> cells fused | Soft agar plating efficiency |
|-----------------------------------------------|----------------------------------|------------------------------|
| NRK49F.T24G3 REF52.H1                        | 11 ± 5                           | <0.003                       |
| NRK49F.H1                                    | 211 ± 14                         | 4 ± 3                        |
| Rat2.H1                                      | 209 ± 7                          | 4 ± 4                        |
| REF52.E1aH1                                  | 28 ± 15                          | 2 ± 1                        |
| Rat2.T24G3                                   | 28 ± 18                          | 0.04 ± 0.05                  |
| NRK49F.H1                                    | 121.3 ± 0.9                      | 1.6 ± 0.3                    |
| Rat2.H1                                      | 175 ± 11                         | 9 ± 2                        |
| REF52.E1aH1                                  | 20 ± 5                           | 17 ± 4                       |

### Table 4. Spontaneous transformation of REF52 cells by ras typically results from recessive changes.*

| Neo<sup>B</sup>parent × Hyg<sup>B</sup>parent | Hybrids/10<sup>6</sup> cells fused | Soft agar plating efficiency |
|-----------------------------------------------|----------------------------------|------------------------------|
| RT1                                           | REF52.H1                         | 11                            | <0.01                        |
| N5 (REF52)                                    | REF52.E1aH1                      | 148                           | 3                            |
| RC6T1                                         | REF52.H1                         | 1                             | <0.01                        |
| 15-26-4                                       | REF52.H1                         | 16                            | 0.2                          |
| N5 (REF52)                                    | REF52.H1                         | 76                            | 0.5                          |
| N5 (REF52)                                    | REF52.E1aH1                      | 4                             | 0.03                         |
| 15-26-4                                       | REF52.H1                         | 6                             | ND                           |
| 15-26-4                                       | REF52.E1aH1                      | 6                             | ND                           |

*ras-Transformed REF52 tumor lines (RT1, RC6T1, RC6T2, and 15-26-4) were fused with the indicated hyg<sup>B</sup> cells. Cell lines and methods are described in Tables 1 and 2 and in text. Fusions between REF52 cells and ras-transformed REF52 cells isolated by in vivo selection failed to produce transformed hybrids. ND, not determined.
of point-mutations or posttranslational alterations that adversely affect the activity of p105Kβ.

Summary

In summary, REF52 cells possess growth controls that prevent a sustained mitogenic stimulus from transforming. These growth controls are dominant and can act in trans to antagonize transformation of rat cells previously transformed by ras. Given that ras and sis initially provoke REF52 cells to proliferate, the antagonist may itself be activated by mitogens as a part of a feed-back mechanism which limits cell proliferation. E1A and SV40 large T are able to circumvent or inactivate the antagonist. The extent to which this relates to the mechanism by which the viral oncproteins enable REF52 cells to grow in the absence of serum is currently under investigation.

Future studies will focus on genes, such as Rb and p53, known to be involved in the negative regulation of cell proliferation in an effort to understand at a biochemical level the information obtained in genetic analyses. In addition, we will examine the effects of ras on various cell-cycle genes. It is interesting that ras causes REF52 and other cells to arrest in G2 upon removal of SV40 large T-antigen (27,37). The control of the cells cycle in G2 has been described in a variety of organisms and can involve a protein kinase identified as: a) a component of maturation promoting factor (MPF), b) a 34 kDa protein in mammalian cells, c) a starfish histone M-phase kinase, and d) products of the CDC2 and cdc28 genes of S. pombe and S. cerevisiae, respectively (38,39). That E1A both induces p34 in baby rat kidney cells (40) and binds to a cyclinlike molecule (41) as well as the observation that ras-expressing REF52 cells arrest in G2 suggests that p34 may influence complementation between these oncogenes. For example, E1A may protect against the repression of p34 expression or activity by ras.

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