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Advances in Brief

The Mammalian Ribonucleotide Reductase R2 Component Cooperates with a Variety of Oncogenes in Mechanisms of Cellular Transformation[1]

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Abstract

Ribonucleotide reductase, which is composed of the two protein components R1 and R2, is a highly regulated enzyme activity that is essential for DNA synthesis and repair. Recent studies have shown that elevated expression of the rate-limiting R2 component increases Raf-1 protein activation and mitogen-activated protein kinase activity and acts as a novel malignancy determinant in cooperation with H-*ras* and *rac*-1. We show that R2 cooperation in cellular transformation extends to a variety of oncogenes with different functions and cellular locations. Anchorage-independent growth of cells transformed with v-*fms*, v-*src*, A-*raf*, v-*fes*, c-*myc*, and ornithine decarboxylase was markedly enhanced when the R2 component of ribonucleotide reductase was overexpressed. In addition, we observed that elevated R2 expression conferred on c-*myc*-transformed NIH 3T3 cells an increased tumorigenic potential in immunoincompetent mice. Taken together, these observations demonstrate that the R2 protein is not only a rate-limiting component for ribonucleotide reduction but that it is also capable of acting in cooperation with a variety of oncogenes to determine transformation and tumorigenic potential.

Introduction

Mammalian ribonucleotide reductase is composed of two different dimeric protein components often called R1 and R2, which are essential for the reduction of ribonucleoside diphosphates to their corresponding deoxyribonucleotides, a rate-limiting process in DNA synthesis (1). Alterations in the activity of the enzyme and the expression of the R1 and R2 genes have been observed in human tumor tissue (2–5), and, for example, after treatment of cells with tumor promoters or with transforming growth factor β_1 (6–8). The enzyme is also important in mechanisms of DNA repair after treatment with chemotherapeutic compounds, and its expression can determine the drug sensitivity characteristics of tumor cells (9, 10). Very recently, observations obtained in gene transfer experiments have indicated that changes in the relative levels of the two components of ribonucleotide reductase can profoundly affect the malignancy properties of tumor cells. These studies demonstrated that deregulated R2 gene expression can cooperate with H-*ras* and *rac*-1 to increase cellular transformation and malignant potential, whereas elevated expression of the R1 component exhibits malignancy-suppressing activity (11, 12). These findings indicate that the two components of ribonucleotide reductase are novel malignancy determinants playing opposing roles in its regulation. This has led to the hypothesis that in addition to functioning as subunits for ribonucleotide reductase activity, R1 and R2 have novel but undefined properties that affect signal pathways important in determining transformation and tumor progression (11, 12). Support for this idea comes from the demonstration that deregulated R2 gene expression activates a Ras/Raf/MAPK[3] pathway, which is important in mediating the expression of genes that participate in the regulation of such important biological events as cell cycle progression, apoptosis, and cellular differentiation (11).

The concept that R2 can synergize with oncogenes to alter malignant potential was first observed in experiments with H-*ras* and *rac*-1 (11). Because it is important to determine whether or not this cooperation is confined to *ras* and *rac* or generally extends to a variety of oncogenes, we have examined this question in cells exhibiting alterations in the expression of genes affecting diverse cellular functions and whose products are found in different intracellular locations.

Materials and Methods

Cell Lines and Cell Culture. The cell lines used in this study and related information are shown in Table 1. Cells were routinely cultured in α-MEM supplemented with 8% FBS (Canadian Life Technologies). Cell lines Src/mR2, Raf/mR2, Fes/mR2, and Myc/mR2 were generated by infecting the Src1, NIH/9IV#5, Fes1, and NIH/hmyc1 parental cell lines, respectively, with human Myc epitope-tagged mouse R2 vector virus (SH/mR2). Control cell lines Src/SH, Raf/SH, Fes/SH, and Myc/SH were generated by infecting the above-mentioned parental cell lines with control vector virus without an R2 sequence (LXSH). Stable infectants (≥1000 clones) were obtained by selection with 400 μg/ml hygromycin and pooled and expanded (11, 13). Cell lines Fms&mR2 and ODC&mR2 were derived from NIH 3T3 cells after cotransfection of pSH/mR2 plasmid plus either expression vector pZip V-*fms* encoding V-Fms (14) or pEUK-ODC222 encoding mammalian ODC (15, 16). A LipofectAMINE kit (Canadian Life Technologies) was used to transfect DNA into cells, and the ratio of pSH/mR2:pZip V-*fms* or pEUK-ODC222 was 1:4 (10–13). More than 100 stable clones were obtained for each transfection after selection with hygromycin and pooled for additional experiments. Growth in soft agar was estimated in 10-cm tissue culture plates containing 15 ml of base agar (0.5% Bacto agar in α-MEM containing 10% FBS) and 10 ml of growth agar (0.33% in α-MEM containing 10% FBS). Cells were obtained from subconfluent cultures, and colonies were scored 14–21 days later (11, 12).

Detection of R2 Protein. Monoclonal 9E10 antibody recognizing a human Myc epitope and a polyclonal antiserum raised against R2 protein were used in Western blot analysis to determine the expression of recombinant and/or wild-type endogenous R2 protein, as we have described previously (11, 13).

Assay for Tumorigenicity. Balb/c *nu/nu* mice (Charles River Breeding Laboratories, Quebec, Canada) were used to assess tumorigenicity as described previously (11, 12, 14). Cells were prepared from subconfluent logarithmically growing cultures and collected by gentle treatment with trypsin/EDTA solution. Tumor latency was determined by s.c. injecting 3×10^5 cells in 0.1 ml of medium and recording the time required to form a tumor (2×2 mm) detectable by palpation. Tumors were removed from the mice, and tumor weight was recorded 21 days later. To confirm that equal numbers of test and control cells were injected, duplicate culture plates containing growth medium were supplemented with 100 cells/plate aliquoted from cell suspensions prepared for injection. The plates were stained with methylene blue, and colonies were scored after 10 days in culture (10).

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[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; ODC, ornithine decarboxylase.

Table 1 *Information about cell lines*

Designation	Parental cell line	Related characteristics and transgene expression	Source or reference
NIH 3T3		Mouse fibroblast, untransformed	ATCC[a]
N3/SH	NIH 3T3	*hph*	Fan *et al.* (12, 13)
N3/mR2	NIH 3T3	hph, mR2[b]	Fan *et al.* (12, 13)
Fms&SH	NIH 3T3	v-*fms*, *hph*	This study
Fms&mR2	NIH 3T3	v-*fms*, *hph*, mR2	This study
ODC&SH	NIH 3T3	ODC, *hph*	This study
ODC&mR2	NIH 3T3	ODC, *hph*, mR2	This study
Src 1	NIH 3T3	v-*src*	Egan *et al.* (14)
Src/SH	Src1	v-*src*, *hph*[c]	This study
Src/mR2	Src1	v-*src*, *hph*, mR2	This study
Fes1	NIH 3T3	v-*fes*	Egan *et al.* (14)
Fes/SH	Fes1	v-*fes*, *hph*	This study
Fes/mR2	Fes1	v-*fes*, *hph*, mR2	This study
NIH/9IV#5	NIH 3T3	A-*raf*	Egan *et al.* (14)
Raf/SH	NIH/9IV#5	A-*raf*, *hph*	This study
Raf/mR2	NIH/9IV#5	A-*raf*, *hph*, mR2	This study
NIH/hmyc1	NIH 3T3	c-*myc*	Egan *et al.* (14)
Myc/SH	NIH/hmyc1	c-*myc*, *hph*	This study
Myc/mR2	NIH/hmyc1	c-*myc*, *hph*, mR2	This study

[a] ATCC, American Type Culture Collection.
[b] mR2, human Myc epitoped-tagged R2 protein.
[c] Selective marker, *hph* (hygromycin phosphohydrogenase gene).

Results

Expression of Recombinant R2 Protein. We investigated the properties of a variety of oncogene-expressing cell lines stably transfected or retrovirally infected with vectors carrying the coding region for the R2 component (Table 1). To distinguish the recombinant R2 gene product from the endogenous R2, a human c-Myc epitope encoding 10 amino acids plus methionine was added to the 5′ end of the R2 cDNA (11, 13). Fig. 1 shows that recombinant R2 was expressed in all the cell lines obtained by either transfection with vector plasmid DNA or after infection using a retrovirus vector. Monoclonal 9E10 antibody detected a Myc epitope-tagged protein of 45 kDa, the predicted size of the recombinant R2 protein, in all cell lines containing the R2 vector, and as expected, no expression of the recombinant R2 protein was detected in the control cell lines (Fig. 1*A*). Polyclonal antibody raised against the endogenous R2 protein detected the wild-type endogenous R2 protein from both R2 vector-containing cells and control cells, and in addition, this antibody also detected the recombinant protein that is slightly larger in size than the endogenous protein in the R2 vector-containing cells (Fig. 1*B*). Previous studies have demonstrated that cell-expressed recombinant R2 protein is biologically active as judged by both enzyme assays and tests for decreased sensitivity to hydroxyurea that specifically target the R2 protein (11, 13). Contrary to an earlier suggestion (16), it is clear that stable expression of R2 protein from its cDNA can be readily achieved in a variety of cell lines.

R2 Expression Cooperates with Oncogenes to Determine Transformation Potential. Cellular transformation is usually accompanied by anchorage-independent growth in cell culture, which can be measured in growth medium containing a low-percentage agar supplement. Anchorage-independent growth generally correlates with tumorigenic potential (12, 14). Fig. 2 shows the results obtained with cells expressing recombinant R2 protein and the altered activities of v-*fms*, v-*src*, v-*fes*, A-*raf*, c-*myc*, or ODC when compared to the parental cell lines without recombinant R2 as controls. Each of the cell lines containing recombinant R2 plus altered oncogene expression was much more efficient in forming colonies in soft agar than the relevant control cell lines. NIH 3T3 cells expressing recombinant R2 alone in the absence of a cooperative oncogene were not capable of forming colonies in soft agar. This result further supports the concept that R2 cooperates with activated oncogenes in mechanisms of cellular transformation, although R2 overexpression alone is not sufficient to mediate cellular transformation. When grown on the surface of plastic tissue culture plates, recombinant R2-expressing cells, with one exception, exhibited growth rates and plating efficiencies identical or very similar to those of control cell lines (data not shown). The only exception was Fes/mR2 cells, which showed a significantly

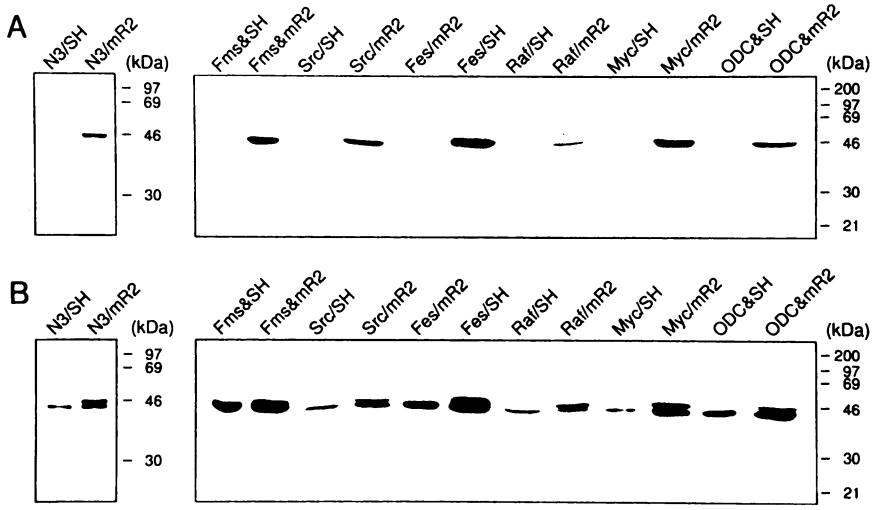

Fig. 1. Western blot analysis detecting the expression of 9E10 epitope-tagged R2 protein in cells containing the R2 vector. Filters in *A* were probed with monoclonal 9E10 antibody that recognizes a human Myc epitope; those in *B* were probed with rabbit polyclonal antibody raised against R2 protein.

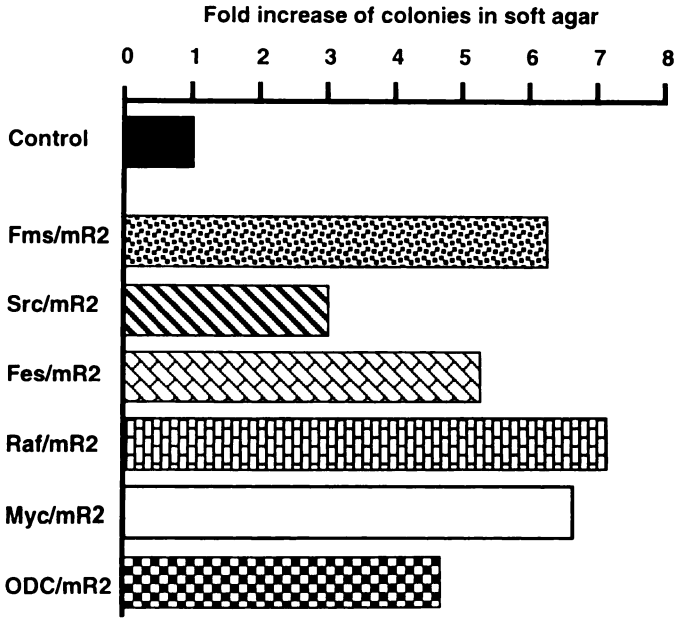

Fig. 2. Recombinant R2-expressing cells form more colonies than control cells in soft agar. The number of colonies formed by the cell lines Fms/mR2, Src/mR2, Fes/mR2, Raf/mR2, Myc/mR2, and ODC/mR2 were compared to that of control cell lines Fms/SH, Src/SH, Fes/SH, Myc/SH, and ODC/SH, respectively. For all cell lines, results were obtained from three to six individual experiments with duplicate plates for each cell line, except those of ODC/SH and ODC/mR2, which were from a single experiment performed in duplicate.

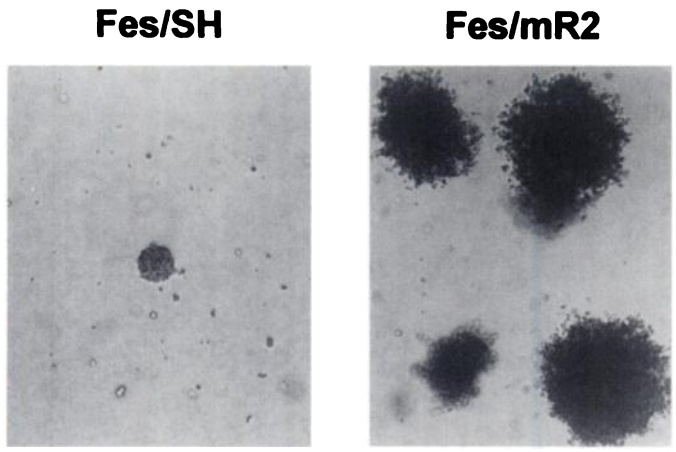

Fig. 3. The Fes/mR2 cell line forms more and larger colonies in soft agar when compared with control Fes/SH cells.

higher plating efficiency than control Fes/SH cells. This shows that in most cases, increased R2 expression in the presence of an activated oncogene can up-regulate signal transduction events involved in controlling anchorage-independent cell growth without modifying the growth properties observed on a solid support during normal culture conditions. The increased plating efficiency observed with Fes/mR2 cells seems to be an additional growth advantage associated with R2 overexpression in this cell line. The increased anchorage-independent growth of Fes/mR2 cells is not simply due to a higher plating efficiency when compared with control cells, because Fes/mR2 cells also exhibited the ability to form much larger colonies in soft agar (Fig. 3).

R2 Overexpression Cooperates with c-*myc* in Determining Tumorigenic Potential *in Vivo*. It is well known that c-*myc* can cooperate with H-*ras* in mechanisms of cellular transformation and tumorigenicity (17). We have observed that R2 overexpression can also cooperate with H-*ras* in cellular transformation and in mechanisms of malignancy (11). Furthermore, deregulated R2 expression leads to activation of a Raf/Ras/MAPK signal pathway (11), and transcription factors such as the product of the c-*myc* gene are downstream of this pathway (18, 19). Because R2 overexpression can cooperate with altered c-*myc* expression to mediate cellular transformation as judged by growth in soft agar (Fig. 2), we directly tested the possibility that R2 overexpression may cooperate with c-*myc* in determining the tumorigenic potential in syngeneic mice. We found that Myc/mR2 cells exhibited a shorter tumor latency period and formed significantly larger tumors than control cells that lacked recombinant R2 expression (Table 2).

Discussion

The oncogenes investigated in this study transform cells by different mechanisms, and their gene products function in a variety of intracellular locations. Although it is likely that deregulated R2 gene expression cooperates with these oncogenes in cellular transformation through a variety of signal pathways, it is also possible that the MAPK pathway has a role to play, at least in part, in the transformation process mediated by R2 in cooperation with most, if not all, of the oncogenes examined. For example, we have previously reported that R2 overexpression can up-regulate Raf translocation to the plasma membrane, where it is activated and in turn stimulates MAPK activity (11). Because activated Raf stimulates the MAPK cascade (11, 20, 21), the cells containing elevated A-Raf expression that were examined in this study would possess increased activities in this pathway. The v-*fms* oncogene encodes a homologue of the cellular receptor for colony-stimulating factor. Its product, v-Fms, is a constitutively active transmembrane tyrosine kinase (22), and cells that contain the v-*fms* oncogene most likely possess an activated Ras/Raf/MAPK cascade, as do cells that contain altered expression of other growth factor receptors (23–25). The v-*src* oncogene codes for a protein kinase whose cellular homologue interacts with an intracellular domain of activated receptors of a wide variety of growth factors and cytokines (26). Like receptor-phosphorylated c-Src, v-Src can modify cytoskeleton organization and stimulate MAPK and other pathways downstream of the receptors (26, 27). Finally, it has been reported that ODC overexpression, which induces anchorage-independent tumor cell growth, also stimulates MAPK activity (15, 28). In contrast to the above-mentioned oncogene products, c-Myc is a nuclear protein and functions biochemically downstream of MAPK in response to a variety of signals. c-Myc is a transcription factor and can, for example, stimulate the expression of cyclins that are important in regulating cell cycle progression (19, 29, 30). Fes is also a nuclear protein, and it functions as a tyrosine kinase (31), but it is also found in the granular and plasma membrane fractions of some cells (32). It is less likely that transformation by v-*fes* requires MAPK activation, although there is some evidence that the v-Fes kinase can phosphorylate the Ras GTPase-activating protein that may result in altered p21 Ras function, and, in turn, modify the regulation of the Ras/Raf/MAPK cascade (33).

In summary, we have found that the R2 component of ribonucle-

Table 2 *Tumorigenic potential of c-Myc-transformed cells expressing recombinant R2*

Cell line	Tumorigenicity assay[a] (s.c. tumors)		
	Frequency	Latency (days)	Weight (g)[b]
Myc/SH	5/5	12.7 ± 2.1	0.27 ± 0.12
Myc/mR2	5/5	9.1 ± 1.4	0.75 ± 0.23

[a] Tumor latency and tumor weight are expressed as an average ± SE.
[b] The differences observed between the two groups are statistically significant ($P < 0.005$).

otide reductase cooperates with a variety of different oncogenes in mechanisms of cellular transformation. We propose that one of the signal pathways that is important in this cooperative interaction between R2 and oncogene activity involves the MAPK cascade. Our results also support the idea that the R2 protein is not only a rate-limiting subunit for ribonucleotide reduction (1), but that it is also capable of participating in other critical cellular functions that are important in determining malignant potential (10–12).

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