The N-ras Proto-oncogene Can Suppress the Malignant Phenotype in the Presence or Absence of Its Oncogene

Roberto Diaz, Daniel Ahn, Lluis Lopez-Barcons, Marcos Malumbres, Ignacio Perez de Castro, Jeffrey Lue, Neus Ferrer-Miralles, Ramon Mangues, Jerry Tsong, Roberto Garcia, Roman Perez-Soler, and Angel Pellicer


ABSTRACT

ras proto-oncogenes have traditionally been associated with the regulation and promotion of cell growth. We have induced thymic lymphomas in N-ras−/− mice and in transgenic mice that overexpress wild-type N-ras and found that the lack of wild-type N-ras alleles favors the development of thymic lymphomas, whereas overexpression of wild-type N-ras protects against thymic lymphomagenesis in the presence or absence of its oncogene. To investigate the inhibitory role of wild-type N-ras in in vitro transformation, we introduced wild-type N-ras in N-ras-deficient tumor cells that lack ras activating mutations and found decreased growth in both low serum and soft agar. Taken together, our results indicate that wild-type N-ras has “tumor suppressor” activity, even in the absence of its oncogenic allele.

INTRODUCTION

ras genes, a large family of small GTPases, play an important role in a variety of differentiation processes and signal transduction, including the regulation of cell growth, vesicle movement, cell survival, T-cell activation, apoptosis, and cytoskeleton (1, 2). Three functional ras genes compose the immediate Ras family: H-ras, K-ras, and N-ras (3). Point mutations at specific codons of the normal ras proto-oncogene produce transforming Ras oncogenic proteins that are locked in the active, GTP-bound state (3, 4). The dogma that ras oncogenes are dominant (3, 4), whereby the mutation of a single allele in a cell will predispose the host cell to transformation regardless of the number of normal alleles present, is being challenged (5, 6). We have observed previously that ras alleles were frequently lost in lymphomas with activated ras genes (7–9), suggesting that the normal ras allele could have some suppressive role in the transformation process by the oncogenic allele (10–12). Because N-ras is the isofrom preferentially activated in myeloid and lymphoid disorders (13–15), we set out to investigate the effects of wild-type N-ras on thymic lymphomagenesis.

MATERIALS AND METHODS

Mice. The mice lines used in this work include: the N-ras oncogene under a truncated MMTV-LTR (line A; Ref. 16); N-ras knockout (17); and the N-ras proto-oncogene under the whole MMTV-LTR (line 5; Refs. 16, 18). We crossed line A with the N-ras−/− and obtained an F2 of N-ras−/− mice, 50% containing the N-ras oncogene and 50% that did not. We crossed these F2 mice with each other and established an F3 of the N-ras oncogene in either an N-ras+/− or N-ras−/− background. We maintained these lines and monitored for the formation of thymic lymphomas. We also crossed line A with line 5 and obtained an F3 of line A5, line A, line 5, and wild-type mice. These F3 mice were monitored for the formation of thymic lymphomas. Care for the animals was maintained in accordance with NIH and our institutional guidelines.

DNA Analysis. For the experiments involving the crosses between the N-ras oncogene and the N-ras−/− mice, the wild-type and N-ras−/− alleles were genotyped by Southern blot as described previously (17). For all other experiments, genomic DNA was extracted from mouse toes as described previously (19). For all of the other experiments using the N-ras knockout, we genotyped the mice by PCR using primers NrasE2F (5′-GCAAGTTGATGTTGTTGTTG-3′), NrasE2R3 (5′-TACACAGAGGACCTCCCTGC-3′), NrasE2F (5′-TTCCTAGCTTCATCCAGGAAAG-3′), and KONrasM2 (5′-GCGATTGACTGAGGTCAC-3′). The wild-type (118-bp) and knockout (350-bp) allele PCR products were amplified using NrasE2F and NrasE2R3, and NrasE2F and KONrasM2, respectively, in separate reactions. Oligonucleotides were used in a 25-μl reaction mixture with 1 μl of DNA and 1 unit of Taq DNA polymerase (Sigma Chemical Co., St. Louis, MO). Cycling conditions were 94°C for 5 min, followed by 40 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 45 s, followed by an elongation cycle of 72°C for 5 min, using a Perkin-Elmer Cetus DNA Thermal Cycler. Screening of animals positive for the transgene (line A or line 5) was performed by PCR using primers specific for the transgene (16). For the crosses between lines A and 5 only, we used two other PCR conditions. To ascertain the presence of the N-ras oncogene (line A), we used primers 618 and 623 under cycling conditions as described previously (18). To identify the mice that carry the full-length MMTV′ promoter (line 5), we used primers MMTVF1 (5′-GCGGT-TCGTTGCTCGAGGGGCTTCCACC-3′) and 749 (16), which yielded a product of 1480 bp. Primer MMTVF1 is a forward primer that recognizes a region that is found in the MMTV but lost in the 5′ truncation of the promoter, and primer 749 is a complementary 3′ primer in the second exon of N-ras. Oligonucleotides were used in a 20-μl reaction mixture with 1 μl of DNA and 0.5 unit of Pfu Turbo polymerase (Stratagene, La Jolla, CA). Cycling conditions were 94°C for 2 min; followed by 5 cycles of 94°C for 1 min, 62°C for 45 s, and 72°C for 2 min; followed by 30 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 2 min; and followed by an elongation cycle of 72°C for 5 min, using a Perkin-Elmer Cetus DNA Thermal Cycler.

Detection of ras Mutations. We performed a mismatch PCR-RFLP analysis in tumor DNA to detect point mutations at codons 61, 12, and 13 of N- and K-ras using the same primers and PCR conditions described previously (18).

Carcinogen Treatments and Analysis of Thymic Lymphomas. Treatment of N-ras knockout mice with MNU was performed as described previously (18). The incidence and latency of thymic lymphoma development were monitored and analyzed as described previously (16). A statistical analysis comparing thymic lymphoma incidence was performed using the χ2 test to study whether there was an association between tumor incidence and transgenic type. The level of significance was established at P < 0.05.

Wild-type and N-ras−/− mice, 6–8 weeks of age, were injected s.c. in the back with 1 mg of MCA (Sigma) dissolved in 0.1 ml of olive oil (Sigma) as described previously (20). The mice were euthanized when tumor volume reached 1 cm3. The tumors were excised, and different samples were frozen and taken for histological analysis and derivation of cell lines. Both groups of

Received 2/1/02; accepted 5/24/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Grant CA 36327 (to A. P.) from the NIH. I. P. C. received a fellowship from the Ministerio de Educación (Madrid, Spain).

2 Present address: Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461.

3 Present address: Centro Nacional de Investigaciones Oncológicas Carlos III, Ctra. Majadahonda-Pozuelo, 28220 Majadahonda, Madrid, Spain.

4 Present address: Microbiology Unit, Universitat Pompeu Fabra, Doctor Aiguader 80, 08003, Barcelona, Spain.

5 Present address: Laboratorio d’Investigacio Gastrointestinal, Institut de Recerca, Hospital de Sant Pau, 08025 Barcelona, Spain.

6 To whom requests for reprints should be addressed, at Department of Pathology and Kaplan Comprehensive Cancer Center, NYU Medical Center, New York, NY 10016.

Address correspondence to Dr. Roberto Diaz, Department of Pathology and Kaplan Comprehensive Cancer Center, NYU Medical Center, New York, NY 10016. Phone: (212) 263-5342; Fax: (212) 263-8211; E-mail: pellata01@med.nyu.edu.

The abbreviations used are: MMTV, Moloney murine virus tumor virus; MNU, N-methyl-N-nitrosourea; MCA, 3-methylcholanthrene.

4514

CANCER RESEARCH 62, 4514–4518, August 1, 2002
mice developed high-grade pleomorphic sarcomas with histology consistent with malignant fibrous histiocytoma, as has been described previously (21).

**Derivation of Tumor Cells, Cell Culture, and Transfection.** Unless otherwise stated, all reagents were used from Invitrogen (Carlsbad, CA). Briefly, the tumor was removed, washed with PBS, and minced in trypsin. The cells were incubated at 37°C under rotation for ~10 min. Tumor cells were plated in Preventive Media (15% fetal bovine serum, 1 mM sodium pyruvate, 100 units/ml penicillin/streptomycin, 50 μg/ml neomycin, and 2.5 μg/ml fungizone in RPMI 1640) for three passages. Afterward, cells were maintained in 10% fetal bovine serum in RPMI 1640 without antibiotics. Cultures were maintained on plastic and incubated in 5% CO₂ and 95% air at 37°C in a humidified incubator. All of the cell lines were assayed with a Mycoplasma Plus PCR Primer Set (Stratagene) and found to be free of Mycoplasma. The two N-ras⁻/⁻ cell lines were transfected with 1 μg of either pcDNA3.1/Zeocin⁺ or with wild-type mouse genomic N-ras subcloned in the pcDNA3.1/Zeocin⁺ vector. Fourteen μg of herring sperm DNA were added as carrier, and the stable transfection was performed using Lipofectamine PLUS. Five hundred μg/ml of Zeocin was used for selection of clones. The stable clones were always maintained with 250 μg/ml of Zeocin.

**Cell Growth, Soft Agar, and Tumorigenicity Assays.** N-ras⁺/⁺ and N-ras⁻/⁻ tumor cell lines at similar passage were grown in 10% fetal bovine serum in RPMI 1640. Five thousand cells were plated in 6-well plates at day 0 and maintained with 1% fetal bovine serum (and 250 μg/ml Zeocin for the clones) in RPMI 1640. Triplicate samples for each cell line were counted at the days shown. The medium was changed every 3–4 days. For soft agar assays, cells at similar passage were resuspended in 0.33% agar in RPMI supplemented with 10% fetal bovine serum (and 250 μg/ml Zeocin for the clones) at a density of 20000/65-mm-diameter plate and seeded onto solidified 0.5% agar-containing culture medium; duplicate samples were used in each experiment. Cultures were fed weekly, and colonies were counted 2 weeks after plating. The tumorigenicity assays involving the injection of N-ras⁺/⁺ and N-ras⁻/⁻ tumor cells in wild-type mice were performed, monitored, and analyzed as described previously (22).

**RESULTS AND DISCUSSION**

**Lack of Endogenous N-ras Alleles Increases Susceptibility to Thymic Lymphomagenesis.** We have characterized previously a transgenic mouse line that expresses the N-ras oncogene at low levels under the control of the truncated MMTV-LTR (line A or N-ras-T), which develops thymic lymphomas with high frequency (16). We crossed line A with N-ras knockout mice (17) and found that thymic lymphomas induced by the N-ras oncogene were more frequent on a N-ras⁻/⁻ background (61.5%) than on a wild-type background (37.5%; Fig. 1A). These results indicate an inhibitory role for the normal allele of N-ras in the tumorigenesis induced by the corresponding oncogene and indicate that the N-ras oncogene is not completely dominant.

To study thymic lymphomagenesis in a context independent of the N-ras oncogene, we treated wild-type and N-ras⁻/⁻ mice with MNU. A shorter latency was associated with the lack of N-ras alleles, although there were no significant differences in the final incidence of thymic lymphomas (Fig. 1B). These thymic lymphomas were analyzed for activating mutations in K-ras and for the loss of the other corresponding allele (Table 1). No mutations were found in codons 13 and 61 of K-ras after MNU treatment. The percentage of mutations in codon 12 of K-ras (K12) was significantly higher among the thymic lymphomas induced in an N-ras⁻/⁻ background (46% versus 11%). The K12 wild-type allele was found together with the mutated one in only 40% of the cases for the N-ras⁻/⁻ mice, suggesting a tendency to lose the wild-type allele. In addition, loss of wild-type K-ras allele was observed in 27% of the N-ras⁻/⁻ mice compared with just 6% of the wild-type mice (Table 1). These findings indicate that cells containing ras mutant alleles, but no normal N-ras alleles, are more efficiently selected for tumor development. In fact, they suggest that inactivation of N-ras could predispose to thymic lymphomas with an activated K-ras oncogene. The data also indicate that in thymic lymphoma development, activation of K-ras and N-ras might be playing a similar role.

**Overexpression of the N-ras Proto-oncogene Protects against Thymic Lymphomagenesis.** Because lack of wild-type N-ras seems to be associated with an increase in thymic lymphomagenesis, we next set out to test whether overexpression of wild-type N-ras has a protective effect on thymic lymphomagenesis induced by the N-ras oncogene and by MNU treatment. We have characterized previously a transgenic mouse line that overexpresses the N-ras proto-oncogene >10-fold (line 5 or N-ras-N) under the control of the MMTV-LTR (16, 18). We crossed line A with line 5 and monitored for the appearance of tumors. Line A developed thymic lymphomas in 46.2% of the cases, whereas in the mice that also overexpress the N-ras proto-oncogene (line A5), the incidence of thymic lymphomagenesis drops to 20.6% (Fig. 2A). These results show that increased ratios of wild-type versus activated N-ras allele produce a reduction in thymic lymphomagenesis, indicating that the N-ras oncogene is not fully dominant over its proto-oncogene.

In addition, we have treated wild-type and line 5 mice with MNU and monitored for the appearance of lymphomas (18). When we focused on thymic lymphoma induction, we found that the wild-type
mice treated with MNU develop thymic lymphomas with an incidence of 57.1%, whereas this drops to 30.3% in line 5 mice treated with MNU (Fig. 2B). Interestingly, the majority of these tumors do not harbor ras activating mutations (18). When we take into account only the tumors that lack ras mutations, we find that the differences in incidence were still significant (51.4% versus 23.3%; \( P < 0.05 \)). These results strongly support that the protective effect against thymic lymphomagenesis by overexpression of wild-type N-ras occurs even in the absence of the ras oncogene.

**Introduction of Wild-Type N-ras in N-ras\(^{-/-}\) Tumor Cell Lines Suppresses the Malignant Phenotype.** To confirm and expand these results, we subsequently set out to investigate whether the N-ras proto-oncogene could protect against malignant properties in a different model of tumorigenesis where we induced fibrosarcomas in N-ras\(^{+/+}\) and N-ras\(^{-/-}\) mice by treatment with MCA. We then derived three N-ras\(^{+/+}\) and two N-ras\(^{-/-}\) cell lines from the tumors and observed that the N-ras\(^{-/-}\) cells are much more fibroblastic and elongated than the N-ras\(^{+/+}\) cells that appeared more transformed (data not shown). We found that the N-ras\(^{-/-}\) cell lines grow at a much faster rate in low serum conditions than the N-ras\(^{+/+}\) cells (Fig. 3A). In fact, the N-ras\(^{-/-}\) cells are able to grow in an anchorage-independent environment (Fig. 3B) and are more tumorigenic when injected into wild-type mice (Table 2). To ascertain whether these differences in tumorigenic potential could be a consequence of the lack of N-ras, we transfected either wild-type N-ras or empty vector in the two N-ras\(^{-/-}\) cell lines and selected for stable clones. Introduction of wild-type N-ras in these two N-ras-deficient tumor cells resulted in a morphological phenotype similar to the N-ras\(^{+/+}\) cells (data not shown). Significantly, the clones transfected with the N-ras proto-oncogene show a marked inhibition of growth in low serum (Fig. 3C) and a drastic reduction in the numbers of colonies in the soft agar assay (Fig. 3D). Because none of the cell lines exhibit any K- or N-ras activating mutations (data not shown), these results further show that wild-type N-ras can reduce malignant properties of tumor cells that lack ras mutations.

It has been observed that GDP-bound ras mutants can inhibit signaling by the ras oncogene (23, 24); therefore, the possibility exists that wild-type ras, which is predominantly GDP bound, could be inhibitory to the ras oncogene (10–12). For instance, it has been shown recently that wild-type K-ras2 itself has tumor suppressor activity over the K-ras2 oncogene. When treated with different chemical carcinogens that cause oncogenic mutations in one K-ras2 gene, mice with only one copy of the K-ras2 proto-oncogene produced many large tumors compared with few small tumors observed when wild-type mice were treated with the same carcinogens (6). It has also been suggested that wild-type ras could compete with the oncogene for similar downstream targets or unknown regulatory molecules (25). The results presented in this study, which involve another system (thymic lymphomas) and another ras gene (N-ras), further extend the overall conclusions of ras proto-oncogenes suppressing the malignant properties of their oncogenic counterpart.

Other reports suggest that the ras oncogene and proto-oncogene may preferentially signal through different downstream effectors (26, 27). In fact, recent findings indicate that ras effectors, such as RASSF1, can inhibit the malignant phenotype of various tumor cells (28–31). In the present report, we have shown that the lack of wild-type N-ras alleles favors thymic lymphomagenesis and in vitro transformation, whereas wild-type N-ras overexpression protects against the development of thymic lymphomas, and its expression in tumor cell lines lacking activating ras mutations reduces the transformed phenotype. Therefore, this work also demonstrates that even in the absence of ras activating mutations, the N-ras proto-oncogene is able to suppress malignant properties both in vivo and in vitro.
indicating that wild-type N-ras could act as a general inhibitor of the transformed phenotype. The observations made in this report should be the basis for understanding the competition between ras oncogenes and their proto-oncogenes and to explore the suppressor pathways induced by the N-ras proto-oncogene. The ability of the Ras proto-oncogenes to counteract some of the tumorigenic properties driven by their oncogene counterparts provides evidence for a possible biochemical or genetic manipulation of the tumors to block the action of the oncogenes.

Table 2 Incidence of tumors produced by N-ras<sup>+/+</sup> and N-ras<sup>−/−</sup> tumor cell lines

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell line</th>
<th>Tumor incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4E31 (+/+)</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>4G21 (+/+)</td>
<td>5/5</td>
</tr>
<tr>
<td>II</td>
<td>4E31 (+/+)</td>
<td>1/5</td>
</tr>
<tr>
<td></td>
<td>4G21 (+/+)</td>
<td>4/5</td>
</tr>
</tbody>
</table>

ACKNOWLEDGMENTS

We thank M. Bang for help in the characterization of transgenic mouse lines; M. P. Vargas for assistance with the tumorigenicity assays; T. Tunney for technical assistance; A. Garcia-Espana for support and encouragement; D. Levy for suggesting experimentation with MCA; and M. Pagano for comments and critical reading of the manuscript.

REFERENCES


The N-ras Proto-oncogene Can Suppress the Malignant Phenotype in the Presence or Absence of Its Oncogene


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/15/4514

Cited articles
This article cites 30 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/15/4514.full.html#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
/content/62/15/4514.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.