Independent Molecular Pathways in Initiation and Loss of Hormone Responsiveness of Breast Carcinomas

SARASWATI SUKUMAR,* WALTER P. CARNEY, MARIANO BARBACID

These studies were set up to determine whether those oncogenes participating in the initiation of mammary carcinogenesis (for example, *ras* oncogenes) play a direct role in the outcome of events associated with the late stages of tumor development such as loss of hormone dependency. Mammary carcinomas induced by a single carcinogenic insult in pubescent rats was selected as an in vivo model system with direct relevance to human breast cancer. Acquisition of hormone-independent growth in these carcinogen-induced tumors was found to be independent of the activation of *ras* oncogenes during the early stages of carcinogenesis. In agreement with these observations, introduction of a human *ras* oncogene into human MCF-7 breast carcinoma cells did not abrogate their hormonal dependency for growth in vivo. These findings suggest that those events responsible for the critical stages of breast cancer development occur independently and in an uncoordinated manner.

HUMAN BREAST CANCERS ARE DEpendent on estrogen for growth. This property has made it possible to devise therapeutic strategies involving the removal of circulating estrogen either by ovariectomy, hypophysectomy, or by treatment with anti-estrogens such as tamoxifen. Many breast cancers evolve into an estrogen-independent growth phenotype, a change that often marks the beginning of a more aggressive phase of the disease and makes them nonresponsive to anti-estrogen therapy. It is, therefore, important to understand those factors that contribute to the escape of breast tumor cells from estrogen dependency in vivo.

Among model systems with which to study mammary carcinogenesis in vivo, induction of breast adenocarcinomas in rats by chemical carcinogens has been the most widely used because this system has close similarities to human breast cancer. The rat tumors are hormone responsive since ovariectomy, hypophysectomy, or injection of anti-estrogens results in their regression (*1–4*). However, a significant proportion becomes independent of hormonal supply during the late stages of tumor progression (*1–4*). We have previously shown that mammary carcinogenesis in rats proceeds by different molecular pathways depending, in part, on the nature of the initiating carcinogen. Whereas more than 85% of rat mammary carcinomas induced by a single dose of nitrosomethylurea (NMU) contain transforming H-*ras* oncogenes, only one in four tumors induced by dimethylbenz[*a*]anthracene (DMBA) contain such oncogenes (*5–6*).

The differential involvement of H-*ras* oncogenes in the induction of breast tumors in rats has made it possible to devise an experimental protocol with which to examine the role of oncogenes in the hormonal requirements of these tumors. DNAs were isolated from 55 carcinogen-induced rat tumors and tested in transfection assays for their ability to transform NIH 3T3 cells. Twenty-three (88%) of 26 NMU-induced tumors were able to induce foci, whereas only 6 (21%) of 29 DMBA-induced carcinomas scored positive. Representative transformants were submitted to Southern blot analysis and shown to carry rat H-*ras* sequences, thus demonstrating that the transforming principle present in each of these mammary tumor DNAs was the H-*ras* oncogene (*5*). Oligonucleotide hybridization analysis of each of the H-*ras* oncogenes present in the NMU-induced tumors demonstrated the presence of the same G→A transition, a mutation diagnostic of NMU-induced mutagenesis (*6*). These results indicate that the oncogenes become directly activated by the initiating carcinogen NMU during the very early stages of tumor development (*6*).

The presence of *ras* oncogenes in tumors of known hormonal status was next investigated. Tumor-bearing rats were ovariectomized and the size of each tumor was monitored for a week. Tumors that regressed to less than half of the original size were considered as regressors. Those that either maintained or increased their size were considered as progressors. As shown in Table 1, 80% of the NMU-induced tumors that remained hormone-dependent carried H-*ras* oncogenes. A similar percentage (83%) of tumors that lost hormone dependency for growth also contained H-*ras* oncogenes. These results illustrate that the presence of H-*ras* oncogenes did not correlate with their hormonal status. A similar conclusion was reached when we examined the results obtained with the DMBA-induced tumors. H-*ras* oncogenes were found in 20% of those tumors that remained hormone-dependent and in 33% of those that acquired hormonal autonomy. Thus, the molecular pathways involved in the initiation of carcinogenesis, such as H-*ras* oncogene activation, do not determine the loss of hormone dependency during the late stages of mammary tumor development.

The tumorigenic properties of *ras* oncogenes can be modulated by their levels of expression (*7*). Therefore, those tumors harboring H-*ras* oncogenes may have lost their hormone growth requirements by a mechanism involving increased expression of this oncogene. To examine this possibility, we measured the levels of H-*ras* oncogene expression in such tumors by immunoblotting (*8*). We used the widely reactive Y13-259 monoclonal antibody to p21 (*9*) as well as a novel monoclonal antibody designated E184 that is specific for $p21^{Glu}$, the product of H-*ras* oncogenes activated by GGA→GAA mutations in their 12th codon (*10*). All tumors examined to date, independently of their hormonal status, expressed very low levels of normal or mutated $p21^{Glu}$ proteins, or both. Moreover, quantitative experiments performed with four cell lines derived

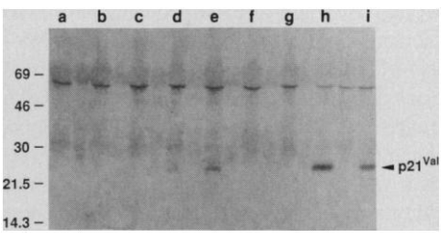

Fig. 1. Expression of $p21^{Val}$ in transfected MCF-7 cells. Cells (10^7) derived from cultures of (a) MCF-7; (b) MCF-*neo*; (c) MCF-H-*ras*; (d and e) MCF-T24; (f) NIH 3T3 cells transformed by a rat H-*ras* oncogene encoding $p21^{Glu}$; (g) normal NIH 3T3 cells; and (h and i) NIH 3T3 cells transformed by MCF-T24 DNA were lysed in RIPA buffer and immunoprecipitated with the monoclonal antibody Y13-259 to p21 (*9*). Immunoprecipitates were subjected to electrophoresis in linear 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose. Filters were incubated for 2 hours with 25 μg/ml of DWP, a monoclonal antibody specific for p21 proteins carrying a Gly→Val substitution in position 12 (*8*), washed three times with TBS (tris 0.01*M* in 0.9% NaCl), and complexed with goat antiserum to mouse immunoglobulin complexed with horseradish peroxidase (Bio-Rad) for 1 hour. The immune complexes were visualized by reacting with 4-chloro-1-naphthol as previously described (*8*). Migration of $p21^{Val}$ is indicated by an arrow. Molecular weight markers included bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), trypsin inhibitor (21,500), and lysozyme (14,300).

S. Sukumar and M. Barbacid, Developmental Oncology Section, Basic Research Program, Frederick Cancer Research Facility, Frederick, MD 21701.
W. P. Carney, E. I. Du Pont de Nemours & Co., Biomedical Products Department, N. Billerica, MA 01862.

*Current address: The Salk Institute, La Jolla, CA 92037.

from hormone-dependent (NMU26 and NMU38) and -independent (NMU58 and NMU68) tumors (*10*) revealed no significant differences in the levels of $p21^{Glu}$ expression.

The possibility that other oncogenes might be involved in the loss of hormone dependency in these tumors was examined by searching for genetic abnormalities in several known proto-oncogenes including *abl*, *erb*B1, *fms*, *fos*, *myb*, and *neu*. Some of these genes are involved in certain human malignancies such as chronic myelogenous and acute lymphocytic leukemias (*abl*), glial tumors (*erb*B1), myeloid leukemias (*myb*), Burkitt's lymphoma (c-*myc*), and mammary carcinomas (*neu*, c-*myc*) (*11*). A total of 40 tumor DNAs, including ten NMU- and DMBA-induced progressor and regressor tumors, were subjected to Southern blot analysis with probes specific for each of the above oncogenes. Thirty-eight of these tumors showed no detectable abnormalities in these loci. An NMU-induced progressor tumor showed an amplified (10×) c-*myc* locus and a DMBA-induced regressor tumor exhibited a similar degree of c-*myb* amplification. No significant genetic alterations could be observed in the locus encoding the rat estrogen receptor. These results indicate that those molecular events responsible for the loss of hormone dependency in carcinogen-induced rat tumors do not involve gross genetic alterations in either the proto-oncogenes tested or in the estrogen receptor itself.

To extend our observations to human breast cancer, we examined whether *ras* oncogenes contribute to loss of estrogen dependency in MCF-7 cells, a well-characterized human breast carcinoma cell line. MCF-7 cells were transfected with a human H-*ras* oncogene isolated from the T24 bladder carcinoma cell line and with a normal H-*ras* gene isolated from human placenta DNA (*12, 13*). Two plasmids, designated pAL8 and pAL11, were generated by inserting the 6.6-kbp Bam HI DNA fragments containing either the T24 oncogene or its normal H-*ras* allele, respectively, at the single Bam HI site of pSV2*neo*. The plasmid pSV2*neo* renders mammalian cells resistant to the antibiotic G418 (*14*). Transfection of 5×10^5 MCF-7 cells with 0.5 μg of pAL8, pAL11, or pSV2*neo* led to the generation of about 300 G418-resistant colonies, none of which had significant morphological alterations. Several of these colonies were examined by Southern blot analysis for the presence of transfected sequences. Three clones from each group, designated MCF-T24, MCF-H-*ras*, and MCF-*neo* were selected for further studies.

The presence of a functionally active H-*ras* oncogene in the MCF-T24 clones was verified by two methods. First, we examined the expression of its gene product, $p21^{Val}$, by immunoblotting analysis with a monoclonal antibody that discriminates between this mutated protein and the normal H-*ras* product, $p21^{Gly}$ (*8*). Expression of $p21^{Val}$ was detected in the MCF-T24 cell lines but not in MCF-7 cells or in the MCF-H-*ras* and MCF-*neo* clones (Fig. 1). Each of these cells expressed similar levels of endogenous p21 proteins, as determined by immunoprecipitation with the widely reactive Y13-259 monoclonal antibody (*9*). Second, DNAs isolated from each of the MCF-T24 cells, but not from the parental MCF-7 cells, induced the appearance of transformed foci in NIH3T3 cells. Each of the NIH3T3 transformants analyzed expressed high levels of the H-*ras* oncogene product, $p21^{Val}$ (Fig. 1). These results unequivocally demonstrate the presence of a functional H-*ras* oncogene in the MCF-T24 cells.

The growth properties of these clones were next examined. In the absence of estrogen, neither the parental MCF-7 cells nor the subclones transfected with the *neo* gene

Table 1. H-*ras* oncogenes in rat mammary tumors of known hormonal status. The growth of mammary carcinomas was monitored for 1 week after ovariectomy. Tumors that regressed to half or less than half their original size were termed hormone-dependent, whereas those that remained steady or increased in size were termed hormone-independent. The H-*ras* oncogenes were detected as described (*5, 6*).

Initiating carcinogen	Hormonal status	Total tumors tested	H-*ras* oncogene	
			Positive	Negative
NMU	Hormone-dependent	5	4 (80%)	1 (20%)
	Hormone-independent	6	5 (83%)	1 (17%)
DMBA	Hormone-dependent	10	2 (20%)	8 (80%)
	Hormone-independent	12	4 (33%)	8 (67%)

Table 2. Growth properties of MCF-7 cells transfected with H-*ras* oncogenes. MCF-7 cells (5×10^5) were transfected with 0.5 μg of pSV2*neo*, pAL11, or pAL8 as previously described (*19*). Agar colony formation efficiency was tested by seeding 10^2, 10^3, or 10^4 cells in 0.33% agarose suspension on a 0.55% agarose underlay in 60-mm petri dishes. The suspension medium was a 1:1 mix of Dulbecco's minimum essential medium and Ham's F12 supplemented with either 10% fetal bovine serum, 10% charcoal adsorbed fetal bovine serum containing estradiol valerate ($5 \times 10^{-9} M$), or 10% charcoal adsorbed fetal bovine serum. Cultures were evaluated 2 weeks later. Colonies consisting of more than 100 cells were counted. Female Swiss nude mice were either left untreated or ovariectomized at 4 weeks of age. Half of the ovariectomized mice received weekly subcutaneous injections of estradiol valerate (15 μg/100 g of body weight) starting the fifth week until the end of the experiment. At the same time, mice from all three groups received a subcutaneous injection of 5×10^6 MCF-7 cells or the sublines shown, in the area of the inguinal mammary fat pad. Growth of the injected cells was monitored at weekly intervals for 12 weeks. Results shown are the composite of two experiments with six animals per group in each experiment.

Transfected plasmid	Oncogene	Cell line	Colony formation in agar*			Nude mice with tumors/total		
			Untreated serum	Charcoal adsorbed serum		Untreated† mice	Ovariectomized mice	
				−Estrogen	+Estrogen		−Estrogen	+Estrogen
None		MCF-7	1.0	<0.001	0.5	0/12	0/12	10/12
pSV2*neo*		MCF-*neo*-1	1.0	<0.001	0.4	0/12	0/12	10/12
		MCF-*neo*-2	0.9	<0.001	0.6	0/12	0/12	11/12
		MCF-*neo*-3	0.6	<0.001	0.2	0/12	0/12	10/12
pAL11	H-*ras* proto-oncogene	MCF-H-*ras*-1	0.6	<0.001	0.2	0/12	0/12	12/12
		MCF-H-*ras*-2	0.7	<0.001	0.3	0/12	0/12	11/12
		MCF-H-*ras*-3	0.7	<0.001	0.5	0/12	0/12	11/12
pAL8	H-*ras* oncogene	MCF-T24-1	5.0	0.8	2.5	0/12	0/12	11/12
		MCF-T24-2	2.5	0.5	1.0	0/12	0/12	12/12
		MCF-T24-3	3.0	0.5	1.0	0/12	0/12	10/12

*Numbers shown are average of two determinations expressed as percentage of cells forming colonies in agar. †Six to eight mice in each of the groups showed tumor outgrowths ranging from 2 to 4 mm in diameter 2 weeks after injection, which regressed completely by the end of the experiment.

or the H-*ras* proto-oncogene grew to form colonies in agar. In contrast, each of the MCF-T24 cell lines carrying the H-*ras* oncogene grew with limited efficiency (less than 1%), forming colonies of more than 100 cells in about 10 days (Table 2). These results are in agreement with previous observations indicating that activated H-*ras* oncogenes conferred growth advantage to MCF-7 cells, partially overcoming their requirement for estradiol supplementation in vitro (*15*). However, injection of MCF-T24 cells in estrogen-depleted (ovariectomized) nude mice did not lead to tumor formation unless they received estrogen supplementation (Table 2). Under these conditions, both the parental MCF cells and those carrying the normal H-*ras* proto-oncogene also led to tumor development (Table 2). These observations indicate that the human H-*ras* oncogene can overcome the hormonal dependency of MCF-7 cells for growth in vitro, but not in vivo.

A previous report has indicated that a *ras* oncogene driven by tandem retroviral long terminal repeats (LTRs) can release MCF-7 cells from their hormonal requirements in vivo, albeit at low frequency (*16*). These findings are likely to be the consequence of overexpression of the *ras* oncogene. It is well known that whereas retroviral *ras* oncogenes are sufficient to induce tumors in immunocompetent animals, their cellular oncogenic counterparts require additional oncogenes to induce the full malignant phenotype. Thus, caution must be exerted when extrapolating results obtained with *ras* oncogenes driven by powerful heterologous regulatory elements.

The present studies were set up to investigate whether *ras* genes, the oncogenes most frequently found in human cancer (*7*), play a role in the losss of estrogen dependency associated with the late stages of progression of breast carcinomas. We used two widely utilized models for human breast cancer, induction of mammary carcinomas by a single carcinogenic insult to pubescent rats (*17*) and the estrogen-dependent MCF-7 human tumor cell line (*18*). The results obtained in both model systems support the concept that *ras* oncogenes, when driven by their own regulatory elements, do not induce growth of mammary carcinoma cells in vivo in the absence of estrogen stimulation. Since *ras* oncogenes are involved in the initiation of a significant fraction of rat mammary adenocarcinomas, our findings suggest that loss of hormone dependency in breast cancer results from genetic events independent of those involved in the initiation of the neoplastic process.

REFERENCES AND NOTES

1. J. Russo, L. K. Tay, I. H. Russo, *Breast Cancer Res. Treat.* **2**, 5 (1982).
2. D. P. Rose, B. Pruitt, P. Stauber, E. Erturk, G. T. Bryan, *Cancer Res.* **40**, 235 (1980).
3. B. M. Arafah, P. M. Gullino, A. Manni, O. H. Pearson, *ibid.* **40**, 4628 (1980).
4. J. C. Williams *et al.*, *J. Natl. Cancer Inst.* **66**, 147 (1981).
5. S. Sukumar, V. Notario, D. Martin-Zanca, M. Barbacid, *Nature (London)* **306**, 658 (1983).
6. H. Zarbl *et al.*, *ibid.* **315**, 382 (1985).
7. M. Barbacid, *Annu. Rev. Biochem.* **56**, 779 (1987).
8. W. P. Carney *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7485 (1986).
9. M. E. Furth, L. J. Davis, B. Fleurdelys, E. M. Scolnick, *J. Virol.* **43**, 294 (1982).
10. Unpublished observations.
11. M. Barbacid, in *Retroviruses and Disease*, M. E. Pullman, H. Hanafusa, A. Pinter, Eds. (Academic Press, Orlando, FL, in press).
12. S. Pulciani *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2845 (1982).
13. E. Santos, S. Tronick, S. A. Aaronson, S. Pulciani, M. Barbacid, *Nature (London)* **298**, 343 (1982).
14. P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* **1**, 327 (1982).
15. R. B. Dickson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 837 (1987).
16. A. Kasid, M. E. Lippman, A. G. Papageorge, D. R. Lowy, E. P. Gelmann, *Science* **228**, 725 (1985).
17. C. W. Welsch, *Cancer Res.* **45**, 3415 (1985).
18. H. D. Soule, J. Vazquez, A. Long, S. Albert, M. Brennan, *J. Natl. Cancer Inst.* **51**, 1409 (1973).
19. F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973).

20. Research sponsored by the National Cancer Institute, DHHS, under contract No. N01-CO-74101 with Bionetics Research, Inc.

23 November 1987; accepted 16 March 1988