Harvey ras Results in a Higher Frequency of Mammary Carcinomas than Kirsten ras after Direct Retroviral Transfer into the Rat Mammary Gland

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ABSTRACT

Exclusive activation of either the Harvey-, Kirsten-, or N-ras gene is often found in human and rodent cancers, although the mechanisms responsible for tissue-specific ras gene activation are poorly understood. In this study, the contribution of ras gene expression and Ras protein activity to the tissue-specificity of ras gene activation was investigated using the rat mammary carcinogenesis model where ras activation, when it occurs, is exclusively in the Harvey ras gene. Differential ras gene expression was examined in mammary tissue from virgin, pregnant, and lactating rats. Harvey ras expression was 1.5-2-fold higher than Kirsten ras or N-ras at each adult stage of development, with the highest ras levels expressed during pregnancy. The modest difference in total mRNA expression found between the independent members of the ras gene family is unlikely to fully account for the exclusive tissue-specificity of Harvey ras activation observed in rat mammary carcinogenesis. Thus, the role of Ras protein specificity was studied by infecting the mammary gland of virgin rats in situ with replication-defective retroviral vectors expressing either the activated or wild-type forms of Harvey- or Kirsten-ras. A 7-14-fold higher number of mammary carcinomas was observed after infection with vectors expressing the G35 to A activated Harvey ras gene product compared with those expressing G35 to A activated Kirsten ras. Mammary carcinomas also developed from infusion of vectors expressing wild-type Harvey ras, but not wild-type Kirsten ras. These data suggest the importance of the Ras protein itself in determining the specificity of the highly homologous Ras family members in organ-specific carcinogenesis.

INTRODUCTION

The Ras family of proteins consists of four primary members: Harvey Ras, Kirsten Ras 2A, Kirsten Ras 2B, and N-Ras. Ras proteins are highly homologous, evolutionarily conserved M, 21,000 proteins that are bound to guanosine 5'-triphosphate in their active form and guanosine 5'-diphosphate in the inactive state, which function as intermediates in signal transduction (reviewed in Ref. 1). Mutations that result in the activation of the Ras proteins are found in many different cancers, supporting an active role of ras in cancer development (reviewed in Ref. 2). Interestingly, within cancers of a particular tissue or organ, these activating events are often restricted to a single ras gene family member (3).

Tissue-specific ras gene activation is frequently observed in rodent carcinogenesis models (3). For example, the Harvey ras gene, but not the Kirsten ras gene, was found mutated in mammary carcinomas from rats exposed to NMU during sexual development (4-6). In contrast, NMU-induced mutations of the Kirsten ras gene have been observed in rat colon carcinogenesis (7). Selective ras gene activation is also associated with many human cancers. For example, activation of the Harvey ras gene is observed in human bladder cancer (8); Kirsten ras gene activation is found in human colon cancer (9, 10) and pancreatic cancer (11); and N-ras gene activation is found in acute myeloid leukemia (12). The mechanisms responsible for tissue-specific activation of the ras gene family members are poorly understood. Therefore, a more complete evaluation of the differences in biological function of the ras family members would aid in understanding their role in cancer development.

Expression of the independent members of the ras family of genes follow both a qualitative and quantitative tissue-dependent pattern. For example, Leon et al. (13) found that in mice, the Harvey-, Kirsten-, and N-ras genes are expressed in all tissues, but the relative levels of each form of ras varies in a tissue-dependent manner and the total levels of ras expressed differs between tissues. In the rat mammary gland, differential expression of the Harvey ras gene has been reported to vary depending on the differentiation status of the gland (14). Interestingly, in carcinogenesis studies where ras is found activated, the ras gene family member found mutated frequently correlates with the form of ras predominantly expressed within the tissue of origin (15). Thus, tissue-dependent expression of the ras genes has been hypothesized to play a role in the organ-specificity associated with ras gene activation.

Although the four members of the Ras proteins are highly homologous, their COOH termini vary, which could allow for distinct functional activities (1). For example, in vitro analyses have demonstrated differences in the efficiency of posttranslational modifications that occur at the COOH terminus for Harvey Ras and Kirsten Ras (16, 17). Also, distinctions between the ras genes are evident in knockout studies of mice, where deletion of the N-ras gene is without apparent consequence (18), whereas Kirsten ras knockouts are developmentally lethal (19). Thus, differences in the roles of the independent ras gene family members at the protein level could account for the tissue-dependent functions of ras in carcinogenesis.

Retroviral gene transfer has been used extensively for the introduction of novel genes to many different tissues, including the rat mammary gland (20). Infection of mammary parenchyma with retroviral vectors expressing the viral Harvey ras gene produces mammary carcinomas that are similar in morphology and temporal development to chemically induced mammary carcinomas (21). In this study, a retroviral mammary ductal infusion methodology was used to introduce Harvey ras and Kirsten ras genes to the rat mammary parenchyma under identical promoters, allowing the determination of the relative potency of the Harvey ras and Kirsten ras genes products in rat mammary carcinogenesis.

MATERIALS AND METHODS

Cloning, Mutagenesis, and Sequencing. Wild-type rat Harvey ras 1 cDNA and rat Kirsten ras 2B cDNA were PCR-amplified from a Sprague Dawley rat brain cDNA library (Stratagene, La Jolla, CA) and subcloned using a TA cloning kit (Invitrogen, Carlsbad, CA). The 5' primer (atgagaataacagctgtaggt) and 3' primer (tcaggaacacagacgatg) used to amplify rat Harvey ras spanned the entire coding region. The 5' primer (atagagtaggtgtgagct-
The rat Kirsten ras 2B sequence was subcloned into the HindIII site of an expression vector for the Kirsten ras 2B gene; JRKrasV contains the viral Kirsten ras gene (containing exon 4A). M7 *, indicates the region of the retroviral packaging signal; TAG, specifies a mutation in the pPol** initiation codon; Δ, denotes the location of activating point mutations found in the ras genes. Constructs are not drawn to scale.

Fig. 1. Retroviral vector constructs. The pJR backbone (21) was used for construction of all ras expression vectors as described in “Materials and Methods.” JRHrasG contains the wild-type Harvey ras coding region; JRHrasA contains the G35 to A-activated form of the Harvey ras gene; JRKrasG contains the first 84 bp of the wild-type Harvey ras gene followed by the coding region for the Kirsten ras 2B gene; JRKrasV contains the viral Kirsten ras gene (containing exon 4A).Ψ⁺, indicates the region of the retroviral packaging signal; TAG, specifies a mutation in the pPol** initiation codon; Δ, denotes the location of activating point mutations found in the ras genes. Constructs are not drawn to scale.

Retroviral Vector Construction and Production. The wild-type and codon 12-activated forms of the Harvey ras and Kirsten ras gene were subcloned into the BamHI and SalI restriction sites of pJR (Fig. 1), producing the plasmids used to generate the replication-defective retroviral vectors used in this study. The construction of JRHrasV and JRgal were described previously (21) and used in these studies as negative and positive retroviral infusion controls, respectively. For the purpose of this study, JRras (21) was redesignated JRHrasV to avoid confusion with the other vectors used in this study. Each retroviral vector plasmid was independently transfected into the ecotropic packaging cell line Psi-CRE (24) using Lipofectin (Life Technologies, Inc., Gaithersburg, MD). Retroviral vectors from these cells were used to infect the amphotropic packaging cell line PA317 (25) to produce replication-defective retroviral vectors for rat mammary gland infusions. Infected PA317 clones were selected using resistance to G418 (Life Technologies, Inc.). High-titer producing clones were expanded in 162 cm² cell culture flasks and grown at 30°.

Fig. 2. Analysis of Harvey-, Kirsten-, and N-ras mRNA expression in the mammary gland of virgin, pregnant, and lactating rats. A, RNase protection assay for N-ras, Harvey ras, Kirsten ras, and β-actin mRNA expression as described in “Materials and Methods.” B, histogram showing results from the RNase protection assay normalized to β-actin expression. *, Harvey ras mRNA levels were significantly higher than Kirsten ras and N-ras levels in the virgin, pregnant, and lactating mammary gland (P < 0.05). Columns, means of two experiments done in triplicate; bars, SE.
Retroviral Infusion. Retroviruses were infused into the central duct of each of the 12 teats of virgin inbred female Wistar-Furth rats, 45–55 days of age, as described previously (21). To promote viral incorporation, mammary glands were stimulated to proliferate by treating rats with 3 mg/kg perphenazine (Sigma Chemical Co., St. Louis, MO) in 0.9% NaCl, 0.02N HCl, and injected s.c. for 2 consecutive days before and 4 h before viral infusion. Viral preparations were thawed on ice, and then polybrene (Sigma Chemical Co.) was added to a final concentration of 80 µg/ml. Either 3 mg/ml indigo carmine (Sigma Chemical Co.) or 0.5 mg/ml fast green (Fluka, Ronkonkoma, NY) was added to visually assess the completeness of viral infusion into the entire mammary ductal structure. Under ether anesthesia, the thoracic, abdominal, and inguinal regions of each rat were shaved to expose the 12 mammary teats. Teats were individually clipped to allow the infusion of 12 µl or 20 µl of viral preparation into the central mammary lacteal using a blunt-ended 27-gauge needle. Occasionally, <12 teats were present, so all available teats were infused.

After viral infusions, rats were housed in plastic, suspended cages receiving lab chow and acidified water ad libitum. Mammary tumor size and location were recorded weekly beginning at 4–5 weeks after viral infusion and continued until study termination at 18 weeks. Tumors reaching 10–15 mm in size before study termination were resected while the animal was under ether anesthesia. Animals were returned to the study after tumor resection. Sections of resected mammary tumor were divided and stored frozen at −80°C for later molecular characterization or fixed in formalin for histopathological analysis.

Tumor Retroviral mRNA Measurement. Retroviral gene expression from the 5' LTR was quantified using a competitive PCR MIMIC assay (Clontech, Palo Alto, CA). Total mRNA was isolated from frozen mammary tumor sections using RNAzolB (Tel-Test). cDNA was generated from this message using a Reverse Transcription Kit (Promega). Fluorescine-labeled PCR primers (U. W. Biotechnology Center, Madison, WI) were used to amplify a 730-bp fragment of the 5’ LTR-generated retroviral message and a 320-bp viral message MIMIC. This product was normalized to a concurrently amplified 980 bp PCR product of the GAPDH message and its 450-bp MIMIC. PCR products were resolved on a 2% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME) and recorded using a FluorImager (Molecular Dynamics). Band intensities from the recorded image were determined using ImageQuant software. Message levels were quantified as the ratio of band intensities for PCR products of viral message to viral-message MIMIC and normalized to the ratio of band intensities for PCR products of the GAPDH message and GAPDH message MIMIC.

Ras Protein Expression Measurement. Total Ras protein was determined using standard protocols for Western analysis (23). Total extracted tumor protein...
Electrophoresed proteins were transferred to an Immunobilon-P membrane (n = 72 glands). The 12 teats of each rat were infused with 20 μl of each viral preparation. JRHrasA was infused at 6.0 × 10^6 CFU/ml (n = 108 glands). JRKrasA was infused at 8.0 × 10^6 CFU/ml (n = 71 glands), and JRGal was infused at 1.8 × 10^6 CFU/ml (n = 131 glands), and JRgal was infused at 3.0 × 10^6 CFU/ml (n = 72 glands). B. mammary carcinoma multiplicity after infusion of 5-fold higher titers of the JRKrasA than the JRHrasA retroviral expression vector. JRHrasA was infused at 6.0 × 10^6 CFU/ml (n = 108 glands), JRKrasA was infused at 30.0 × 10^6 CFU/ml (n = 131 glands), and JRGal was infused at 1.8 × 10^6 CFU/ml (n = 72 glands). The 12 teats of each rat were infused with 20 μl of each viral preparation.

RESULTS

Harvey-, Kirsten-, and N-ras Expression in the Virgin, Pregnant, and Lactating Mammary Gland. Over the life span of an adult female mammal, the mammary gland goes through various stages of development and proliferation. To quantify ras mRNA levels during different stages of mammary gland development, an RNase protection assay was used to determine the relative levels of Harvey-, Kirsten-, and N-ras mRNA in the virgin, pregnant, and lactating mammary gland (Fig. 2A). All ras mRNA levels in the mammary gland were significantly increased during pregnancy (P < 0.05). Only the Harvey ras mRNA level was found to be significantly lower during lactation in comparison with its expression in the virgin gland (P < 0.05). Harvey ras mRNA levels were 1.5–2.0-fold higher than Kirsten- and N-ras mRNA levels in all differentiation states of the mammary gland (Fig. 2B), and the ratios of the Harvey-, Kirsten-, and N-ras mRNA were constant in all developmental stages examined.

Mammary Carcinogenesis Induced by Different Forms of Harvey ras. To compare the relative mammary carcinogenicity of Harvey ras in its wild-type and activated forms, retroviral vectors were produced for infection of rat mammary parenchyma that express either the wild-type Harvey ras gene (JRHrasG; Fig. 1), the G35 to A-activated form of the Harvey ras gene (JRHrasA; Fig. 1), or the activated viral Harvey ras gene (JRHrasV; Fig. 1). After 18 weeks, the average number of mammary carcinomas that developed after infusion of JRHrasA and JRHrasV were similar in multiplicity and latency of development, thus the different activating mutations present in these forms of Harvey ras may have equivalent mammary carcinogenic potency (Fig. 3A-B).

Carcinomas were also produced after infusion of the wild-type Harvey ras gene (Fig. 3). These neoplasms were unique in that they were smaller and slower growing in comparison with those resulting from infusion of the activated forms of the ras genes (data not shown). Furthermore, the latency was significantly greater for carcinomas induced with wild-type Harvey ras compared with those that developed after infusion of JRHrasA and JRHrasV which were similar in multiplicity and latency of development, thus the different activating mutations present in these forms of Harvey ras may have equivalent mammary carcinogenic potency (Fig. 3A-B).

Mammary Carcinogenesis Induced by Different Forms of Kirsten ras. Most cancer types with an activated ras gene have activating mutations present in these forms of Kirsten ras genes (P = 0.59; Fig. 4), suggesting that the activating mutations present in these forms of Kirsten ras are comparable in mammary carcinogenicity. The average number of mammary carci-
nomas resulting from JRKrasA infusion were significantly less than the number resulting from infusion of the JRHrasV vector (P < 0.05; Fig. 4). Mammary carcinomas did not result from infusion with vectors expressing the wild-type form of Kirsten ras (Fig. 4).

Relative Potency of Activated Harvey- and Kirsten-ras in Mammary Carcinogenesis. To more accurately determine the relative carcinogenic potency of the activated forms of Harvey ras and Kirsten ras, the JRHrasA and JRKrasA vectors, which both produce Ras proteins with the same glycine to glutamic acid activating substitution at the 12th amino acid, were directly compared. Infusion of equivalent volumes and titers of either JRHrasA or JRKrasA resulted in mammary carcinoma development (Fig. 5A). Eighteen weeks after retroviral infusion, the JRHrasA vector resulted in the development of 0.48 mammary carcinomas/gland, whereas the JRKrasA led to the development of only 0.07 mammary carcinomas/gland, which was significantly different (P < 0.05).

Because the number of carcinomas generated by the JRHrasA vector was significantly higher than the number observed after infusion with equal titers of the JRKrasA vector, an additional retroviral infusion study was performed using a 5-fold higher titer of the activated Kirsten ras gene expressing vector over the activated Harvey ras gene expressing vector. In this study, infusion of JRHrasA led to the development of 0.42 carcinomas/gland and JRKrasA at 5-fold higher titers led to 0.15 carcinomas/mammary gland 18 weeks after viral infusion (Fig. 5B). Although the activated Kirsten ras expressing vector was infused at a 5-fold higher viral titer, JRHrasA led to more than double the number of mammary tumors 18 weeks after viral infusion, which was found to be significantly different (P < 0.05). Thus, in this experiment when linearly corrected for titer, the G35 to A-activated form of the Harvey ras gene-expressing vector was found to be approximately 14-fold more potent than the comparably activated form of the Kirsten ras gene-expressing vector in rat mammary carcinogenesis.

Histopathological Examination of Mammary Carcinomas Produced by Harvey- and Kirsten-ras Expressing Retroviral Vectors. Although the multiplicity of carcinomas resulting from infusion of activated Harvey ras expressing retroviral vectors was higher than the number resulting from infusion of vectors expressing the Kirsten ras genes, no significant difference in the histopathology of resultant carcinomas was observed (data not shown). Carcinomas produced by JRHrasV, JRKrasV, JRHrasA, and JRKrasA were most often papillary adenocarcinomas with comparable histopathologies (Fig. 6A-D). In contrast, on histopathological analysis of the carcinomas produced after wild-type Harvey ras gene infusion several unique morphological characteristics were observed compared to those resulting from infusion of the activated forms of the ras genes. For example, carcinomas produced by wild-type Harvey ras infusion more often showed localized invasion of adjacent muscle (Fig. 6E) and also uniquely possessed regions of squamous metaplasia (arrow) in a mammary carcinoma produced by the wild-type Harvey ras expression vector (×230).
the retroviral promoter, which was controlling their expression, retroviral gene expression in resultant mammary carcinomas was determined using a competitive PCR and MIMIC assay. mRNA levels in JRHrasA-induced (0.43 ± 0.17-fold GAPDH levels) and JRKrasA-induced (0.57 ± 0.32-fold GAPDH levels) mammary carcinomas did not differ significantly (P = 0.36; Fig. 7A). Thus, differential feedback from expression of the mutant ras genes on the expression of the 5' LTR of the retroviral vector did not result in significantly different levels of vector message expression from JRHrasA or JRKrasA-induced mammary carcinomas. Arrow indicates the location of p21 Ras, which may be comprised of multiple bands (40).

DISCUSSION

The mechanisms responsible for the tissue-specificity of ras gene activation are not well defined, although the high frequency with which these genes are found activated in some cancers suggests that they participate in cancer development (reviewed in Refs. 1-3). Factors that may contribute to the tissue-specific activation of the independent members of the ras family of genes include gene expression levels (13, 15) and tissue-dependent Ras protein activity (3). In this study, variations in the levels of expression of the independent ras gene family members during different stages of mammary gland differentiation were modest and could not solely account for the complete absence of activation of the Kirsten- and N-ras genes in rat mammary carcinogenesis. In contrast, a comparison of the carcino-

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differences in the carcinogenicity of the Ras proteins in the mammary gland were more substantial. A 7-fold higher number of carcinomas was found after infusion of retroviral vectors expressing the activated Harvey ras gene product. Even when the vector expressing activated Kirsten ras was infused at 5-fold higher titers than the activated Harvey Ras expressing vector, the number of carcinomas that developed from activated Harvey ras was more than double the number of carcinomas that developed with activated Kirsten ras (14-fold higher using linear extrapolation). Differences in message and protein expression levels are unlikely to solely account for the potency of the Harvey ras gene at inducing rat mammary carcinomas because vector message and total Ras protein levels were found to be equivalent in both Harvey- and Kirsten-ras generated carcinomas. Thus, based on the tumor multiplicity resulting from vector infusion, the carcinogenicity of activated Harvey ras was found to be approximately an order of magnitude higher than with activated Kirsten ras in the rat mammary gland.

In this study, retroviral vectors were also produced that express the wild-type forms of Harvey ras and Kirsten ras. Interestingly, mammary carcinomas resulted from infusion of vectors expressing wild-type Harvey ras, but not wild-type Kirsten ras, further supporting the higher potency of Harvey ras in mammary carcinogenesis. Carcinomas produced by wild-type Harvey ras had a longer latency and were slower growing than mammary carcinomas that developed from the activated forms of the ras genes, although the final tumor multiplicity resulting from the wild-type Harvey ras expressing vector was not significantly different from the number that developed with the activated vectors. Also, regions of localized muscle invasion were more often observed in these neoplasms. In addition, many of these carcinomas had areas of squamous metaplasia. Squamous metaplasia is rarely observed in mammary carcinomas from adult rats exposed to chemical carcinogens or in mammary carcinomas resulting from retroviral infusion with the activated forms of ras. However, adenosquamous carcinoma has been observed in rats genetically resistant to chemical carcinogens or in mammary carcinomas resulting from retroviral infusion with the activated forms of ras. Harvey ras, and adenosquamous carcinoma was found after infusion of retroviral vectors expressing the activated Harvey ras gene.

Infection of mammary cells with the wild-type Harvey ras expressing vector should lead to overexpression of the Harvey ras gene. Furthermore, expression of the wild-type Harvey ras gene from this vector is controlled by the retroviral 5' LTR and would, thus, be physiologically deregulated compared with the endogenous Harvey ras gene. Therefore, Harvey ras gene overexpression and/or deregulation may be contributing factors to Ras-induced rat mammary carcinogenesis. Also, it is noteworthy that activating mutations in the ras genes are rarely observed in human breast cancer whereas overexpression of total Ras has been reported (36–39). The development of mammary carcinomas by wild-type Harvey ras as observed in this model may, thus, be more analogous to the action of wild-type Harvey ras in the development of some breast cancers in humans.

In summary, we presented data showing that in the rat mammary gland Harvey ras is expressed 1.5–2.0 times the level of Kirsten ras and, at equal levels of expression, is approximately 10 times more potent than Kirsten ras at inducing mammary carcinomas. Both the increased expression of Harvey ras relative to Kirsten ras and the much higher potency of wild-type and activated Harvey Ras proteins relative to Kirsten Ras proteins in their role in mammary carcinogenesis may contribute to the specificity of Harvey ras activation in chemically induced mammary carcinomas. However, we feel that because the magnitude of difference in the expression of Harvey ras versus Kirsten ras is modest in comparison to the differences in carcinogenic potency of these proteins, that the latter plays a more central role in the exclusive organ specificity of Harvey ras in rat mammary carcinogenesis.

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