Regulation of Growth and Tumorigenicity of Breast Cancer Cells by the Low Molecular Weight GTPase Rad and Nm23

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ABSTRACT

Rad is the prototypic member of a family of novel Ras-related GTPases that is normally expressed in heart, skeletal muscle, and lung and that has been shown to exhibit a novel form of bidirectional interaction with the nm23 metastasis suppressor. In the present study, we have investigated the expression of Rad in normal and neoplastic breast tissues by Western blot and immunohistochemistry and the functional effect of altered Rad expression in breast cancer cell lines. We found that, although Rad is frequently expressed in normal breast tissue (23/30 Rad+ve), expression is usually lost in adjacent invasive carcinoma (8/30 Rad+ve; P < 0.0001). However, where Rad expression persists in a small proportion of tumors, it is associated with higher grade, larger size, and extensive auxillary nodal involvement (n = 48; P = 0.035, P = 0.016, P = 0.022, respectively). Furthermore, Rad is also highly expressed in a breast cancer cell line with high tumorigenic and metastatic potential (MDA-MB231). To further examine the role of Rad in breast cancer, we stably transfected a Rad-ve breast cancer cell line (MDA-MB435). We observed an increase in growth and marked increased colony formation in soft agar in vitro (P < 0.05) and an increase in tumor growth rate in nude mice (P < 0.05). Moreover, coexpression of nm23 with wild-type Rad inhibited the effect of Rad on growth of these cells in culture and markedly inhibited tumor growth in vivo. Additional transfection studies with mutated Rad cDNAs revealed that the growth-promoting effects of Rad appeared to be mediated through its NH2- and COOH-terminal regions, rather than its GTPase domain, and might involve acceleration of cell cycle transition. These findings suggest that Rad may act as an oncogenic protein in breast tissues and demonstrate a potential mechanism by which interaction between Rad and nm23 may regulate growth and tumorigenicity of breast cancer.

INTRODUCTION

Development and progression of breast cancer is a complex process involving both hormonal and genetic factors. Among the several hormones known to stimulate both normal and malignant mammary cell proliferation are steroids, such as estrogen (1) and progesterone (2), and peptide growth factors, such as prolactin (3), insulin (4), and insulin-like growth factor-1 (5). Alterations of a number of genes in breast cancer have also been identified, some of which have been proposed as molecular markers to help predict the prognosis. These include breast cancer susceptibility genes BRCA-1 and -2, p53, Her-2/neu (c-erbB-2), and some regulatory proteins of cell cycle such as cyclin D1 and p27Kip1. Still further altered genes may emerge from investigations centered on chromosomal regions showing loss of heterozygosity (for review, see Ref. 6). Despite the recognition of these factors, the molecular mechanisms of formation of breast cancer still remain unclear, and identification of regulatory genes in the process of tumorigenesis and metastasis is one of the major goals of cancer research.

Rad is a Mr 35,000 small GTPase that was initially cloned by subtractive cloning as a mRNA overexpressed in skeletal muscle of some type-2 diabetic humans and is normally highly expressed in heart and lung (7). It is the prototypic member of a newly emerged Ras-related GTPase family with several unique characteristics, including Gem/Kir, Rem, Rem2 and Ges. Gem/Kir was found by its overexpression in activated T lymphocytes (8) and in v-abl-transformed pre-B cells (9). Rem was cloned as a product of PCR amplification using oligonucleotide primers derived from conserved regions of Rad and Gem/Kir as a mRNA that was repressed by lipopolysaccharide in mice (10). Rem2 mRNA is expressed in rat brain and kidney and possesses a novel cellular localization signal that is different from most Ras-related proteins (11). Ges is expressed in the endothelium and functions as a promoter of cytoskeleton reorganization (12). All of these G proteins possess several structural features that are distinct from other Ras-related GTPases, including major NH2- and COOH-terminal extensions, a lack of typical prenylation motifs, and several nonconservative changes in the sequence of the GTP-binding domain. The NH2 terminus of Rad is extended by 88 amino acids, and the COOH-terminus is extended by 31 amino acids as compared with Ras. As a result of the lack of a prenylation motif, Rad is primarily a cytosolic protein that associates with the cytoskeleton in a nonlipid-dependent manner (13). Rad, Gem, and Rem differ from each other and from other Ras-like molecules in the pputative effector (G2) domain. They also contain residues in the G3 consensus sequence for guanine nucleotide binding that are divergent from Ras (7). By expression cloning and coimmunoprecipitation, Rad can be shown to interact with CalM, CalMKII (14), and β-tropomyosin (15). These interactions are enhanced by an increase in calcium influx and favor the GDP-bound form of Rad. Overexpression of Rad in 3T3-L1 adipocytes and C2C12 myocytes causes a marked reduction in insulin-stimulated glucose uptake (16). However, the exact function of Rad is still unknown.

Our laboratory has recently identified a novel form of bi-directional interaction between Rad and nm23 (17). In this complex, nm23 acts as both a GTPase-activating protein and a guanine nucleotide exchange factor for Rad, determining the balance between GTP-Rad and GDP-Rad. The first nm23 gene (nm23-M1) was originally identified by subtractive cloning in murine melanoma cell lines as a putative tumor metastasis suppressor (18). Since then, an additional murine nm23 gene, nm23-M2 (19), and five human nm23 genes, namely nm23-H1 (20), nm23-H2 (21), DR-nm23 (22), nm23-H4 (23), and nm23-H5 (24), have been identified. The metastasis suppressor function of nm23 has been demonstrated by both in vivo and in vitro experiments that show reduced incidence of primary tumor formation and a significant reduction in metastatic potential on transfection of nm23-M1 and nm23-H1 cDNA into highly metastatic murine melanoma cells (25) and human breast cancer cells (26), respectively. In addition,...

Received 5/22/00; accepted 1/10/01.

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1 Supported by the NIH Grant DK-45935 (to C. R. K.). P. H. W. is supported by a Scientist Award from the Medical Research Council of Canada. The National Cancer Institute of Canada-Manitoba Breast Tumor Bank is funded by the National Cancer Institute of Canada.

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EFFECTS OF Rad AND Nm23 ON BREAST CANCER CELLS

nm23 transfection inhibits motility of human and murine tumor cells in response to different factors (27). Nm23 possesses several enzymatic activities, including a nucleoside diphosphate kinase activity (28), a histidine kinase activity (29), and a serine protein kinase activity (30). Rad has been shown to enhance the nucleoside diphosphate kinase activity of nm23 and decrease its autophosphorylation (17).

In addition to its potential function on suppression of tumor metastasis, several reports have suggested that nm23 has a role in cell differentiation and proliferation (for review, see Ref. 31). Nm23 H2 has been found to be identical to the c-myctranscription factor, PuF (32). The homologue of nm23 in Drosophila is the Awd (abnormal wing discs) protein (33). Mutation in awd causes abnormal structures of imaginal disc during wing development (34). A correlation between increased nm23 expression and cell proliferation has also been suggested by other investigations. The levels of nm23 expression strictly correlate with cell growth rate and DNA synthesis in the human breast epithelial cell line, MCF-10A (35, 36). Overexpression of nm23 in rat pheochromocytoma PC12 cells enhances nerve growth factor-induced sympathetic neuronal cell differentiation by delaying cell cycle transition and increasing neurite outgrowth (37). Nevertheless, the biochemical mechanism of nm23 action is unknown to date.

In this report, we show that Rad is expressed in some human breast cancer and breast cancer cell lines and that expression is related to features of poor prognosis in vivo. In cultured cells, overexpression of Rad causes a marked increase in growth and increased colony formation in soft agar, and these effects are inhibited by nm23. Moreover, similar effects are seen when these cells are injected into nude mice. These findings suggest that the Rad-nm23 interaction may regulate growth and tumorigenicity of human breast cancer cells.

MATERIALS AND METHODS

Human Breast Cancer Specimens. All of the breast tumor cases used for this study were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As described previously (38), tissues are rapidly collected and processed to create matched formalin-fixed-embedded and frozen tissue blocks for each case. The histology of every sample in the bank is uniformly interpreted by a pathologist in H&E-stained sections from the face of the paraffin tissue block. For each case, interpretation data include an estimate of the cellular composition of the section used for study, tumor type, and tumor grade (Nottingham score; Ref. 39). Steroid receptor status was determined for all of the cases by ligand binding assay performed on an adjacent portion of tumor tissue. Tumors with estrogen and progesterone receptor levels above 3 fmol/mg and 15 fmol/mg of total protein, respectively, were considered estrogen-receptor or progesterone-receptor positive.

Two cohorts of tumors were selected. The first cohort comprised a series of 24 invasive ductal tumors selected only to ensure >25% tumor cells/section. Frozen sections from these cases were cut and used for protein extraction and Western blot analysis in our preliminary survey of Rad expression. The second cohort of 48 invasive ductal carcinomas was selected to comprise tumors with approximately equivalent numbers of each tumor grade [low, intermediate, and high (15, 16, and 17 cases, respectively)] and a range of estrogen receptor, progesterone receptor, nodal status, and tumor sizes (Table 1). Additional selection criteria also included high tissue quality, presence of invasive tumor within >25% of the cross-section of the paraffin block, and, where possible, normal ducts or lobules adjacent to the tumor to allow comparison between tumor and normal tissue.

Immunohistochemistry. Immunohistochemistry was performed using polyclonal anti-Rad antibody (1:200 dilution) and the AEC Kit (DAKO EnVision System, Toronto, Ontario, Canada) following the manufacturer’s instructions. Slides were counterstained with H&E. Rad expression was assessed by brightfield microscopic examination at low (10× objective) magnification with reference to negative control tumor sections run with each batch. Levels of expression were scored semiquantitatively by assessing the average signal intensity (on a scale of 0 to 3) and the proportion of tumor cells showing a positive signal (0, none; 0.1, less than one tenth; 0.5, less than one half; 1.0, greater than one half). The intensity and proportion scores were then multiplied to give an overall score. Tumors with a score equal to or higher than 1.0 were deemed positive.

Cell Culture and Transfection of Cell Lines. Human breast carcinoma cell lines MDA-MB435 expressing pCMV vector or pCMVnm23-H1 construct (cell lines C-100 and H1–177, respectively; Ref. 26) were generous gifts from Dr. Patricia S. Steeg (National Cancer Institute, Bethesda, Maryland). These cells were transfected with pBabe puromycin resistance vector only (Puro) or expressing full-length human WT Rad cDNA, the Rad S105N mutant, the Rad N88 mutant, the Rad C249 mutant, or the full-length cDNA of Gem by calcium phosphate method (14, 15). Stable cell lines were established by selection in puromycin-containing media (2 μg/ml). Cells were maintained in DMEM containing 10% fetal bovine serum in a 5% CO2 environment.

Immunoblotting. Cells grown on a 100-mm dish were washed twice with ice-cold PBS and scraped into 1 ml of lysis buffer as described previously (14). For the preparation of tissue extracts, about 2 mg of normal or tumor human breast tissues from frozen samples were homogenized in 400 μl of lysis buffer. Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Lysates (50 μg) were subjected to SDS-PAGE followed by Western immunoblotting using specific antisera and detection with chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Rad polyclonal antiserum was used at 1:1000 dilution as described (15). Monoclonal antibody against human nm23 H1 was purchased from Santa Cruz (Catalogue #SC-465; Santa Cruz, CA) and used at 1:500 dilution.

Growth Assays. For anchorage-dependent growth assay, 1 × 105 cells were plated/well in multiple 24-well plates and incubated at 37°C for varying times, and the numbers of cells were determined by trypsinization and counting in a hemocytometer or Coulter particle counter. Soft-agar colonization assay was performed using 3 × 104 cells in 0.5 ml of medium containing 0.5% (w/v) agar over a 0.5 ml plug of medium containing 0.5% (w/v) agar. Colonies were counted after 14 days of incubation. All of the data were obtained from three independent experiments with each group represented by triplicate wells.

Cell Cycle Analysis by Flow Cytometry. Cells (1.5 × 106) were plated in a 60-mm dish and grown for overnight. The cells were washed twice with PBS, kept in serum-deprived medium for 84 h, and then reexposed to 10% fetal bovine serum for 12 or 24 h. Cells were collected by trypsinization followed by centrifugation, washed once with PBS, and resuspended in 0.2 ml of ice-cold PBS. The cells were fixed in 70% ethanol [in 50 mm glycine buffer (pH 2.0)]. After at least 24 h of fixation, DNA was stained with 2.5 μg/ml propidium iodide, and the content was analyzed in a FACScan at the Dana-Farber Cancer Institute flow cytometry facility (Boston, MA).

Tumor Growth Assay. A suspension of 5 × 105 (in 0.2 ml of PBS) MDA-MB435 cells expressing vector alone (Puro), WT Rad, or Rad S105N in the absence or presence of coexpression of nm23 was injected s.c. into the left hind flank of 5-week-old female NIH Swiss Nude mice (Taconic, German-

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Table 1  Rad expression status relative to the clinical-pathological features of the cohort of invasive breast tumors studied

<table>
<thead>
<tr>
<th>ERb</th>
<th>Total No.</th>
<th>Rad−ve</th>
<th>Rad+ve</th>
<th>P*</th>
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<tr>
<td>ERb</td>
<td>19 (37.7%)</td>
<td>10 (20%)</td>
<td>9 (18%)</td>
<td>ns</td>
</tr>
<tr>
<td>PRb</td>
<td>22 (44%)</td>
<td>12 (24%)</td>
<td>10 (21%)</td>
<td>ns</td>
</tr>
<tr>
<td>Grade</td>
<td>I</td>
<td>15 (31%)</td>
<td>9 (19%)</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>II</td>
<td>16 (33.5%)</td>
<td>10 (21%)</td>
<td>6 (12%)</td>
<td>0.022</td>
</tr>
<tr>
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<td>17 (35.5%)</td>
<td>11 (23%)</td>
<td>6 (12%)</td>
<td>0.016</td>
</tr>
<tr>
<td>Sizec</td>
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<td>6 (12%)</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>2-5</td>
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<td>17 (35.5%)</td>
<td>5 (10.5%)</td>
<td></td>
</tr>
<tr>
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<td>7 (14.5%)</td>
<td>3 (6%)</td>
<td>4 (8.5%)</td>
<td></td>
</tr>
<tr>
<td>NSd</td>
<td>Unknown</td>
<td>7 (14.5%)</td>
<td>4 (8.5%)</td>
<td>3 (6%)</td>
</tr>
</tbody>
</table>

* P, χ2 test for trend.

b ER, estrogen receptor status; PR, progesterone receptor status; NS, nodal status; ns, not significant.
c Numbers in parentheses represent approximate percentage values.
d Tumor size in cm3.

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RESULTS

Rad Was Highly Expressed in a Tumorigenic Breast Cancer Cell Line and a Number of Breast Cancer Tissues. Many small G proteins may act as positive or negative effectors of cell growth; however, the exact function of the small GTPase Rad remains unknown. To explore a possible role for Rad in breast cancer, Western blot analysis was performed on protein extracts of breast tissue samples from a number of patients with breast cancer. This demonstrated a variable level of Rad expression, from very low or undetectable in most cases to very high in a few (Fig. 1B). To examine Rad expression further, immunohistochemical analysis was conducted in a second cohort of 48 human breast cancer specimens, selected to allow exploration of the relative cellular expression of Rad in breast. These included normal and neoplastic components in the majority of cases. This revealed that Rad was expressed within both normal breast epithelium and tumor cells. Rad expression was often present and at high levels in normal ductal and lobular epithelium (23/30 cases). By contrast, expression was commonly lost in adjacent invasive carcinoma in the same patient (8/30 Rad+ve; P < 0.0001; Wilcoxon matched pairs test). Nevertheless, where persistence of high levels of Rad expression was present in breast tumors, this was associated with higher grade, larger size, and extensive axillary nodal involvement (n = 48; P = 0.035, P = 0.016, P = 0.022, respectively; χ² test for trend; Fig. 1C; Table 1).

In an attempt to find cell models to study Rad action further, several cultured cell lines were screened for Rad expression. Whereas the levels of Rad expression were low in the C2C12 myocytes and in the breast cancer cell line MDA-MB435, Rad was highly expressed in MDA-MB231 breast cancer cells (Fig. 1A).

Rad Induces Both Anchorage-dependent and Anchorage-independent Growth in Breast Cancer Cells, and Nm23 Blunts These Effects. Our laboratory has recently demonstrated a novel form of bi-directional interaction between Rad and nm23 (17). To determine whether the Rad-nm23 interaction may have a role in growth of breast cancer cells, we established cell lines overexpressing Wt Rad in the presence or absence of coexpression of nm23 in the MDA-MB435 breast cancer cells, which have a low level of endogenous Rad. Drug-resistant clones were pooled together, and both anchorage-dependent and anchorage-independent growth were monitored in these cells. Rad expression resulted in accelerated cell growth on tissue culture plastics by almost 3-fold, and an increased number of colonies formed in soft agar by almost 2.5-fold (Fig. 2, A and B). Coexpression of nm23 slightly increased the basal levels of growth in both of these in vitro assays, although these increases were not statistically significant. More strikingly, however, nm23 almost completely blocked the growth-promoting effects of Rad. Similar results were obtained from isolated individual clones (data not shown). These data suggested that Rad was able to accelerate both anchorage-dependent and anchorage-independent growth in the breast cancer cells and that this effect may be modified by its interaction with nm23.

The Growth-promoting Effects of Rad Required Its NH₂- and COOH-terminal Regions. To further study the structure-function relationship of Rad on cell growth, we generated several cell lines with either Wt Rad or a series of mutants of Rad in the MDA-MB435
cells. These mutants included the Rad S105N mutant, the Rad N88 mutant, and the Rad C249 mutant (Fig. 3A). The Rad S105N mutant contained a Ser to Asn mutation at position 105, which is analogous to the S17N mutation in Ras and results in a loss of GTP-binding activity favoring GDP binding (15). The other two mutants, Rad N88 and Rad C249, have deletions in the NH2-terminal 88 amino acids or the COOH-terminal 59 amino acids (residues 249–308), respectively. Both regions have been shown to be important for CaM binding (14). These constructs were transfected into the MDA-MB435 cell with or without coexpression of nm23. Western blotting revealed an over 100-fold overexpression for both Wt Rad and Rad S105N, about a 40-fold increase for Rad C249, and about a 30-fold increase for Rad N88. The extra bands comigrating with Rad C249 and Rad N88 were nonspecific and were not shown when 125I-labeled protein A detection system was used (14). The relatively lower levels of expression of the latter two were consistent with our previous studies demonstrating that the NH2 terminus and possibly the COOH terminus of Rad may be critical for antibody recognition, protein expression, and/or stability (Ref. 14; Fig. 3B).

Analysis in tissue culture revealed that Wt Rad, as well as the Rad S105N mutant, were able to accelerate cell growth (Fig. 3C, Left Panel). This occurred primarily by shortening the lag time required for entering exponential growth, rather than altering the doubling time (Fig. 3, D and E). Interestingly, the GDP-bound form of Rad (S105N) had an effect equal to or greater than that of the Wt protein. This is similar to our previous observation that Rad S105N is more potent in interacting with CaM, CaMKII (14), and tropomyosin (15), all of which favor the GDP form of Rad. Cells overexpressing either the NH2- or COOH-terminal truncation mutant of Rad, in which their interactions with CaM were affected, did not show any change in lag time as compared with the control cells. Similar structure-function relationships were observed in the soft agar colony assay (Fig. 3F). Overall, coexpression of nm23 increased slightly, but not significantly, both anchorage-dependent and anchorage-independent growth as shown in both Fig. 2 and Fig. 3. These data agreed with other investigations on a positive correlation of levels of nm23 expression and cell growth rates (35). Taken together, our data suggested that the growth-promoting effects of Rad might be mediated through its NH2- and COOH-terminal regions and CaM binding, rather than being dependent on the GTPase activity of Rad. Gem, another member of the Rad family of GTPases that binds CaM (40), was also able to shorten the lag time when transfected into the MDA-MB-435 cells (Fig. 3D). However, it was unable to promote colony formation in soft agar (Fig. 3F). No significant difference was observed in growth rates of all cell lines during exponential growth (Fig. 3E). Again, coexpression of nm23 blunted the growth-accelerating effects of Rad in these cells (Fig. 3, D–F).

Rad Promoted Cell Growth by Accelerating Cell Cycle Transitions. Potential mechanisms by which Rad might regulate cell growth in vitro include induction of the expression of autocrine factors, increasing plating efficiency, and/or acceleration of cell cycle. To see if Rad induced the expression of autocrine factors, we collected conditioned media from the Puro, Rad Wt, or Rad S105N cultures, added them to control Puro cells, and measured growth. No significant difference was found among these culture media (data not shown). In addition, we treated the cells with mitomycin-C to prevent DNA replication and then replated these cells into tissue culture plates to look for the possibility of changing the plating efficiency of cells by Rad. Again, no difference in plating efficiency was observed among the Puro, Rad Wt, and Rad S105N cells (data not shown). Finally, we determined if Rad had any effect on cell cycle transition. The cells were synchronized by 84 h of serum-starvation (Fig. 4, A–C) and then treated with serum for 12 or 24 h, and cell cycle distributions were determined by flow cytometry of propidium iodide-stained cells. Overexpression of Wt Rad or the S105N mutant caused a large portion of cells to shift into S/G2/M phases after 24 h of serum treatment (Fig. 4, H and I). This suggests that the mechanism by which Rad promotes cell growth involves, at least in part, induction of cell cycle transitions rather than secretion of some autocrine growth factors or change in plating efficiency.

The Rad-Nm23 Interaction Regulated Tumor Formation in Nude Mice. The above experiments indicate that Rad is able to increase numbers of colonies formed in soft agar, and this effect is blunted in the presence of nm23 (Fig. 2B and Fig. 3F). To determine whether Rad and/or nm23 could affect the growth of tumors derived from human breast cancer cells in vivo, a suspension of 5 × 105 MDA-MB435 cells expressing either Wt Rad or Rad S105N in the absence or presence of coexpression of nm23 was injected s.c. into the left hind flank of 5-week-old, female NIH Swiss Nude mice, and tumor growth was monitored. Mice that received cells overexpressing either Rad Wt or Rad S105N in the absence of nm23 developed larger tumors than mice receiving the control cells (Fig. 5, A and B). A significant increase was observed in the percentage of Rad Wt or Rad S105N mice that developed detectable tumors at early times after injection (day 33 and day 27; Fig. 5C). Furthermore, by day 33 there was a significant increase in the size of tumors in the Rad Wt positive/nm23 negative group relative to the corresponding Puro control (Fig. 5D). This is in agreement with the in vitro data that also showed Rad affecting the early time points of cell growth (Fig. 3D). Interestingly, as in the in vitro experiments, the growth-promoting
effect of Rad was blocked in the cells coexpressing nm23, suggesting that nm23 may play a role as a dominantly negative regulator for Rad in tumor growth (Fig. 5, A, B, and D). Moreover, nm23 caused significant decreases in the probabilities of tumor formation at days 27 and 33, and this effect appeared to be independent of the presence of Rad WT or Rad S105N (Fig. 5C). With only one exception, spontaneous metastases were not evident at 67 days after injection, at which time the mice were sacrificed under animal welfare guidelines, because of the primary tumor sizes of mice.

The histological appearance of tumor resembled typical medullary carcinoma, consistent with the origin of the cells (41). Histological analysis of cross-sections of tumors also revealed that Rad WT tumors showed a relatively larger area (approximately 30–40% of the cross-section area) with degenerative changes (picnotic nuclei, necrosis, and hemorrhage) as compared with the Puro controls that contain only about 10% of the area involved with degenerative changes (Fig. 6).
DISCUSSION

Ras-related GTP-binding proteins comprise a superfamily of molecules that play important roles in a wide variety of cellular processes including cell proliferation and differentiation (42), apoptosis (43), intracellular vesicular trafficking (44), cytoskeletal rearrangement (45), cell cycle regulation (46), and glucose transportation in cells (16, 47). Activating mutations of Ras occur in about 30% of all of human tumors, including breast cancer (48, 49). Rad itself has not been implicated in tumor development, although another member of the Rad family, Gem, was originally identified by its overexpression in mitogen-stimulated T lymphocytes and v-abl-transformed pre-B cells (8, 9). In the present study, we have demonstrated not only that Rad is present in some human breast cancers, but also that it is able to accelerate growth of breast cancer cells in vitro and increase the tumorigenicity of these cells when injected into nude mice.

Rad was originally identified to be highly expressed in the skeletal muscle of some type-2 diabetic humans and is normally also highly expressed in heart and lung (7). When overexpressed in skeletal muscle, Rad alters contractility and potentiates high fat diet-induced insulin resistance. In transgenic mice with overexpression of Rad in the heart, there is cardiac hypertrophy and an increase in metabolic

Fig. 5. Effects of Rad and nm23 on tumor growth in nude mice. Five-week old NIH Swiss Nude mice received injections in the left hind flank with $5 \times 10^5$ cells expressing either Wt or mutant Rad with or without coexpression of nm23. A, shows representative mice from each treatment group at day 67 after injection. Arrows indicate tumors. B, growth curves of tumors in nude mice. Data are presented as average volume of tumors from mice receiving either control or Rad overexpressors in the presence or absence of coexpression of nm23 ($n = 10$ or 11). C, percentage of mice with visible tumors; * = $P < 0.05$ as determined relative to corresponding Puro control, statistical significance was determined using the Fisher’s exact test; † = $P < 0.05$; †† = $P < 0.001$ as determined relative to corresponding nm23 negative. D, distribution of individual tumor volumes at day 33. Significance was determined relative to the control using a nonparametric Mann-Whitney test; * = $P < 0.05$; ** = $P < 0.01$.

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Moreover, epidemiological evidence has suggested hyperinsulinemia and insulin resistance found in type-2 diabetes mellitus are risk factors for the development of breast cancer (50, 51). In vivo experiments using euglycemic, hyperinsulinemic clamps have shown that insulin is able to stimulate Rad expression (52). This raises the possibility that Rad overexpression found in some patients with type-2 diabetes mellitus may be the result of hyperinsulinemia rather than the cause of insulin resistance. Taken together with the data presented in this study, which indicate a growth-accelerating role of Rad in human breast cancer, these data suggest that in type-2 diabetes, Rad may be more closely related to changes in cell growth than changes in cell metabolism.

Studies on the structure-function relationship reveal that the growth-promoting effects of Rad in vitro may be mediated through its NH₂- and COOH-terminal regions. Previously (14), we have identified a CaM-binding region in residues 278–297 at the COOH terminus of Rad and a potential negatively regulatory region of Rad-CaM interaction in the NH₂ terminus of Rad which, when removed, increases the binding of Rad to CaM. In the current study, we found that deletions of either the CaM-binding domain (C249) or the CaM-inhibitory domain (N88) of Rad results in losing its growth-promoting effect. Gem, which can also bind CaM (40), also accelerated breast cancer cell growth in vitro but differed from Rad in its inability to promote colony formation in soft agar. These data suggest that interaction of these GTPases with CaM through their COOH-terminal extensions may be important for their ability to accelerate anchorage-dependent cell growth, although additional sequences in the NH₂ terminus may also be critical. It has been shown that CaM regulates the G₁-S transition of the cell cycle by increasing the activities of cyclin-dependent kinase 4, cyclin-dependent kinase 2, and retinoblastoma protein phosphorylation (53). In this study, we also found that overexpression of Rad in the MDA-MB435 cells results in acceleration of cell cycle transitions in response to serum stimulation. Taken together, these data suggest that Rad may regulate the growth of breast cancer cells through its interaction with CaM and an acceleration of cell cycle transition.

Another potential mechanism by which Rad may regulate cell growth is related to changes in cytoskeletal adhesion and cell motility. This is suggested by its ability to interact with CaM, CaMKII (14), and β-tropomyosin (15). Tropomyosin clearly plays a role in contraction and cytoskeletal organization, both of which contribute to cell motility (54). CaM and CaMKII have also been shown to be involved in calcium-mediated cell movement (55). Interestingly, there are several lines of well-documented evidence concerning the involvement of small G proteins in cell adhesion and migration (for review, see Ref. 56). Aberrations in these events lead to cell transformation, tumor invasion, and metastasis; e.g., the Rho family proteins, including cdc42, Rac1, and RhoA, have been suggested by multiple studies (57, 58) to play an important role in cytoskeletal rearrangements, cell adhesion, tumor invasion, and metastasis. It seems likely that Rad may regulate growth of tumor cells via similar mechanisms used by the Rho family small G proteins. This is an interesting topic for future studies.

Data from our in vitro and in vivo studies suggest that nm23 may act as a dominant negative regulator of Rad and that coexpression of nm23 with Rad abolishes its growth-promoting effects. Nm23 possesses several enzymatic activities and mediates a number of biological functions, including proliferation, differentiation, cell motility, and suppression of metastasis (31). We have demonstrated previously (59) a coordinate, bi-directional, and bimolecular interaction between Rad and nm23. In the present study, we find that this interaction appears to play a significant role in control of tumor cell growth. This
is particularly interesting because the expression of nm23 gene has been shown to inversely correlate with metastatic potential in several tumor types (60, 61). To date, the exact mechanism of these effects has been unclear. It has been shown that nm23 may mediate part of its effects by altering cell motility (27). Taken together with the fact that Rad may also affect motility via interactions with CaM, CaMKII (14), and β-tropomyosin (15), it is possible that the Rad-nm23 complex acts in a coordinated manner to regulate growth of tumor cells via pathways involved in cytoskeletal organization and cell motility. Furthermore, in this complex, nm23 functions as a GTPase-activating protein and a guanine nucleotide exchange factor for Rad, determining the balance between GTP- and GDP-Rad (17). However, the GTPase activity of Rad does not appear to be critical to its effects on in vitro growth of breast cancer cells because this effect appears to favor the GDP-bound form of Rad (the S105N mutant) more than the WT protein. This is similar to the ability of Rad to interact with CaM, CaMKII, and tropomyosin, all of which favor the GDP form of Rad (14, 15). However, both the WT Rad and the S105N mutant have similar effects on promoting tumor growth in vitro. These phenomena may be explained by having distinct cellular environments between in vitro and in vivo, which may differentially regulate the activities of Rad and nm23.

In the present study, we have also shown that Rad expression in cell lines significantly increases the tumor growth and the percentage of mice that develop tumors in vivo at early times after injection of breast cancer cells. This agrees with the in vivo studies where we have observed that high levels of Rad expression are significantly associated with several features indicative of more aggressive tumors, including larger size, higher grade, and more extensive nodal metastasis. Interestingly, the latter features are also those with which loss of nm23 was initially associated (62). However, whereas subsequent investigation has not always confirmed these associations (63), in some of the larger studies, a relationship between persistence of nm23 and longer disease-free and metastasis-free survival has emerged (60, 61). Our current findings, that the positive effects of Rad on tumor growth can be abrogated by interaction with nm23 and the implication that the interaction between these proteins may be significant in prognosis, may be important in resolving the discrepancies between such studies to determine the role of nm23. However, a more extensive study on a much larger cohort of cases than the cohort used in this study will be required to determine the in vivo significance in human tumors of an interaction with nm23 and the prognostic significance of Rad expression.

In summary, we have shown that some human breast cancers express the small G protein, Rad, and that Rad is able to regulate growth of breast cancer cells both in vitro and in vivo. Furthermore, we have shown that this effect of Rad is blocked by coexpression of nm23. These results suggest a novel mechanism by which interaction between Rad and nm23 may play an important role in the regulation of growth and tumorigenicity of breast cancer. Rad may also provide both a new diagnostic test for staging of breast cancer and a new therapeutic target for its treatment.

ACKNOWLEDGMENTS

We thank S. E. Curtis for assistance in injection of cells into nude mice. We are also grateful to T-L. Azar and J. Konigsberg for excellent secretarial assistance.

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