Rad6 Overexpression Induces Multinucleation, Centrosome Amplification, Abnormal Mitosis, Aneuploidy, and Transformation

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ABSTRACT

We have isolated by differential RNA display a cDNA that is up-regulated in metastatic mammary tumor lines. This cDNA corresponds to HR6B, the yeast homologue of Rad6, a ubiquitin-conjugating enzyme, and a key player in postreplication repair and induced mutagenesis in the yeast. We show that Rad6 protein expressed in metastatic tumor lines is wild type and functional, because it is able to catalyze the transfer of ubiquitin to histone H2b and is predominantly localized in the nucleus as compared with cytoplasmic localization in normal or nonmetastatic mammary cells. This pattern of Rad6 protein expression/localization is not restricted to breast cancer cell lines, because human breast carcinomas display similar patterns of Rad6 up-regulation and nuclear localization suggesting that deregulation in expression of Rad6 may be an important step in transformation to malignant phenotype. Constitutive overexpression of exogenous human HR6B cDNA into normal-behaving MCF10A human breast epithelial cells induced cell-cell fusion that resulted in generation of multinucleated cells, centrosome amplification, multipolar mitotic spindles, aneuploidy, and ability for anchorage-independent growth. Double immunofluorescence labeling experiments demonstrated the colocalization of Rad6 protein with γ-tubulin on centrosomes. This physical association of Rad6 with centrosomes is maintained throughout the interphase and mitotic phases of the cell cycle. The Rad6 protein exhibits notable alterations in distribution during interphase and mitotic stages of the cell cycle that are compatible with its function as a transcription factor. These findings suggest that Rad6 is an important ubiquitin-conjugating enzyme that may play a significant role in the maintenance of genomic integrity of mammalian cells and that an imbalance in the levels and activity of Rad6 could lead to chromosomal instability and transformation in vitro.

INTRODUCTION

Mutagenesis is considered to be a major pathogenetic factor in the progression of human epithelial neoplasia because mutations are thought to inactivate cellular defenses against uncontrolled proliferation and cell migration. Some of these mutations are likely to have occurred during physiological cell replication as a result of rare failures to correct DNA synthesis errors (1). High accuracy is maintained by the combined action of mechanisms that include 3’ to 5’ exonuclease editing of mismatched nucleotide insertions, DNA polymerase preference for correct base-pairs, and postreplication mismatch repair. Despite demonstration of numerous genomic alterations in human breast carcinoma cells, little is known about the mechanisms(s) responsible for genetic instability.

The Rad6 group is concerned with postreplication or “error-prone” repair (2). The Rad6 gene encodes a M, 17,000 protein (3), which is one of a group of ubiquitin-conjugating (E2) enzymes (4) that covalently add ubiquitin to selected lysine residues. The Rad6 pathway appears to be regulated by post-translational modification of target proteins with ubiquitin, which commits them to rapid proteolysis. The Rad6 gene of Saccharomyces cerevisiae is required for a variety of cellular functions including DNA repair, induced mutagenesis, and sporulation (5, 6). rad6 mutant phenotypic effects include slow growth, severe defects in induced mutagenesis, extreme sensitivity to UV, X-ray, and chemical mutagens, and hypersensitivity to antifolate drug metabolites (7). The diversity of the phenotypes of rad6 mutants suggests that the Rad6 gene product is central to cell cycle regulation. All of the functions performed by the Rad6 protein appear to result from ubiquitination, because replacement of the conserved Cys 88 with serine produces a totally null phenotype. Although all of the E2s characterized to date are structurally related, they fall into several functionally distinct categories. The yeast CDC34 (UBC3) E2 is required for G1 to S phase transition of the cell cycle (8), whereas the yeast Rad6/UBC2/E2 is involved in a variety of processes including DNA repair, mutagenesis, and cell proliferation (6).

Rad6 is highly conserved among eukaryotes. Two closely related human DNA repair genes, HRHR6A and HRHR6B (human homologues of yeast Rad6), encode ubiquitin-conjugating enzymes (E2), and complement the DNA repair and UV mutagenesis defects of the S. cerevisiae rad6 mutant (9). HRHR6A and HRHR6B share 95% identical amino acid residues and are localized on human chromosome Xq24-q25 and 5q23-q31, respectively (10). Inactivation of the gene encoding the mammalian homologue of yeast Rad6, HR6B, in mice leads to male sterility (11). Experiments described here show for the first time that HR6B is overexpressed in mouse and human breast cancer lines and tumors, and that constitutive overexpression of HR6B induces formation of multinucleated cells, centrosome amplification, abnormal mitosis, aneuploidy, and transformation.

MATERIALS AND METHODS

Mouse Mammary Metastasis Model. Tumor sublines 67, 168, 66c4, 4T07, and 4T1 were isolated from a single spontaneously arising mammary tumor from the Balb/c3H mouse (12). The subpopulations were classified based on their ability to metastasize spontaneously from the orthotopic site. Subline 66c4 spontaneously metastasizes to the lung via the lymphatics, whereas subline 4T1 spontaneously metastasizes to the lung and liver via the hematogenous route (12). Sublines 67, 168FAR, and 4T07 are highly tumorigenic but fail to metastasize from primary lesions through different end points in the dissemination; however, injection of 4T07 cells into the tail vein results in the formation of tumor nodules in the lung and liver (13).

Human Breast Epithelial Cell Lines. MCF10A, MCF10AT, MCF10ADCIS.com, MCF-7, and MDA-MB-231 cells were also used. MCF10A cells are normal-behaving human breast epithelial cells that lack tumorigenicity in nude mice and are unable to support anchorage-independent growth (14), whereas MCF10AT and MCF10ADCIS.com are T24-Ha-ras-transformed MCF10A cells that produce preneoplastic (15) or DCIS (16) lesions, respectively, when implanted in immunodeficient mice. MCF-7 (tumorigenic) and MDA-MB-231 (metastatic) human breast cancer cells were

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3 The abbreviations used are: DCIS, ductal carcinoma in situ; Gas, growth arrest-specific; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; CPE, colony-forming efficiency; PCNA, proliferating cell nuclear antigen.
obtained from Cell Resource Core of Karmanos Cancer Institute or purchased from American Type Culture Collection (Manassas, Virginia), respectively.

**Cell Culture.** Mouse mammary tumor sublines were grown in DMEM supplemented with 5% FCS, 5% newborn calf serum, 1 mM nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. MCF10A and MCF10AT cells were maintained in DMEM/F-12 medium supplemented with 2.5% horse serum, 0.02 µg/ml epidermal growth factor, 0.5 µg/ml hydrocortisone, 10 µg/ml of insulin, 0.1 µg/ml cholera toxin, 100 units/ml penicillin, and 100 µg/ml streptomycin. MCF10A/ADCIS.com and MDA-MB-231 cells were grown in DMEM supplemented with 10% FCS, and MCF-7 cells were maintained in DMEM/F-12 supplemented with 5% FCS and 10 µg/ml insulin.

**mRNA Differential display.** Total cellular RNA was isolated using the Trizol reagent (Life Technologies, Inc., Grand Island, NY) following the manufacturer’s protocol. Before mRNA differential display, DNase I treatment was performed on the RNA samples using the Message Clean kit (GenHunter Corp., Nashville, TN) and differential display of cDNA fragments was performed as essentially described by Liang et al. (17). The radioactive PCR products were electrophoresed on 6% acrylamide-8 M urea gels in Tris-borate/EDTA buffer (pH 8.0) at 35 W for 3 h, and the gel was dried and subjected to autoradiography. Selected bands were identified, and the corresponding slices on the dried gels were excised and eluted by incubation in 50 µl of 10 mM Tris-HCl-1 mM EDTA buffer (pH 8.0) at 60°C for 1 h. Gel pieces containing differentially displayed bands of interest were reamplified by PCR, subcloned into pCR-telomeric repeat amplification protocol vector and sequenced using primers provided in the pCR-telomeric repeat amplification protocol cloning system (GenHunter Corp.).

**RT-PCR Analysis of HR6B/E2F8/Rad6 Expression.** Total RNAs (2 µg) from 67, 168FAR, 66cl4, and 4T1 tumor sublines, and normal BALB/c mouse liver using the forward and reverse primers 5'-AGGTGACCGT-3' and 5'-GGAATCTCGCTCCTGGAAGA-3' (accession no. NM_009458), respectively. PCR amplification was performed for 25 cycles at 95°C for 1 min, 52°C for 2 min, and 72°C for 3 min. The PCR products were additionally characterized by Southern blot analysis using: (a) the 240-bp cDNA fragment isolated from differential display; and (b) a full-length HR6B cDNA prepared from the normal human breast epithelial cell line MCF10A using primers +330/+348 and +935/+914 (Accession no. M74525). The amplified cDNAs were sequenced by Cyclist DNA sequencing kit (Stratagene, La Jolla, CA) and sequence data subjected to similarity search at nucleotide and amino acid levels using the GenBank databases.

**RT-PCR of Rad6 Antibody and Western Blotting.** Antibody to Rad6 was generated by multiple immunization of New Zealand White rabbits with a synthetic peptide (K plus amino acid residues 131–152, Accession no. NM_009458) that is conserved 100% in mouse and human HR6B, and 91% in human HR6A. For Western blotting, cell lysates from exponentially growing mouse and human breast cells were prepared in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml each of leupeptin, pepstatin, aprotinin, and 1 mM sodium orthovanadate, and proteins (50 µg) from each lysate were separated by SDS-PAGE and transblotted onto Immobilon P membranes. The blots were stained with anti-Rad6 antibody. After detection, blots were stripped and reprobed with a rabbit polyclonal Gasl protein antibody, a kind gift from Dr. G. Del Sal (International Center for Genetic Engineering and Biotechnology, Consortium for Interuniversity, Biotechnology Laboratories, Trieste, Italy). Loading of protein was monitored by reprobing stripped membranes with mouse anti-β-actin antibody. Rad6 (and Gas1) and β-actin protein bands were visualized with antirabbit or antimouse IgG coupled to HRP, respectively, using enhanced chemiluminescence kit (Amershams, Arlington Heights, IL). The relative amounts of Rad6 (HR6A/HR6B) protein(s) to β-actin bands were quantitated with a scanner-densitometer (Molecular Dynamics).

**Immunochemistry.** Cryostat or formalin-fixed, paraffin-embedded human breast carcinoma tissue sections were incubated with anti-Rad6 antibody followed by biotinylated antirabbit IgG secondary antibody and HRP-conjugated streptavidin. Nuclei were counterstained with hematoxylin. Control sections were stained with secondary antibody only.

**Rad6-associated Histone H2B Ubiquitination Activity.** Cell extracts of mouse tumor sublines 67, 168FAR, 4T07, 4T1, and 66cl4 containing equivalent amounts of total protein (100 µg) were immunoprecipitated with anti-Rad6 antibody or with an equivalent amount of normal rabbit IgG. Immune complexes were pelleted after incubation with protein A/G-Sepharose, washed in lysis buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride], and the washed pellets rinsed with reaction buffer [50 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl2, 2 mM ATP, 0.2 mM DTT, 100 µg/ml BSA]. The rinsed pellets were resuspended in the same buffer supplemented with 0.5 µg Histone H2B (Sigma Chemical Co., St. Louis, MO), 0.5 µg ubiquitin (Sigma Chemical Co.), and 0.2 µg ubiquitin activating enzyme, E1 (Boston Biochem, Boston, MA) and incubated for 30 min at 30°C. Some reactions were performed with the same assay buffer but lacking ATP. The reaction mixtures were run on reducing 17% polyacrylamide gels, electroblotted, and subjected to Western analysis with mouse antiguaiacylase antibody (Zymed Labs). Ubiquitinated histone H2B and free ubiquitin were visualized with antimonous IgG coupled to HRP using an enhanced chemiluminescence kit (Amershams Corp.).

**Generation of Stable HR6B Transfectants and Analysis.** Stable wild-type HR6B cDNA was subcloned into the BamHI site of the mammalian expression vector pCMVneo (Promega Corp., Madison, WI) for high-level expression under the transcriptional control of the cytomegalovirus promoter. Circular plasmid DNA (either empty vector or sample construct) was transfected into MCF10A cells by Mirus Trans IT-I transfection reagent (PanVera Corp., Madison, WI). Stable transfectants were selected by resistance to G418 selection (500 µg/ml) for 3 weeks. Selected resistant colonies were cloned, expanded, and maintained in the presence of G418. Expression of the transfected gene was monitored by RT-PCR with primers specific for the vector and the exogenous gene on DNase-treated RNA samples, Northern, and Western blot analysis.

**Growth in Soft Agar.** Vector-transfected or HR6B-overexpressing MCF10A clones (2 x 104 cells) were seeded in 2 ml of 0.33% agar in...
DMEM-F12-supplemented medium as described above for propagation of MCF10A cells. This suspension was layered over 1 ml of 0.9% agar medium base layer and dishes incubated at 37°C in 5% CO₂/95% O₂ for 4 weeks with twice weekly medium changes. All of the cultures were examined 24 h after plating, and cell aggregates that might bias final results were marked. Plates with >10 aggregates were discarded. CFE was calculated by dividing the number of colonies >50 μm (sized using a calibrated ocular grid) by the number of cells seeded. Ten microscopic fields were counted to calculate the total number of colonies/well from the whole well (19). Reported values are the average count from triplicate wells. The number of colonies in different size ranges (50–100 μm and >100 μm) was calculated in the same manner.

**RESULTS**

**Isolation and Sequence Analysis of Rad6.** Taking advantage of a genetically related mouse mammary metastasis model system, we compared the cDNAs differentially displayed from mRNAs of mouse mammary tumor subpopulations 67, 168FAR, 66cl4, 4T07, and 4T1 of variant metastatic capacities. Two cDNAs that were overabundantly expressed or down-regulated in the most metastatic cell line, 4T1, were isolated and additionally characterized by sequence and expression analysis (Fig. 1a). A BLAST search for sequence homologues in the GenBank database revealed that the abundantly expressed 240-bp cDNA is the mouse homologue of the yeast ubiquitin-conjugating enzyme Rad6 and matched 93% and 100% with the human HR6B (Accession no. M74525) and mouse E2B (Accession no. NM_009458) genes, respectively, whereas the down-regulated 290-bp cDNA matched 100% with the mouse gene Gas1 (18).

**HR6B mRNA and Protein Are Overexpressed in Metastatic Mammary Tumor Cells.** To test whether the pattern that appeared on differential display fingerprints faithfully reflects the real expression profile of HR6B/E2B transcript in the mouse mammary tumor subpopulations, 20 μg total RNA isolated from sublines 67, 168FAR, 66cl4, 4T07, and 4T1 cells were subjected to Northern analysis using the 240-bp Rad6 cDNA as the probe. Weakly hybridizing signals were detected, suggesting the presence of very low steady-state levels of HR6B mRNA in these cells. For RT-PCR analysis of HR6B expression, oligonucleotide primers designed (encompassing base 17–574) to amplify the entire coding region were used. A 588-bp fragment was detected in all of the tumor sublines, and the identity of the 588-bp fragment was confirmed by Southern blot hybridization to both the 240-bp Rad6 cDNA (isolated from differential display) and the full-length human HR6B cDNA amplified from MCF10A cellular RNA (data not shown). The relative levels of HR6B mRNA expression in the mouse mammary tumor sublines were confirmed by RT-PCR using primers for the mouse Rad6 (+17/+33 and +114/+97) and GAPDH cDNAs (+186/+206 and +320/+302), and PCR conditions that yielded detectable products with a minimum number of cycles. Results of RT-PCR (Fig. 1b) are in agreement with those from differential display (Fig. 1a). HR6B mRNA levels in 4T07 and 4T1 cells were 35-fold higher than line 67, and approximately 15–20-fold, respectively, higher than lines 168FAR and 66cl4 (Fig. 1b).

Cell lysates prepared from mouse mammary tumor sublines (67, 168FAR, 4T07, 66cl4, and 4T1) or human breast cells MCF10A (normal), MCF10AT (preneoplastic), MCF10A/DCIS.com (DCIS lesions), MCF-7 (tumorigenic), and MDA-MB-231 (metastatic) were subjected to SDS-PAGE and Western blot analysis with rabbit anti-Rad6 antibody. As expected for the mouse HR6B/E2B/Rad6 protein, the antibody recognized a M<sub>ᵣ</sub> 17,000 protein from all of the cell extracts. Levels of the Rad6 protein detected in the lysates of mouse tumor sublines are in agreement with those observed by RT-PCR (Fig. 1b). The 66cl4, 4T07, and 4T1 cells expressed ~10-, 15-, and 25-fold higher steady-state levels of Rad6 protein, respectively, when compared with lines 67 and 168FAR (Fig. 1c). The human HR6A and...
HR6B proteins share 95% identical amino acid residues. Because the synthetic peptide used for generation of the HR6B antibody differs from HR6A by only two amino acid residues, levels of Rad6 protein expressed by HR6A and B forms are not distinguishable. Thus, the Mr 17,000 immunoreactive protein(s) detected in the human cells are referred to as Rad6 rather than HR6A or B. Western blot analysis of human breast cells revealed approximately 3–4-fold higher levels of Rad6 protein in MCF10A, MCF-7, and MDA-MB-231 breast cancer cells as compared with MCF10A and MCF10AT cells after normalizing for loading with β-actin antibody (Fig. 1d).

Overexpressed HR6B mRNA Is Wild Type. HR6B cDNAs were amplified by RT-PCR from 67 (low Rad6 expressor), 4T1 (Rad6 overexpressor), and normal BALB/c mouse liver using primers designed to yield full-length HR6B cDNAs as described in “Materials and Methods.” The amplified cDNAs were subjected to direct sequence analysis, and sequences of 67 and 4T1 Rad6 cDNAs compared with those from the normal mouse liver. No alterations were detected (data not shown), and the sequence matched 100% to that reported for the mouse HR6B/E2B/Rad6 mRNA indicating that the Rad6 overexpressed in the highly metastatic subline 4T1 is wild type.

Immunocytochemical Localization of Rad6 Protein in Mammary Tumor Cells and Human Breast Carcinomas. Results of immunofluorescence microscopy not only confirmed the abundant presence of Rad6 protein in metastatic 4T1 cells (Fig. 1c) but also showed a dramatic difference in cellular distribution of Rad6 between normal, nonmetastatic and metastatic breast epithelial cells. Whereas MCF10A and 168FAR cells show predominant localization of Rad6 in the cytoplasm with diffuse or punctate staining in the nucleus, Rad6 protein is predominantly localized as large aggregates in the nuclei of metastatic 4T1 cells (Fig. 2a).

Immunohistochemical localization of Rad6 was performed on cryostat and paraffin-embedded sections from 30 human breast carcinoma tissues and five human breast DCIS lesions, respectively. Examples of the results obtained are shown in Fig. 2b. Low levels of Rad6, predominantly localized in the lumen, were observed in the ducts of normal breast (Fig. 2b, panel A), and in ducts of normal mammary epithelium adjacent to tumor (Fig. 2b, panel E). Breast tissues with adenosis revealed moderate Rad6 immunoreactivity (Fig. 2b, panel D) or invasive cancer (Fig. 2b, panels E and F) exhibited intense Rad6 reactivity. Whereas Rad6 immunostaining is localized in the cytoplasm of cells from normal or benign breast tissues, note the presence of distinct patterns of Rad6 distribution in a DCIS: cytoplasmic staining (long black arrow) in the peripheral cells versus nuclear staining (●) in the core (D). A, B, C, and E, bars, 10 μm; D and F, bar, 4 μm.
Rad6 OVEREXPRESSION INDUCES CHROMOSOMAL INSTABILITY

Fig. 3. Ubiquitin-conjugating activity of endogenous Rad6 in mouse mammary tumor sublines of variant metastatic capacities. Total cell lysates containing 100 μg protein from tumor sublines 67 (Lane 2), 168FAR (Lane 3), 4T07 (Lane 4), 4T1 (Lanes 1, 5, and 7), or 66cl4 (Lane 6) were immunoprecipitated with anti-Rad6 antibody (Lanes 2–7) or with an equivalent amount of corresponding nonimmune IgG (Lane 1) and subjected to ubiquitin conjugation assay in the presence of ATP (Lanes 1–6) or absence of ATP (Lane 7) as described in “Materials and Methods.” Positions of mono- and di-ubiquitinated histone H2b are indicated by * beside position of unconjugated ubiquitin. The blot on the bottom is a Western blot analysis of cell extracts with anti-Rad6 antibody.

stage I, 3, stage II), nuclear in 2 (stage III), and combined nuclear and cytoplasmic in 4 (1, stage 1; 1, stage II; and 2, stage III).

Ubiquitin-conjugation Activity of Rad6 Protein in Tumor Cells.

To determine whether the mouse tumor sublines of variant metastatic capacities and Rad6 expressions differed in their Rad6-mediated ubiquitin-conjugating activity, we tested the ability of Rad6 immunoprecipitated from cell lysates to ubiquitinate histone H2b. Results of Fig. 3 demonstrate both the validity of the anti-Rad6 antibody and the presence of functionally active Rad6 in the extracts of tumor sublines as immunoprecipitable Rad6 from all of the tumor sublines, regardless of their metastatic capacity, had the ability to conjugate one or two molecules of ubiquitin to histone H2b. However, contrary to expected results, metastatic sublines exhibited lower histone ubiquitin-conjugating activity as compared with nonmetastatic sublines that was not proportional to levels of endogenous Rad6. The histone ubiquitin-conjugating activity is dependent on Rad6 enzymatic activity, because assays performed in the absence of ATP or extracts immunoprecipitated with the corresponding normal IgG fail to conjugate ubiquitin to histone.

Stable HHR6B Expression in Normal MCF10A Cells.

to determine the effect of constitutive overexpression of HHR6B, normal behaving breast epithelial cell line MCF10A was stably transfected with HHR6B expression construct. Rad6 expression levels in the G418-selected clones were evaluated by Western blot analysis, RTPCR using primers specific to the vector and the exogenous gene, and Northern analysis. All of the clones overexpressed HHR6B mRNA as compared with MCF10A cells containing the empty vector (data not shown); however, of the six clones analyzed for Rad6 protein expression, four clones showed overexpression of Rad6 protein that ranged from approximately 10–50-fold higher levels than those of vector-transfected MCF10A cells (Fig. 4a). Clones 1 and 5 overexpressing Rad6 mRNA (50-fold higher than vector-transfected parental cells) and protein displayed an increase in the number of cells exhibiting nuclear pleiomorphism, multinucleated phenotypes, and supernumerary nuclei (Fig. 4b). The generations of multinucleated cells are probably the result of both abnormal mitosis and cell-cell fusion. Evidence for the latter event is deduced from the observation that adjacent nuclei of two or more Rad6-overexpressing cells are found frequently to polarize toward one another apparently promoting cell-cell fusion with the cell membranes separating them often becoming less prominent (Fig. 4b). It is interesting to note that whereas parental MCF10A cells showed no cells with polarized nuclei on day 4 of plating, Rad6-overexpressing clones 1 or 5 frequently displayed cells with two or more polarized nuclei that widely ranged from 5 to 25%, which is probably reflective of dynamic cell behavior. Quantitative analysis of multinucleated cells revealed that in contrast to parental MCF10A cells that had <0.4% of cells containing multiple or giant nuclei, both Rad6-overexpressing clones 1 (14.5 ± 4; P < 0.005) and 5 (25.2 ± 8.9; P < 0.001) exhibited a significant increase in the percentage of cells containing multiple or giant nuclei. Although a proportion of these cells undergo senescence, the majority retained their proliferative capacity as observed by expression of PCNA (Fig. 4c). Colocalization of Rad6 with PCNA (Fig. 4c) was observed in some cells implicating the intimate association that Rad6, a DNA repair protein, might have with DNA replication factors during the S phase of the cell cycle.

Constitutive Overexpression of HHR6B Protein Induces Centrosome Amplification and Abnormal Mitosis. Because HHR6B-overexpressing MCF10A cells exhibited an abnormal increase in the number of multinucleated cells and cells with pleiomorphic nuclei, we tested whether these cells displayed alterations in centromere number. Quantitative scoring of centrosomes with anti-γ-tubulin antibodies in mitotic and nonmitotic nuclei revealed the presence of supernumerary centrosomes in constitutively HHR6B-overexpressing MCF10A clones 1 and 5 as compared with cells transfected with the empty vector. Whereas ~98% of parental MCF10A cells contained two or three centrosomes per cell, ~25% of HHR6B-overexpressing clones displayed more than four centrosomes per cell including a significant proportion with >10 centrosomes in clone 5 (Fig. 5, a and b). These cells contained either a single giant nucleus or multiple multinucleated nuclei. Double immunofluorescence labeling and image-merging experiments demonstrated colocalization of Rad6 with centrosomes in interphase cells and at each spindle pole in mitosis (Fig. 5c). Centrosome amplification observed in HHR6B-overexpressing clones was associated with a 4–8 fold increase in the number of multipolar mitosis (more than three and up to six spindle poles) as compared with MCF10A cells transfected with control vector (Fig. 6, a and b).

Rad6 Protein Is Not Associated with Mitotic Condensed Chromosomes. Because Rad6-overexpressing clones exhibit a greater tendency for abnormal mitosis, we determined whether Rad6 protein was associated with mitotic condensed chromosomes. Dividing MCF10A or HHR6B-overexpressing MCF10A cells were fixed and stained for Rad6, and representative cells at different stages during mitosis were examined. Rad6 is distributed fairly uniformly throughout the interphase nuclei either diffusely with notable exception from the nucleolus during G1/G0 phases (Fig. 7, panel A) or focally concentrated in the nucleolus during S and/or G2-phases (Fig. 7, panel B). On entry into mitosis, i.e., after the breakdown of the nuclear membrane, Rad6 diffuses throughout the cytoplasm, and the pattern of fluorescence corresponds to the shape of the cell. However, the fluorescence from Rad6 is clearly reduced in correspondence to the volume occupied by the condensed chromosomes indicating that Rad6 is not associated with DNA during mitosis (Fig. 7, panels C–F). After cell division and reformation of nuclear membranes (Fig. 7, panels G and H), Rad6 protein is redistributed throughout the nucleus suggesting that the protein is concentrated in the nuclei by passage through the nuclear membrane.

HHR6B Overexpression Induces Aneuploidy as Evidenced by SKY. To determine whether the multinucleated phenotypes and abnormal mitosis observed in HHR6B-overexpressing MCF10A cells (Fig. 4) correlated with chromosomal rearrangements, HHR6B-overexpressing clones 1 and 5, and the parental vector-transfected MCF10A cells were analyzed separately by SKY. Although clones 1 and 5 retained the translocation markers t(5;9), t(19;6), and t(3;17), and trisomy 20 observed in parental cells, it is interesting to note that both the HHR6B-overexpressing clones exhibited a tendency to lose chromosomes, because their chromosomal numbers ranged from 42–48 for clone 1 and 31–48 for clone 5 as compared with 46–48 for vector-transfected MCF10A cells (Table 1). Several new aberrations
not observed in parental cells were also found. These included translocations 6;9, 16;1 in 100% of clone 1 and trisomy 1 in 40% of mitotic clone 5 cells.

**HHR6B-overexpressing MCF10A Clones Exhibit Anchorage-independent Growth.** The HHR6B transfectants were characterized in regard to in vitro growth and their potential for transformation. Consistent with previous reports (14), results from soft agar assays showed that the vector-transfected MCF10A cells failed to form colonies in soft agar even if grown for longer periods of time. On the other hand, HHR6B-overexpressing MCF10A cells grew in soft agar as discrete colonies. Under the conditions used in our experiments, HHR6B-overexpressing MCF10A clone 1 had a CFE of ~10% with 50% of the colonies ranging 50–100 μm in diameter. HHR6B-overexpressing clone 5 had a CFE of 40% with ~60% of the colonies ranging from 50–100 μm and a third of them with diameter >100 μm. Interestingly, whereas a majority of the colonies formed by clone 5 cells remained viable and proliferative, colonies formed by clone 1 exhibited an increase in tendency to undergo apoptosis. These data suggest that constitutive Rad6 overexpression confers the ability for anchorage independent growth; however, continued survival and proliferation of the colonies are dependent on additional chromosomal alterations incurred by cells.

**DISCUSSION**

Using a well-characterized mouse mammary metastasis model system, we have demonstrated for the first time that the yeast homologue of Rad6, a ubiquitin-conjugating enzyme and a key player in postreplication repair and induced mutagenesis in the yeast, is overexpressed in metastatic tumor sublines and exhibit predominant localization in the nuclei of metastatic cells as compared with prevalent cytoplasmic distribution in nonmetastatic or normal mammary cells. This abnormal pattern of Rad6 protein expression/localization is not restricted to breast cancer cell lines, because human breast carcinomas (DCIS and...
invasive cancers) display similar patterns of Rad6 up-regulation and nuclear localization suggesting that deregulation in expression of Rad6 may be an important step in transformation to malignant phenotype.

Because the antibody used in our studies probably fails to distinguish between HR6A and B forms of Rad6, it is not clear whether the elevated levels of Rad6 protein observed in metastatic cells are derived from either one or both forms of Rad6 genes. It is also not clear whether the high levels of Rad6 protein and its predominant (aberrant?) localization in the nuclear subcompartment is indicative of its role as a direct contributor of genomic instability and, hence, progression, or whether the presence of elevated levels of Rad6 simply reflect the increased mutation rates in malignant tumors. Interestingly, Rad6 protein exhibits notable alterations in its distribution in the interphase and mitotic nuclei that is compatible with its function as a transcription factor. Stable constitutive overexpression of HHR6B in near diploid normal-behaving MCF10A cells resulted in generation of multinucleated cells, abnormal centrosome numbers, multipolar mitosis, and transformation in vitro. Although the mechanism(s) responsible for generation of multinucleated cells (i.e., abnormal mitosis and/or cell-cell fusion) are yet to be determined, one possibility is that high levels of Rad6 may mediate increased ubiquitin-mediated degradation of proteins on cell membrane.

Whereas a small subset of cancers exhibit genetic instability primarily at the nucleotide level, most breast cancers exhibit instability at the chromosomal level resulting in losses and gains of whole chromosomes, or large portions thereof (22, 23). That Rad6 overexpression results in aneuploidy is confirmed by SKY analysis, because HHR6B-overexpressing clones 1 and 5 were found to have fewer chromosomes ranging from 42–48 or 31–48, respectively, than the parental vector-transfected MCF10A cells that had 46–48 chromosomes. Generation of aneuploidy appears to be an initiation step in these cells, because the parental MCF10A cells are near-diploid and lack the ability to support anchorage-independent growth in soft agar (14). In contrast, Rad6-overexpressing MCF10A cells exhibit a deviation from the normal number of chromosomes, and show centrosome amplification, multipolar mitosis, and the ability to grow in soft agar. Because the clones analyzed overexpress up to 50-fold greater levels of Rad6 than vector-transfected parental cells and ~10-fold greater levels than breast cancer cell lines, it will be interesting to verify whether cells expressing ectopic Rad6 at levels observed in breast cancer cell lines exhibit aneuploidy and whether these effects can be negated by overexpression of mutant rad6.

Aneuploidy is the predominant class of genomic instability found in breast, colorectal, prostate, and other solid cancers in general (24–27). Given the relationship of centrosome function to cell polarity and to maintenance of genomic integrity, understanding the mechanisms that lead to aberrant centrosomes, their interaction with specific proteins, and the degree and nature of centrosomal defects may have predictive value in regard to patient prognosis. Centrosome amplification or dysregulation in centrosome duplication results in assembly of aberrant mitotic spindles that result in misregulation of chromosomes and aneuploidy (28). That Rad6 may play an important role in the maintenance of genomic integrity is strengthened by the observation that it is associated with centrosomes throughout the interphase and mitotic phases of the cell cycle, and displays striking changes in its distribution during different stages of the cell cycle. A noteworthy
consequence of Rad6 overexpression is abnormal mitosis. It is conceivable that the loss of chromosome(s) evidenced by SKY analysis resulted from multidirectional forces impacted on a single chromosome in a multipolar spindle (29). Consequently, the daughter cells would receive abnormal numbers of chromosomes and become aneuploid as supported by our SKY data. These data show that HHR6B overexpression can cause aberrant chromosomal partitioning at mitosis culminating in a catastrophic loss or gain of chromosomes that result in either cell death or survival through malignant transformation.

Centrosomes are abnormal in number, form, and function in a number of human tumors although the mechanisms by which

Fig. 6. Abnormal mitosis in HHR6B-overexpressing MCF10A cells. a. HHR6B-overexpressing MCF10A clone 5 cells were fixed and stained for γ-tubulin (red; A–C, E, and G–I) or DNA with DAPI (blue; D–F), and mitotic cells with established spindle poles were examined. Arrows indicate cells with normal bipolar spindle (A, D, C, and F). Note the presence of monopolar (C), tetrapolar (B, E, and H), tripolar (G), and hexapolar (I) spindles. Image-merging of γ-tubulin and DNA staining is shown in E. Bars, 10 μm. b. Summary of aberrant mitoses induced by HHR6B overexpression. Multipolar (abnormal) mitosis (3 and ≥3 spindle poles) were counted in vector-control (MCF10A-neo) and HHR6B-overexpressing (MCF10A-Rad6 clone 5), and results expressed relative to the total number of mitotic nuclei scored from 300 cells. Data are the means of three independent determinations; bars, ± SD. *, P < 0.005.

Fig. 7. Rad6 protein is not associated with mitotic condensed chromosomes. Dividing MCF10A (A and E–H) or HHR6B-overexpressing MCF10A (B–D) cells were fixed and stained for Rad6, and representative cells at interphase (A, G1/G0; B, S/G2) and different stages during mitosis (metaphase, C and D; anaphase, E and F; and telophase, G and H) were examined. Note the exclusion of Rad6 from the nucleolus in A (→) and its appearance in the nucleolus in B (△), which coincides with its colocalization with PCNA (Fig. 4c). Also note that after the breakdown of the nuclear membrane, Rad6 is distributed uniformly throughout the cytoplasm, and the pattern of green fluorescence corresponds to the shape of the cell. However, the fluorescence from Rad6 is clearly reduced in correspondence to the volume occupied by condensed chromosomes (→), indicating that Rad6 is not associated with DNA during mitosis. After cell division and reformation of nuclear membranes (G and H), note that Rad6 is redistributed uniformly throughout the nucleus. D is a phase contrast micrograph of C.
centrosomal anomalies arise is unknown. Phosphorylation of centrosomal proteins in Drosophila (29) and vertebrates (30, 31) have been reported to influence microtubule nucleation and dynamics at the centrosomes (32). In mammalian cells, cdc2, NIMA, and PLK1 kinases have been implicated in centrosome duplication, maturation, and separation (33–36). Because Rad6 protein is associated with centrosomes throughout the interphase and mitotic phases of the cell cycle, Rad6 protein may play an important regulatory role via regulated ubiquitination and proteolytic degradation of centrosomal proteins. Thus, an imbalance in the levels of Rad6 protein in the cell or that associated with centrosomes could lead to defects in centrosome duplication, maturation, and function, which could induce aneuploidy. Taken together, these data suggest that centrosomes may provide a platform for assembly and functioning of several activities, and that alteration or dysregulation in the activities of specific molecules could directly impact centrosome function as part of tumorigenic process. Identification of natural substrates of Rad6 on centrosomes will help in understanding its role in maintenance of centrosome structure and function, the disruption of which could result in anomalous centrosome amplification and chromosome segregation in tumor cells. These may, in turn, provide new insights in development of new drugs for therapy of tumors with chromosome instability.

In summary, our findings suggest that Rad6 is an important ubiquitin-conjugating enzyme that may play a significant role in the maintenance of genomic integrity of mammalian cells and that an imbalance in the levels and activity of Rad6 could lead to chromosomal instability and transformation in vitro.

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REFERENCES


Table 1 SKY analysis of vector-control versus HR6B-overexpressing clones of MCF10A cells

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*Alterations involving chromosomal translocations are not shown here.*


Rad6 Overexpression Induces Multinucleation, Centrosome Amplification, Abnormal Mitosis, Aneuploidy, and Transformation

Malathy P. V. Shekhar, Alex Lyakhovich, Daniel W. Visscher, et al.


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