The Adenovirus E4orf6 Protein Inhibits DNA Double Strand Break Repair and Radiosensitizes Human Tumor Cells in an E1B-55K-independent Manner*

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The adenoviral protein E4orf6 has been shown to inhibit both in vitro V(D)J recombination and adenoviral DNAconcatenation, two processes that rely on cellular DNA double strand break repair (DSBR) proteins. Most of the known activities of E4orf6 during adenoviral infection require its interaction with another adenoviral protein, E1B-55K. Here we report that E4orf6, stably expressed in RKO human colorectal carcinoma cells or transiently expressed by adenoviral vector in U251 human glioblastoma cells, inhibits DSBR and induces significant radiosensitization in the absence of E1B-55K. Expression of a mutant form of E4orf6 (L245P) failed to radiosensitize RKO cells. E4orf6 reduced DSBR capacity in transfected and infected cells, as measured by sublethal DNA damage repair assay and phosphorylated H2AX (γ-H2AX) levels, respectively. Consistent with the inhibitory effect of E4orf6 on DSBR, expression of wild-type but not mutant E4orf6 reduced recovery of a transfected, replicating reporter plasmid (pSP189) in 293 cells but did not increase the mutation frequency measured in the reporter plasmid. The kinase activity of DNA-PKcs (the DNA-dependent protein kinase catalytic subunit) toward heterologous substrates was not affected by expression of E4orf6; however, autophosphorylation of DNA-PKcs at Thr-2609 following ionizing radiation was prolonged in the presence of E4orf6 when compared with control-infected cells. Our results demonstrate for the first time that E4orf6 expression hinders the cellular DNA repair process in mammalian cells in the absence of E1B-55K or other adenoviral genes and suggest that viral-mediated delivery of E4orf6, combined with localized external beam radiation, could be a useful approach for the treatment of radiosensitive solid tumors such as glioblastomas.

DNA double strand breaks (DSBs) occur naturally during DNA replication and V(D)J recombination but are also produced during the treatment of human malignancies with ionizing radiation (IR) and genotoxic drugs. In mammalian cells, the predominant pathway for repairing DSBs is non-homologous end joining (NHEJ). The DNA-dependent protein kinase (DNA-PK) complex is required for NHEJ. This complex, which includes Ku70, Ku80, and the 450-kDa DNA-PK catalytic subunit (DNA-PKcs), recruits several other repair proteins, including the MRE11/Rad50/NBS1 complex, XRCC4, and ligase IV (1). Despite the large number of proteins and seemingly redundant pathways involved in the processing of DNA damage, targeted gene deletions or mutations of any of the NHEJ proteins in mice results in growth deficiency, immune deficiency from defective V(D)J recombination, hypersensitivity to IR, neuronal apoptosis, and in some cases, tumorigenesis due to increased genomic instability (1, 2). These consequences of defective NHEJ demonstrate the importance of DSBR proteins in maintaining genomic integrity and cellular viability. Conversely, the hypersensitivity of DSBR-deficient cells to IR makes repair proteins an attractive target for radiosensitization of tumor cells with localized beam radiation approaches (3).

Interestingly, adenoviral proteins have recently been shown to interfere with the NHEJ pathway to prevent host cell-mediated ligation of the linear adenoviral DNA, which would otherwise lead to viral DNA concatemer formation (4, 5). During adenoviral infection, E4orf6 cooperates with E1B-55K to target p53 and MRE11 for degradation, preventing a p53-mediated anti-viral response and inhibiting the NHEJ-dependent process of concatemer formation (4—6). It is likely that the ability of E4orf6 and E1B-55K to cooperate in suppressing concatemer formation may have evolved as a means of bypassing host cell defenses against viral DNA replication and productive infection. E4orf6, a 34-kDa protein encoded by open reading frame 6 of the E4 gene, has been reported to interact with DNA-PKcs and to inhibit V(D)J recombination in vitro (7), an NHEJ-dependent process that generates immunological diversity through DNA rearrangements. Since these experiments were performed in 293 cells, which express E1B-55K, it is unknown whether E4orf6 alone can inhibit cellular DSBR or DNA-PKcs activity. The inhibition of NHEJ proteins in suppressing con-
catemer formation and V(D)J recombination makes E4orf6 an attractive tool for inhibiting cellular NHEJ. Considering that radiation therapy is a frequent treatment for solid tumors and proficient DSBR limits tumor cell kill in response to IR treatment (8, 9), the inhibition of DSBR proteins is an attractive approach for tumor radiosensitization (10, 11). Therefore, we examined the ability of E4orf6 to inhibit NHEJ and to radiosensitize tumor cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All cell lines (American Type Culture Collection, Manassas, VA) were incubated at 37 °C with 5% CO2. U251 cells were maintained with RPMI 1640 medium, RKO cells were maintained with McCoys5A, and M059 J and K cell lines were maintained with Dulbecco’s modified Eagle’s medium/F12, each supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. The M059 cell lines were also supplemented with sodium bicarbonate (1.5 g/liter), non-essential amino acids, and sodium pyruvate. RKO stable clones were established by transfection of plasmids (Lipofectamine Plus, Invitrogen) and selection with neomycin (300 µg/ml). Harvested RKO clones were maintained in medium supplemented with 100 µg/ml neomycin.

**Clonogenic Survival Assay**—Clonogenic survival assays were performed as described previously (12). For survival assays involving infections, U251 cells were plated in 35-mm plates in triplicate at equal density, infected the next day at a multiplicity of infection determined to yield maximal infectivity, and irradiated 48 h later with a 137Cs source (dose rate of 4.41 Gy/min) at a range of IR doses. When unirradiated control plates were near confluency (at or near day 7 after IR), the cells were fixed and stained with crystal violet as described previously (12). Crystal violet was solubilized in 33% acetic acid, and the absorbance at 540 nm was measured in triplicate for each well as described by Bernardi et al. (13).

**Immunoblotting**—RKO clones and infected cells were harvested as described previously (12). For E4orf6, E1B-55K, p53, MRE11, and β-actin immunoblots, lysates (60 µg of total protein) were resolved on 12% SDS-PAGE gels, transferred to polyvinylidene difluoride membrane, blocked for 30 min at room temperature, and incubated for 1 h at room temperature with the following antibodies: anti-E4orf6 antibody (monoclonal antibody 3 at 1:100), anti-E1B-55K (9C10 at 1:4), anti-p53 (DO-1 at...
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FIG. 2. Stable expression of functional E4orf6 radiosensitizes RKO cells. The RKO clones (closed circles, neo-mycin (neo) vector; open circles, E4orf6 clone 4; closed triangles, E4orf6 clone 1; and open triangle, E4orf6-L245P clone 2) were plated at low density and treated with ionizing radiation. The surviving fraction was normalized by plating efficiency and plotted on a semilog scale. Error bars represent S.E. values.

RESULTS

Stable Expression of E4orf6 Radiosensitizes RKO Cells—To examine the effects of E4orf6 on human tumor cell radiosensitivity, we first established clones of RKO human colorectal
carcinoma cells stably expressing wild-type E4orf6, a control neomycin resistance gene, or E4orf6-L245P, a non-functional variant during adenovirus infection (15). The wild-type E4orf6 expressed in the RKO cells directed E1B-55K to the nucleus (Fig. 1A) and cooperated with E1B-55K to target p53 and MRE11 for degradation (Fig. 1B), indicating that functional protein was expressed. In contrast, the E4orf6-L245P mutant failed to direct E1B-55K to the nucleus and failed to cooperate with E1B-55K to target p53 and MRE11 for degradation (Fig. 1). Interestingly, reduced levels of E1B-55K protein were observed in the control (neomycin) and L245P cell lines infected with the E4-mutant virus dl1014 (Fig. 1B). Because all the cells were equally infected (data not shown), it seems likely that the decreased E1B-55K protein levels may have resulted from diminished stability of the E1B-55K protein in the absence of a functional E4orf6 protein. To our knowledge, this is the first reported case of stable E4orf6 expression in mammalian cells.

To determine whether E4orf6 increased the radiosensitivity of the RKO cells, clonogenic survival was analyzed after exposure to a range of IR doses. Two independently isolated clones expressing wild-type E4orf6 were significantly more radiosensitive (10-fold at 8 Gy) than either a vector-transfected clone or an E4orf6-L245P mutant clone (Fig. 2). E1B-55K is not expressed in the clones, indicating that E4orf6 radiosensitizes RKO cells without affecting p53 or MRE11 protein levels. The radiosensitization effect was quantified as a dose enhancement ratio of ~1.4 over the range of IR doses.

**Transient Expression of E4orf6 Radiosensitizes U251 Cells**—Although E4orf6 radiosensitized two independent RKO clones, it remained possible that random integration of the foreign DNA in the RKO clones, rather than E4orf6 expression, contributed to the radiosensitization effect (19). To test this, we measured the ability of E4orf6 to radiosensitize human tumor cells following transient expression from a non-replicating and non-integrating adenovirus, a viral vector that is frequently used in cancer gene therapy modalities. Both control virus (vCMV-null) and E4orf6-expressing virus (vCMV-E4orf6) lack the E1 gene, which renders them non-replicative (Fig. 3A) (20). Although the vCMV-null virus contains an intact E4 promoter and gene, expression of the E4orf6 protein from the endogenous gene was not detected by either immunoblotting (Fig. 3B) or immunofluorescent microscopy (Fig. 3C), as expected in the absence of the E1 gene products. The vCMV-E4orf6 virus expresses E4orf6 from a CMV promoter and lacks the endogenous viral E4 gene (20), and therefore, lacks expression of the other E4 gene products. U251 glioblastoma cells infected with vCMV-E4orf6 were significantly more radiosensitive than those infected with vCMV-null virus (Fig. 4). Thus, transient expression of E4orf6 from a non-integrating, non-replicating adenoviral vector significantly radiosensitizes U251 cells, indi-

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**Fig. 3.** Transient expression of E4orf6 via infection with a non-replicating adenoviral vector. **A,** diagram of E4orf6-expressing and control adenovirus genomes. Both viruses lack the E1 region, rendering them non-replicative. **B** and **C,** E4orf6 expression from vCMV-E4orf6 and not vCMV-null virus. U251 glioblastoma cells were infected with either vCMV-null or vCMV-E4orf6 at a multiplicity of infection sufficient to infect more than 95% of the cells. **B,** at 48 h after infection, the cells were harvested for immunoblotting for E4orf6 protein levels. **B** and **C,** at 48 h after infection, the cells were fixed for indirect immunofluorescence microscopy to identify E4orf6-expressing cells. Cells were counterstained with Hoechst 33342.
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E4orf6 Expression Does Not Increase Mutation Frequency—We have demonstrated that E4orf6 radiosensitizes by inhibiting DSBR (measured by sublethal damage repair assay), leading to the accumulation of DSBs (measured by γ-H2AX levels); however, it has been suggested previously that expression of E4orf6 itself may be mutagenic based on the hprt test for mutagenicity (32). To determine whether the decreased capacity for DSBR was due to increased cellular mutation frequency, 293 cells were transfected with empty expression vector or a vector expressing the wild-type E4orf6 or the mutant L245P gene and a replication-competent reporter vector, pSP189. In a representative experiment (Table I), 293 cells transfected with the empty expression vector and the pSP189 reporter vector exhibited a mutation frequency of $3.5 \times 10^{-7}$ mutations/bp, consistent with the background mutation frequency reported for this cell line (33). Expression of the wild-type E4orf6 or mutant L245P variants did not increase the mutation frequency. By contrast, exposure of vector-transfected cells to mutagenic levels of UV light (50 J/m$^2$) increased the mutation frequency by nearly 2 orders of magnitude. Similar results were obtained in two independent experiments. Thus, although E4orf6 reportedly increases the mutation frequency 2-fold at the chromosomal hypoxanthine guanine phosphoribosyl transferase (hprt) locus in the D422 Chinese hamster ovary cell line (32), it does not elicit point or frameshift mutations in a replicating plasmid. However, the amount of reporter plasmid recovered from 293 cells expressing the E4orf6 protein was significantly reduced. The results of two independent experiments indicated that expression of E4orf6 in 293 cells for 48 h reduced the amount of recovered pSP189 reporter by 50% when compared with that recovered from cells transfected with the empty expression vector or the L245P construct (Table I). This result is consistent with the ability of the E4orf6 but not L245P protein to block DSBR as naturally occurring DNA breaks are formed and repaired during plasmid replication.

E4orf6 Does Not Inhibit the Kinase Activity of DNA-PKcs but Prolongs Its Autophosphorylation Following IR—Our results described thus far support a model in which E4orf6 radiosensitizes cells by inhibiting DSBR, perhaps by inhibiting the DNA-PK complex through a direct interaction with DNA-PKcs (7). The effect of E4orf6 on DNA-PK activity was assessed by measuring the ability of DNA-PK to phosphorylate a p53-related peptide in vitro. When expressed in 293 cells by an adenoviral vector, E4orf6 did not affect DNA-PK kinase activity toward a heterologous substrate (Fig. 6A). However, it remains controversial whether p53-phosphorylation is an appropriate measure of DNA-PK activity with regard to DNA repair and radioresistance because DNA-PK-dependent end-joining activity and radiation resistance can be compromised, whereas DNA-PK is still able to phosphorylate p53 (34, 35). Recently, the autophosphorylation of DNA-PKcs has been identified as a critical factor in maintaining radioresistance, with either hypophosphorylation of DNA-PKcs resulting in radio-
sensitization (14, 36, 37). Regulation of the autophosphorylation of DNA-PKcs within a cluster of six serine and threonine sites is important in maintaining radioresistance (14). Thus, we hypothesized that E4orf6 may affect DNA-PK autophosphorylation, which in conjunction with subsequent dephosphorylation is necessary for radioresistance.

Following the treatment of infected U251s with IR, autophosphorylation of DNA-PKcs at Thr-2609 was measured over time by immunoblotting with a phospho-specific antibody. Comparable amounts of Thr-2609-phosphorylated DNA-PKcs accumulated in U251 cells infected with either vCMV-E4orf6 or vCMV-null at early time points after irradiation (Fig. 6B). However, in contrast to vCMV-null-infected cells, in which dephosphorylation was near completion 6 h after irradiation, Thr-2609-phosphorylated DNA-PKcs remained up-regulated in E4orf6-expressing cells, which is consistent with a radiosensitive phenotype. The experiment was repeated two more times with similar results. Reactive bands from all three experiments were quantified and normalized to total DNA-PKcs levels (Fig. 6C).

DISCUSSION
Our data identify the adenoviral protein E4orf6 as a radiosensitizer of human tumor cells. We have demonstrated both stable and transient expression of E4orf6 in two different tumor cell lines. This radiosensitization is occurring in the absence of E1B-55K, the adenoviral protein that interacts with E4orf6, an interaction that is required for the degradation of MRE11 and p53 and the inhibition of adenoviral DNA concatemer formation (5, 6). Our data reveal that E4orf6 is able to radiosensitize tumor cells by inhibiting cellular DNA DSBR in the absence of E1B-55K, and therefore, independent of MRE11 and p53 protein levels.

A recent study reported that transient transfection of E4orf6 in a variety of tumor cell lines, including RKO cells, did not result in significant radiosensitization (19). We believe that this discrepancy may result from the fact that following transient transfection, not all of the cells express E4orf6, which would be important to accurately assess clonogenic survival following treatment with clinically relevant doses of IR. Furthermore, the lack of radiosensitization by the E4orf6-L245P mutant suggests that the wild-type activity or the proper conformation of E4orf6 is necessary for its radiosensitizing capabilities (the L245P mutation was introduced to disrupt a critical amphipathic α helix in the E4orf6 protein) (15). Finally, the similar radiosensitization effect measured in two independently selected wild-type E4orf6 clones, as well as the ability of adenoviral-expressed non-integrated E4orf6 to produce a similar effect, suggest that the radiosensitizing property was not
the result of positional effects from genomic integration.

Our results also argue against the possibility that E4orf6 is mutagenic. The levels of γ-H2AX (DSBs) are similar between vCMV-null- and vCMV-E4orf6-infected cells in the absence of IR, and E4orf6 does not increase the frequency of mutations (point or frameshift) in 293 cells. However, E4orf6 plasmid recovery is reduced when compared with control plasmids, which is consistent with our theory of an inhibition of cellular DSBR by E4orf6. More specifically, during episomal DNA replication, type II topoisomerases transiently introduce double strand breaks in the nascent molecules (38). If any of the plasmids acquire a double strand break during this process, the inhibition of DSBR by the E4orf6 protein may prevent rejoining of the DNA, thereby reducing the number of intact, replicated plasmids recovered.

We have found that expression of E4orf6, although not affecting the kinase activity of DNA-PKcs with regard to heterologous substrates, prolongs the autophosphorylation of DNA-PKcs at Thr-2609. These data lead us to propose a model (Fig. 7) in which E4orf6 inhibits the late stages of repair as DNA-PKcs remains able to phosphorylate itself in response to DNA damage and repair, but dephosphorylation of DNA-PKcs at Thr-2609 is prevented. DNA-PKcs autophosphorylation is Ku-dependent (14) and is proposed to occur as a signal for the repair complex to undergo a conformational change conducive to ligation and/or post-ligation dissociation from the DNA (34, 35). Two possible modes of action for E4orf6 include either (a) a physical interaction with DNA-PKcs or (b) an inhibition of protein phosphatase 5, the phosphatase implicated in the de-

| Plasmid      | Mutation frequency | Plasmid recovery |
|--------------|--------------------|------------------|
| Vector       | $3.5 \times 10^{-7}$ | Vector           |
| E4orf6       | $4.3 \times 10^{-7}$ | E4orf6           |
| E4orf6-L245P | $2.3 \times 10^{-5}$ | E4orf6-L245P     |
| Vector/UV    | $2.5 \times 10^{-5}$ | No plasmid       |

**TABLE I**

*Mutation frequency and plasmid recovery*

**Fig. 6.** E4orf6 does not inhibit the kinase activity of DNA-PKcs toward heterologous substrates but prolongs autophosphorylation of DNA-PKcs at Thr-2609. A, mock-, vCMV-null-, or vCMV-E4orf6-infected U251 cells were tested for in vitro kinase activity on a p53 peptide. Wortmannin (30 μM for 3 h) treated U251 cells served as a positive control for kinase inhibition, and M059J and M059K cell lysates served as negative and positive controls, respectively, for the function of the assay. B, U251 cells infected with either vCMV-null or vCMV-E4orf6 were irradiated with 20 Gy and harvested at the given time points after irradiation for immunoblotting with a Thr-2609 phospho-specific antibody (indicated by P-Thr2609) of DNA-PKcs. A 20-Gy IR dose was chosen for this experiment, due to the relatively low affinity of the phospho-specific antibody. An in vitro kinase reaction sample was loaded as a positive control for the Thr-2609 phospho-specific antibody, and total DNA-PKcs was used as a loading control. C, the immunoreactive bands from three independent experiments were quantified using Scion Image software and normalized (Norm.) to total DNA-PKcs. Consistent with previously published reports (36), in some cases, the Thr-2609 phospho-specific antibody became partially degraded before 360 min after IR, in which case we included both bands (450 and 250 kDa) in our analysis of both viruses. The data were analyzed for significance by a two-tailed Student’s t test ($n = 3$, *, $p < 0.001$).
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improving radiation therapy, especially in radioreistant tumors such as glioblastomas. It is conceivable that E4orf6 delivered by a viral vector or by non-viral methods could substantially improve the cytotoxic effects of localized beam radiation therapy, such as Gamma knife radiosurgery.

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FIG. 7. Model of the inhibition of the late stages of DNA repair by E4orf6. In the presence of E4orf6, cells retain their ability to phosphorylate H2AX at Ser-139 (an early event) and undergo Ku-dependent DNA-PKcs autophosphorylation at Thr-2609 (a late event). The autophosphorylation step is thought to induce a conformational change in the repair complex, preparing it for ligation and dissociation of the proteins from the DNA (34, 35). However, these proteins remain phosphorylated at times when efficient repair would be complete and H2AX and DNA-PKcs would otherwise be dephosphorylated. Therefore, E4orf6 is most likely inhibiting the repair process at the late stages by preventing ligation and/or inhibiting the dephosphorylation which would otherwise signal complete repair and result in cell survival. Our model is in agreement with and partially adapted from Block et al. (35).

phosphorylation of DNA-PKcs at Thr-2609 (36). Although E4orf6 has been shown to require E1B-55K to degrade MRE11 and prevent concatamer formation (3), our model is consistent with these findings in that regardless of the state of DSB ligation, damage-induced signaling is sustained by phosphorylation of H2AX at Ser-139 and DNA-PKcs at Thr-2609 in the presence of E4orf6 alone. It has been recently proposed that sustained autophosphorylation of DNA-PKcs results in a radiosensitive phenotype (14, 34), possibly by signaling incomplete DNA repair and initiating growth arrest or cell death. Our data are in agreement with this model.

In summary, we have demonstrated that the adenovirus E4orf6 protein radiosensitizes human tumor cells by inhibiting DSB repair without affecting p53 or MRE11 protein levels. E4orf6 promotes the accumulation of DSBs as observed by prolonged phosphorylation of both H2AX at Ser-139 and DNA-PKcs at Thr-2609. Our results show for the first time that an adenoviral protein has radiosensitizing properties by inhibiting the repair of damaged host cell DNA in vivo. A model consistent with our data and that of other investigators is that E4orf6 promotes viral DNA replication by inhibiting cellular NHEJ in an effort to prevent concatenation of viral DNA (4, 5, 7). Consequently, overexpression of E4orf6 is sufficient to inhibit the NHEJ response to IR-induced cellular DSBs, thereby radiosensitizing the host cell. The ability of E4orf6 to inhibit the activity of repair proteins other than DNA-PK has not yet been explored. It remains possible that E4orf6 is able to inhibit MRE11 function without degrading it (a process that requires E1B-55K). Future experiments will test such possibilities.

The significant radiosensitization achieved at clinically relevant doses (2–8 Gy) by E4orf6 has promising implications for