Finkel-Biskis-Reilly Mouse Osteosarcoma Virus v-fos Inhibits the Cellular Response to Ionizing Radiation in a Myristoylation-dependent Manner*

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DNA damage is recognized as a central component of carcinogenesis. DNA-damaging agents activate a number of signal transduction pathways that lead to repair of the DNA, apoptosis, or cell cycle arrest. It is reasoned that a cell deficient in DNA repair is more likely to acquire other cancer-promoting mutations. Despite the recent interest in the link between DNA damage and carcinogenesis, retroviral oncogenes have not yet been shown to affect the DNA damage-signaling pathway. In this report, we show that Finkel-Biskis-Reilly mouse osteosarcoma virus (FBR) v-fos, the retroviral homologue of the c-fos proto-oncogene, inhibits the cellular response to ionizing radiation. Cells that express FBR v-Fos show a decreased ability to repair DNA damage caused by ionizing radiation, and these cells showed decreased survival in response to ionizing radiation. In addition, FBR v-Fos inhibits DNA-dependent protein kinase, a kinase specifically activated upon exposure to ionizing radiation. These effects were specific to ionizing radiation, as no effect of FBR v-Fos on the UV light signaling pathway was seen. Last, these effects were dependent on a lipid modification required for FBR v-Fos tumorigenesis, that of myristoylation of FBR v-Fos. A non-myristoylated mutant FBR v-Fos caused none of these effects. This study suggests that a retroviral oncogene can lead to an increased genomic instability, which can ultimately increase the carcinogenic potential of a cell.

A hallmark of neoplasms is the loss of genomic integrity (1). Since cancer arises from a stepwise progression of mutations in DNA, a loss in genomic integrity increases the likelihood of acquiring cancer-promoting mutations. This is highlighted in hereditary non-polyposis colon cancer in which cancer cells have a mutator phenotype caused by a defect in DNA mismatch repair (2, 3). In addition, the human papilloma virus oncoproteins, E6 and E7, have been shown to cause the cell to lose its cell cycle checkpoint controls, ultimately leading to genomic instability (1). Despite the heightened interest in the link between genomic instability, DNA repair, and cancer, the only retroviral oncogene that has been shown to affect DNA repair is the HTLV-I tax protein (4). In this paper, we show that FBR v-Fos, the retroviral homologue of the c-fos proto-oncogene, can inhibit cellular signaling and DNA repair in response to ionizing radiation.

Previously our laboratory has been interested in the mechanism by which FBR v-Fos promotes transformation. FBR v-fos differs from c-fos both by an N-terminal viral gag sequence and a C-terminal mouse c-Fox sequence (5). FBR v-Fos heterodimerizes with c-Jun, but displays a loss of function at AP-1 sites (6, 7). This loss of function can be mapped to the myristoylation of FBR v-Fos. When the myristoylation site is mutated (G2A v-Fos), the non-myristoylated G2A v-Fos regains the ability to transactivate AP-1 sites (6). Cellular transformation is not affected by FBR v-Fos’ myristoylation, but myristoylation of FBR v-Fos increases its carcinogenic potential in vivo. Transgenic mice that express G2A v-Fos only develop lipomas, while mice that express FBR v-Fos develop a wide range of mesenchymal tumors, including rhabdomyosarcomas, liposarcomas, chondrosarcomas, and osteosarcomas (8). The mechanism by which FBR v-Fos causes this increased carcinogenesis is unclear.

In addition to its function in AP-1 transactivation, c-Fos has also been shown to be an integral component of the cell’s stress response (9). Since DNA damage has been shown to be an essential component of carcinogenesis, we hypothesized that FBR v-Fos might cause a generalized defect in the stress response and DNA repair. In this paper, we show that FBR v-Fos causes a decreased capacity to repair double-strand DNA breaks caused by ionizing radiation, but it does not cause a decreased capacity to repair DNA damage caused by UV radiation. We show that cells that express FBR v-Fos show decreased survival in response to ionizing radiation, but normal survival when exposed to UV radiation. In addition, FBR v-Fos expression causes decreased activity of DNA-dependent protein kinase (DNA-PK), a kinase specifically activated by ionizing radiation. Last, all of these effects are dependent on the myristoylation of FBR v-Fos as cells that express G2A v-Fos do not show any of these effects. These data imply that while both FBR v-Fos and G2A v-Fos cause efficient transformation, only FBR v-Fos leads to a genomic instability, which increases a cell’s carcinogenic potential.

MATERIALS AND METHODS

Cell Culture and Transfections—HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine (Sigma) and 1% antibiotics/antifungal (Sigma). Stably transfected cell lines were created by calcium phosphate precipitation with 18 μg of FBR v-Fos or G2A v-Fos and 18 μg of RSV-neo (for RSV-neo only stable cell lines, 2 μg of RSV-neo and 18 μg of pGEM-4Z was used). Cells were then selected for 14 days in medium containing 300 μg/ml G418 (Life Technologies, Inc.). Approximately 500–1000 clones were pooled and shown to express FBR v-Fos or G2A v-Fos by radioimmunoprecipitation and Western blotting.

CAT Assays—DNA repair CAT assays were performed as described previously (9, 10, 11). Briefly, SV40-CAT was damaged by UV radiation previously (9, 10, 11). Briefly, SV40-CAT was damaged by UV radiation.

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1 The abbreviations used are: FBR, Finkel-Biskis-Reilly mouse osteosarcoma virus; CAT, chloramphenicol acetyltransferase; neo, neomycin resistance gene; DNA-PK, DNA-dependent protein kinase; Gv, gray.

(0, 100, 250, and 400 J/m2 (emission peak: 254 nm, Stratagene)) or by ionizing radiation (0, 3, 7, 15 Gy (37Cs, 2 Gy/0.7 min)). Activity of these constructs was assayed 12 h later. At 12 h, DNA repair has not taken place, so the activity of the construct is directly related to the amount of DNA damage that has been sustained (9–11). 12 h after transfection, cell lysis was performed by four alternating cycles of freezing and thawing. Transfection efficiency was then standardized by equal expression of β-galactosidase via methods described previously (12). Following standardization, CAT activity was measured as described previously (13). The TLC plate was developed by autoradiography, and counts were measured by a PhosphorImager (Molecular Dynamics). 400 J/m2 and 15 Gy were judged to be the optimal doses of DNA-damaging agent. These plasmids were then assayed at 48 h after transfection (a time at which DNA repair has taken place) to assay for these constructs’ ability to be repaired. CAT activity was indistinguishable between irradiated DNA and unirradiated DNA at 48 h. Cells were transfected with these constructs and 5 µg of FBR v-Fos or G2A v-Fos, and CAT activity was assayed 48 h after transfection as described above. Activities were standardized to unirradiated construct alone + expression vector. For instance, FBR v-Fos’ effect on repair was determined by standardizing FBR v-Fos + irradiated reporter to FBR v-Fos + unirradiated reporter.

DNA-dependent Protein Kinase Assays—Cells were transfected with 5 or 10 µg of c-Fos, FBR v-Fos, or G2A v-Fos expression vector. 48 h later, cells were washed twice in phosphate-buffered saline and suspended in Buffer P (10 mM HEPES (pH 7.2), 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, 0.1 mM EDTA). Cells were set on ice for 5 min and then centrifuged at 14 K for 10 min to obtain soluble lysate. Soluble lysate was allowed to sit on ice for 30 min to allow all proteins to mix. Transfection efficiency was standardized by β-galactosidase assay, and protein concentration was standardized by Bio-Rad protein assay. Cell Irradiation—Cell irradiation was performed as described previously (14). [γ-32P]ATP was added in 25 mM HEPES (pH 7.4), 70 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 0.2 mM EGTA, 0.1 mM EDTA, and 0.25 mM ATP to a total volume of 40 µL. Double-stranded calf thymus (10 µg/ml) DNA was added to one tube, while a second reaction was run in parallel without DNA added. After 30 min at 30 °C, the DNA-PK phosphorylation site in p55 (PESQEAFDLWKK) as described previously (14). FBR v-Fos Causes Decreased Repair of Double-strand DNA Breaks—Since the cellular proto-oncogene c-fos has been shown to be an integral component of a cell’s stress response (9), and since the stress response is tightly linked to DNA repair and cancer (2, 3), we hypothesized that FBR v-Fos could cause a defect in DNA repair. To test this hypothesis, DNA repair assays were performed (9–11) using either UV light or ionizing radiation as the insulting agent. These plasmids were then assayed at the given dose. For UV irradiation, medium was removed before irradiation. The same medium was then added to the dish immediately following irradiation. Cells were then cultured for 10 days. They were stained with Giemsa and colonies were counted. Relative survival was determined by comparing cell survival of cells irradiated at a given dose to unirradiated cells plated at the same density and cultured for the same amount of time.

RESULTS

FBR v-Fos Causes Decreased Repair of Double-strand DNA Breaks—Since the cellular proto-oncogene c-fos has been shown to be an integral component of a cell’s stress response (9), and since the stress response is tightly linked to DNA repair and cancer (2, 3), we hypothesized that FBR v-Fos could cause a defect in DNA repair. To test this hypothesis, DNA repair assays were performed (9–11) using either UV light or ionizing radiation as the insulting agent. These plasmids were then assayed at the given dose. For UV irradiation, medium was removed before irradiation. The same medium was then added to the dish immediately following irradiation. Cells were then cultured for 10 days. They were stained with Giemsa and colonies were counted. Relative survival was determined by comparing cell survival of cells irradiated at a given dose to unirradiated cells plated at the same density and cultured for the same amount of time.

(cpm disc with DNA added − cpm disc without DNA) × (1000 pmol/min)

incubation time × 5 µL × 1.2 × 10−3 mol of ATP/5 µL

(Eq. 1)

FBR v-Fos Inhibits Radiation Signaling—Since FBR v-Fos causes a defect in a cell’s ability to repair double-strand DNA breaks, we were interested in determining whether FBR v-Fos affected the repair process itself or if FBR v-Fos affected the signaling pathway that leads to repair of double strand DNA breaks. To delineate between these possi-
A cell responds to exposure to ionizing radiation by activating a variety of integrating signaling pathways. To varying extents, DNA-PK (reviewed in Ref. 21), phosphatidylinositol 3-kinase (22), c-Raf (23), pp90 

^rsk (24), c-Abl (25, 26), and protein kinase C (27, 28) have been shown to be activated in response to ionizing radiation, RSV-neo cells show a decrease in cell survival as the radiation dose increases (Fig. 3A), but this decrease is small, indicating that HeLa cells may survive exposure to ionizing radiation. Relative to RSV-neo cells, FBR v-Fos cells show a significant decrease in cell survival at all doses of ionizing radiation (Fig. 3A). G2A v-Fos-expressing cells retain wild-type sensitivity to radiation (Fig. 3A), so FBR v-Fos’ effect on cell survival may be myristoylation-dependent. In contrast to the results seen with ionizing radiation, these cells show no differences in response to UV light (Fig. 3B). Even at doses that cause 90% lethality, no difference in survival is seen. These data support the findings seen with the damaged CAT reporter plasmid and the DNA-PK activity inhibition. FBR v-Fos’ effect is specific to ionizing radiation, the process in which DNA-PK has been implicated as performing a critical function.

**DISCUSSION**

Several important findings were noted in the experiments described above. First, FBR v-Fos inhibits radiation signaling, as evidenced by a decrease in DNA-PK activity, and this decrease is again dependent on FBR v-Fos’ myristoylation (Fig. 2B).

**FBR v-Fos Affects Cell Viability in Response to Ionizing Radiation**—To assess the in vivo effect of FBR v-Fos’ on double-strand break repair, the stable cell lines expressing RSV-neo, FBR v-Fos, and G2A v-Fos were utilized. FBR v-Fos, G2A v-Fos, and RSV-neo cell lines were plated at equal cell densities and exposed to various doses of irradiation from a 137Cs source or a UV source. Colonies were allowed to grow for 10 days before they were stained and counted. Because we were concerned about the effects that expression of a viral oncogene would have on normal cell growth, FBR v-Fos, G2A v-Fos, and RSV-neo cells’ growth were measured relative to themselves. For example, at a given radiation dose, FBR v-Fos cells’ survival was measured relative to an unirradiated plate of FBR v-Fos cells plated at the same density and grown for the same amount of time. In response to ionizing radiation, RSV-neo cells show a decrease in cell survival as the radiation dose increases (Fig. 3A), but this decrease is small, indicating that HeLa cells may survive exposure to ionizing radiation. Relative to RSV-neo cells, FBR v-Fos cells show a significant decrease in cell survival at all doses of ionizing radiation (Fig. 3A). G2A v-Fos-expressing cells retain wild-type sensitivity to radiation (Fig. 3A), so FBR v-Fos’ effect on cell survival is myristoylation-dependent.

In contrast to the results seen with ionizing radiation, these cells show no differences in response to UV light (Fig. 3B). Even at doses that cause 90% lethality, no difference in survival is seen. These data support the findings seen with the damaged CAT reporter plasmid and the DNA-PK activity inhibition. FBR v-Fos’ effect is specific to ionizing radiation, the process in which DNA-PK has been implicated as performing a critical function.

**DISCUSSION**

5-fold decrease in DNA-PK activity (Fig. 2A). Next, HeLa cell lines, which stably expressed RSV-neo, G2A v-Fos, or FBR v-Fos, were constructed. Briefly, cells were transfected with either FBR v-Fos or G2A v-Fos and the neoR gene in a 9:1 ratio. 500–1000 clones from each respective cell line were pooled. A control cell line containing only neoR was also constructed (RSV-neo cells). The FBR v-Fos and G2A v-Fos stable cell lines expressed the 75-kDa FBR v-Fos or G2A v-Fos protein, and neither of these proteins were present in the RSV-neo only stable cell lines (data not shown). DNA-PK activity was measured in these cell lines. Again, FBR v-Fos causes a significant decrease in DNA-PK activity, and this decrease is again dependent on FBR v-Fos’ myristoylation (Fig. 2B).
to ionizing radiation. Of these kinases, DNA-PK has been shown to induce a number of transcription factors including c-Jun (29), Egr-1 (27), and NF-κB (30). Despite the increasing knowledge of a cell’s response to ionizing radiation, no oncogenes have yet been shown to affect this signaling pathway.

In this work, we have analyzed the effect of the retroviral oncogene, FBR v-Fos, on this pathway. We have found that cells that express FBR v-Fos show a decreased capacity to repair double-strand DNA breaks (Fig. 1). These cells show decreased DNA-PK activity (Fig. 2), and they show a decreased ability to survive exposure to ionizing radiation (Fig. 3). These data imply that FBR v-Fos could be causing a genomic instability in cells in which it is expressed. This could in turn lead to a mutator phenotype, making it more likely that a cell which expresses FBR v-Fos becomes carcinogenic.

FBR v-Fos’ role in cancer has been a mystery. It was originally isolated by virtue of its ability to increase the frequency of bone cancers in mice exposed to ionizing radiation (31). Upon discovery of c-Fos and c-Fos’ role in AP-1 transactivation, it was assumed that FBR v-Fos mimicked c-Fos’ transactivation of AP-1. This was shown not to be true, however, as AP-1 activity assumed that FBR v-Fos mimicked c-Fos’ transactivation of AP-1. This was shown not to be true, however, as AP-1 activity was measured relative to the unirradiated cells within a cell line. Relative survival and S.E. values are as follows. RSV-neo: 1 Gy, 0.89 ± 0.09; 2 Gy, 0.70 ± 0.03; 4 Gy, 0.67 ± 0.13; 6 Gy, 0.52 ± 0.04. FBR v-Fos: 1 Gy, 0.35 ± 0.03; 2 Gy, 0.25 ± 0.11; 4 Gy, 0.15 ± 0.01; 6 Gy, 0.08 ± 0.04. G2A v-Fos: 1 Gy, 1.16 ± 0.06; 2 Gy, 1.00 ± 0.11; 4 Gy, 0.58 ± 0.08; 6 Gy, 0.47 ± 0.09. B, survival in response to UV irradiation was performed in triplicate as described above. Relative survival and S.E. values are as follows. RSV-neo: 50 J/m², 1.17 ± 0.07; 100 J/m², 0.26 ± 0.02; 250 J/m², 0.19 ± 0.01; 400 J/m², 0.11 ± 0.05. FBR v-Fos: 50 J/m², 0.96 ± 0.25; 100 J/m², 0.39 ± 0.17; 250 J/m², 0.22 ± 0.12; 400 J/m², 0.09 ± 0.01. G2A v-Fos: 50 J/m², 0.95 ± 0.31; 100 J/m², 0.36 ± 0.13; 250 J/m², 0.26 ± 0.14; 400 J/m², 0.14 ± 0.02.

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