Involvement of p54(nrb), a PSF partner protein, in DNA double-strand break repair and radioresistance

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ABSTRACT

Mammalian cells repair DNA double-strand breaks (DSBs) via efficient pathways of direct, nonhomologous DNA end joining (NHEJ) and homologous recombination (HR). Prior work has identified a complex of two polypeptides, PSF and p54(nrb), as a stimulatory factor in a reconstituted in vitro NHEJ system. PSF also stimulates early steps of HR in vitro. PSF and p54(nrb) are RNA recognition motif-containing proteins with well-established functions in RNA processing and transport, and their apparent involvement in DSB repair was unexpected. Here we investigate the requirement for p54(nrb) in DSB repair in vivo. Cells treated with siRNA to attenuate p54(nrb) expression exhibited a delay in DSB repair in a γ-H2AX focus assay. Stable knockdown cell lines derived by p54(nrb) miRNA transfection showed a significant increase in ionizing radiation-induced chromosomal aberrations. They also showed increased radiosensitivity in a clonogenic survival assay. Together, results indicate that p54(nrb) contributes to rapid and accurate repair of DSBs in vivo in human cells and that the PSF–p54(nrb) complex may thus be a potential target for radiosensitizer development.

INTRODUCTION

The RNA recognition motif (RRM) is one of the most common protein domains in eukaryotes (1). Early work identified the motif in a variety of RNA binding proteins (2,3). More recent work has broadened our concept of the function of RRM-containing proteins. The RRM domain itself serves as a platform for RNA–protein, DNA–protein and protein–protein interactions (1). Although most RRM-containing proteins function in RNA metabolism, others bind to specific chromosomal loci (4–6), regulate transcription (7), mediate the response to DNA damaging agents (8) or modify chromatin (9). Polypyrimidine tract-binding protein associated splicing factor (PSF) and 54 kDa nuclear RNA binding protein [p54(nrb)] are members of a subfamily of RRM proteins defined by tandem RRM domains flanked by an additional region of homology. PSF and p54(nrb), which form a stable complex in vivo, have previously been implicated in nuclear retention of A-to-I edited RNA (10), nuclear retention of other RNAs (11), pre-mRNA 3' end formation (12) and transcriptional activation mediated by the androgen, thyroid hormone and retinoid X receptors and by the transducers of regulated CREB activity proteins (13–16).

In addition to its functions in RNA metabolism, early studies showed that PSF promotes annealing of single-stranded DNA with homologous double-stranded DNA (17,18). Recently, PSF was shown to cooperate in vitro with the homologous recombination (HR) protein, Rad51, to promote homologous DNA pairing and strand displacement (19). We have also shown that the purified PSF–p54(nrb) complex stimulates DNA non-homologous end joining (NHEJ) 10-fold in a reconstituted cell-free DNA double-strand break (DSB) repair assay (20). PSF–p54(nrb) binds the repair substrate in vitro, internal to the ends, and cooperates functionally with Ku to form a preligation complex (21,22). HR and NHEJ are the two major pathways of DSB repair in human cells, and the biochemical data suggest that PSF or the PSF–p54(nrb) complex might be involved in both.

Prior studies of the role of PSF–p54(nrb) in the DNA damage response have used reconstituted in vitro systems. Here, we report the results of a direct genetic investigation of whether p54(nrb) is involved in the DNA damage...
response in vivo in human cells. We show that attenuation of p54(nrb) expression produces a DSB repair-deficient, radiosensitive phenotype.

MATERIALS AND METHODS

Cells and culture conditions

HeLa cervical carcinoma cells were grown in Dulbecco’s Minimal Essential Medium supplemented with 10% FBS, 2 mM glutamine and antibiotics. IMR-90 normal diploid human lung fibroblasts were grown in modified Eagle’s Minimal Essential Medium supplemented with 10% FBS, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and antibiotics. Experiments were performed at population doubling level of 32 or lower. HCT 116 near-diploid colorectal carcinoma cells were grown in McCoy’s 5A medium supplemented with 10% FBS and antibiotics.

siRNA treatment and p54(nrb) expression detection

Human NONO mRNA (accession no. NM_007363) was evaluated using the Ambion ‘siRNA Target Finder’ web tool (http://www.ambion.com). We synthesized complementary 21-mer oligonucleotides containing 19 nucleotides of complementary RNA sequence flanked at the 3′ side by two thymidylate residues (sense strand, GG CUGAGCUAAUUGACCUGATT; antisense strand UCA GGUCAAUAGUCAAGCTT). These were annealed and transfected into IMR-90 human diploid fibroblasts or HeLa cervical carcinoma cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Control cell populations were transfected in parallel using Silencer Negative Control #1 siRNA (Ambion, Austin, TX). The concentration of siRNA was 30 nM. Attenuation of mRNA levels was determined by real-time reverse transcriptase PCR using β-actin mRNA as an internal standard. Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA). DNase treatment was performed to remove residual DNA before reverse transcriptase PCR using β-actin mRNA as an internal standard. 25 μl using the Qiagen OneStep RT-PCR kit (QIAGEN, Valencia, CA). Reactions contained 800 ng of input RNA. Primers for p54(nrb) were d(TTGTGGGAAATCTTCCTCCGAC A) and d(GGGTTTCCAAGGGATAAAGCCA); primers for β-actin were d(AGTCTGTGGCATCCACGATGAGG) and d(AGTCTGTGGCCATCGTACAGGTCCT TGG). Levels of p54(nrb) protein were determined by immunofluorescence and immunoblotting using anti-p54 mouse monoclonal antibody (1:2000, Clone 3, BD Biosciences Pharmingen, San Diego, CA) and Alexa Fluor 488- or enzyme-conjugated secondary antibodies. Other antibodies were: mouse anti-β-actin (1:5000, Sigma-Aldrich, St. Louis, MO), mouse anti-Ku80 antibody (1:200, clone 111, Lab Vision Corporation, Fremont, CA), mouse anti-Ku70 antibody (1:200, clone N3H10, Lab Vision Corporation, Fremont, CA) and mouse anti-XRCC4 antibody (1:250, BD Bioscience, San Jose, CA).

γ-H2AX focus formation assay

At 36 h post-transfection, cells received 0.5 Gy of 137Cs gamma radiation at a dose rate of 1 Gy min⁻¹. They were allowed to recover for the indicated times, fixed with 4% paraformaldehyde, permeabilized and blocked by incubation in PBS containing 0.5% Triton X-100, 15% goat serum, 0.2% fish skin gelatin and 0.03% NaCl. Cells were then stained with mouse monoclonal anti-γ-H2AX antibody (1:500, clone JBW301, Millipore Corporation, Billerica, MA) and Alexa Fluor 488-conjugated secondary antibody and counterstained with 4,6-diamidino-2-phenylindole (DAPI). A minimum of 120 nuclei per experimental group were scored by a blinded observer.

Chromosomal aberration assay

To create stable cell lines, d(TGCTGTATAACAGAAAC GTATGTACGGTTTGGCCTACTGACTGGTCAC ATAGTTCGTGTATA) and d(CCTGTATACAGAAC TATGTACGGTGACGTGCTGGCCAAACCGTAC TACCGTTTCTGGTATA) were annealed and inserted into pcDNA6.2-GW/EmGFp-miR (Invitrogen). This vector and pcDNA6.2-GW/EmGFp-miR-neg control plasmid were transfected into HCT 116 cells, incubated overnight in McCoy’s 5A medium without antibiotics, and subcultured in complete McCoy’s 5A medium supplemented with 18 mg/ml Blasticidin (Invitrogen). Cultures were culured for 2 weeks with replacement of the Blasticidin-containing medium every 3–4 days. EmGFp-expressing colonies were captured on Whatman 3 MM filter paper (GE Healthcare) and expanded with subculturing in Blasticidin-containing medium. Proteins were extracted and analyzed by immunoblotting.

HCT 116-derived stable cell lines were subcultured in T-25 flasks and allowed to reach 60–80% confluency, irradiated using the 137Cs source, and incubated in fresh medium for 18 h. Colcemid was added to 0.1 µg/ml, and incubation was continued for 2 h at 37°C. Cells were trypsinized, collected and resuspended in 2 ml of prewarmed 0.075 M KCl. The suspension was diluted to 45 ml with 0.075 M KCl and incubated for 45 min at 37°C. Five drops of freshly prepared fixative MeOH:HOAc [3:1 (v:v)] were added, and cells were collected by centrifugation. Supernatant was removed, leaving 0.5 ml, and 5 ml of fixative was gradually added. Cells were collected, washed twice in fixative and stored at 4°C until slides were prepared. The cell suspension was dropped onto slides and air-dried, then stained with Giemsa. One hundred metaphase spreads were scored by a cytogeneticist for abnormalities, including chromosome fragments, dicentric chromosomes and translocations. Significance was evaluated using Fisher’s exact test.

Clonogenic survival assay

Clonogenic survival assays were performed essentially as described (23). Cultures were trypsinized and 500–4000 cells were seeded in six-well plates, allowed to attach and irradiated using a 137Cs source at a dose rate of 0.85 Gy min⁻¹. Cultures were incubated at 37°C in a
humidified 5% CO₂ atmosphere for 7 days with one change of medium. Staining was with 0.25% crystal violet and 3.7% formaldehyde in 80% methanol. Colonies of ≥50 cells were counted. Survival rates were determined as a function of radiation dose [\( S(D) \) = colonies/cells plated, where D is expressed in units of Gray (Gy)]. Surviving Fractions [\( SF = S(D)/S(0) \)] were calculated, where S(0) is survival at 0 Gy (mock-irradiated). The radiation survival curves were fitted according to the linear-quadratic model: \( S(D)/S(0) = \exp(\alpha D + \beta D^2) \), where \( \alpha \) and \( \beta \) parameters are determined by weighted, stratified and linear regression (23). Differences between treatment groups were evaluated for significance based on an F test. The mean inactivation dose (MID, integral under survival curve from 0 to infinity) was calculated by numeric integration using the Gaussian-Laguerre quadrature (24). The sensitizing enhancement ratio (SER) was defined as MID(control miRNA)/MID[p54(nrb)RNA].

RESULTS

Attenuation of p54(nrb) expression with siRNA

To investigate the function of the PSF∗p54(nrb) complex in vivo, we first determined the ability to attenuate expression of each subunit using siRNA. We chemically synthesized and annealed 21-nucleotide double-strand RNAs corresponding to the coding region of each gene and transfected them individually into IMR-90 human diploid fibroblast cells. Attenuation of PSF expression was variable and incomplete (data not shown) and was not investigated further. A previous report also described difficulty in attenuating PSF expression in cultured cells (12). Transfection with p54(nrb) siRNA, by contrast, consistently reduced mRNA expression by about 75%, as measured by real-time reverse transcriptase PCR with β-actin mRNA as an internal standard (Figure 1A). There was a similar reduction in p54(nrb) protein levels (Figure 1B). Transfection with p54(nrb) siRNA did not affect PSF, Ku70, Ku80 or XRCC4 protein levels, indicating specificity. Transfection with p54(nrb) siRNA attenuated p54(nrb) expression in at least 80% of cells in the IMR-90 population, with no apparent affect on morphology or viability (Figure 1C). Essentially identical RT-PCR and immunoblotting results were obtained using the same siRNAs to transfect HeLa cells (Supplementary Figure S1).

Attenuation of p54(nrb) expression delays DSB repair

Prior in vitro studies predict that attenuation of p54(nrb) expression should affect DSB repair. To investigate the phenotype associated with attenuation of p54(nrb), we induced DSBs by exposure to ionizing radiation and monitored their fate using a histone γ-H2AX focus assay. Phosphorylation of H2AX serine 139 creates the γ-isof orm, producing foci that can be visualized by immunostaining using anti-γ-H2AX antibody. These foci correspond 1:1 with unrepaired DSBs (25,26). An advantage to the use of this single-cell assay for siRNA-treated cells is that a small fraction of non-transfected cells (if present) would not alter the modal number of foci per cell.

Figure 1. Attenuation of p54(nrb) expression using siRNA. (A) Attenuation of p54(nrb) mRNA in IMR-90 cells 36 h after transfection with control or p54(nrb) siRNA. Total RNA was quantified by real time RT-PCR using an actin internal control. Bars denote standard error based on 3 replicate transfections. Asterisks indicate significant differences between groups at *\( P < 0.05 \) and **\( P < 0.01 \) levels based on Student’s t-test. (B) Attenuation of p54(nrb) expression detected by immunoblotting. Cells were transfected with siRNA as in A and extracts were analyzed as described in ‘Materials and Methods’ section. (C) Attenuation of p54(nrb) expression detected by immunofluorescence. Fixed cells were stained for anti-p54(nrb) expression and counterstained with DAPI.

Following transfection with p54(nrb) or control siRNA, IMR-90 cells were treated with 0.5 Gy of 137Cs gamma radiation. Figure 2A shows the appearance of typical immunostained cells either without irradiation or with irradiation and a 0.5 h or 2 h recovery period. Figure 2B and C provide quantification based on scoring by a blinded observer. Transfected cell populations had a background of about 3 foci/cell in the absence of irradiation. This was the same for p54 siRNA and control siRNA. Transfected cells had a mode of about 14 foci/cell at 30 min after irradiation, roughly consistent with the expected number of DSBs at this radiation dose (27,28). Again, there was no difference between p54 siRNA and control siRNA. As the cells recovered, a marked difference between the two populations emerged. At the 2 h time point, control cells showed significant recovery, but p54(nrb)-attenuated cells did not. At the 4 h time point, the difference between the control and p54(nrb) groups persisted, although it had narrowed. At the 8 h time point, only a few repair resistant foci remained, with again no significant difference between groups. Results
suggest that although attenuation of p54(nrb) has no effect on the initial level of DNA damage, it delays DSB repair.

Attenuation of p54(nrb) expression increases the frequency of radiation-induced chromosomal aberrations

The finding that p54(nrb) siRNA treatment delayed, but did not abolish, DSB repair, suggests that the primary effect is on the rapid, initial phase of DSB repair, which is believed to be mediated by the NHEJ pathway. Prior studies have shown that most DSBs are repaired eventually, even in severely compromised genetic backgrounds [e.g. DNA ligase IV-null (29)]. However, this delayed repair, which has been attributed to ‘backup’ or ‘alternative’ pathways, may be less accurate and does not assure reproductive survival (30–33).

To investigate whether the effect on the rapid, initial phase of DSB repair was biologically significant, it was necessary to establish stable cell lines with reduced p54(nrb) expression. Because IMR-90 cells have a limited lifespan in culture, they were unsuitable for creating stable cell lines. Instead, we used HCT 116 a, p53-positive human colorectal cancer cell line. HCT 116 cells have a near-diploid karyotype and are suitable for

Figure 2. Delay in resolution of γ-H2AX foci in IMR-90 cells treated with p54(nrb) siRNA. IMR-90 cells were irradiated with 0.5 Gy at 36 h post-transfection, allowed to recover for indicated times, fixed and stained with anti-γ-H2AX. The antibody detects phosphorylation at H2AX Ser 139, a marker of unrepaired DNA double-strand breaks. (A) Representative nuclei treated as indicated. (B) Histogram showing number of foci in mock-irradiated cells. (C) Histograms showing number of foci in cells that were exposed to 0.5 Gy of ionizing radiation and allowed to recover for the indicated times at 37°C.
Attenuation of p54(nrb) decreases clonogenic survival following irradiation

To measure radiation sensitivity, we performed clonogenic survival assays in stable miRNA-expressing HCT 116 cell lines as described in ‘Materials and Methods’ section. Colony size was appreciably smaller for p54(nrb) miRNA-expressing cells (Figure 4A), and the plating efficiency in the absence of radiation was lower (16.3% versus 45%). The reduced colony size indicates slower growth of the clones under the conditions used. Slower growth and the lower plating efficiency cannot be attributed to changes in DSB repair, because they were seen even in the absence of exposure to ionizing radiation. There must be some other cause, such as a defect in RNA synthesis, consistent with previous reports that p54(nrb) is involved in various aspects of RNA biogenesis (see ‘Introduction’ section).

The radiation survival data are quantified in Figure 3B. The survival curve for the control-transfected population is typical for mammalian cells, with a ‘shoulder’ in the 0–2 Gy region, reflecting repair of radiation-induced DNA damage. The data can be fitted almost exactly with a linear-quadratic model. By contrast, the p54(nrb) miRNA-transfected population shows a nearly log-linear response, with no evident shoulder. The difference between the two cell clones is highly significant ($P < 0.001$). Similar results were obtained with the other stable cell lines (data not shown). The effectiveness of a radiosensitizing treatment can be quantified based on the horizontal shift in the survival curve at an arbitrarily chosen level of survival (dose enhancement ratio), or more robustly, by taking the ratio of the integrals under the survival curves from zero to infinity (sensitization enhancement ratio) (24). The inset shows the survival data replotted to illustrate these integrals; the sensitization enhancement ratio thus determined is 1.27, indicative of a moderately strong effect.

We have also investigated the effect of p54(nrb) on clonogenic survival independently in siRNA-transfected IMR90 human diploid fibroblasts (Supplementary Figure 2S). There was an approximately 1.5-fold, statistically significant, decrease in survival at all radiation doses tested. In this experiment, the plating efficiencies were low, the shapes of the survival curves were atypical, and we were not able to fit the curves with a quantitative model. We believe that there was an interaction between toxicity...
of the transient transfection procedure and the stress of the clonogenic survival assay that limited the ability to perform a quantitative analysis. Despite this, results were qualitatively consistent with those obtained using the HCT 116 stable cell lines.

**DISCUSSION**

We have investigated the effect of p54(nrb) deficiency on repair and survival following induction of DNA double strand breaks. DSBs, which are produced by ionizing radiation and certain recombination enzymes, are more difficult to repair, more cytotoxic and more destabilizing to the genome than most other DNA damage (34). Three lines of evidence indicate that attenuation of p54(nrb) expression causes a deficiency in DSB repair in *vivo*: delay in resolution of γ-H2AX foci, an increase in chromosomal aberrations at the first metaphase following radiation exposure, and a decrease in clonogenic survival. These repair and survival phenotypes were seen in two different cell types and with two different approaches for gene silencing (siRNA and miRNA directed against distinct sequence regions). The genetic studies presented here complement previous findings that PSF\textsuperscript{*}p54(nrb) stimulates DNA end joining in a reconstituted biochemical system (20,21). Taken together, the evidence strongly supports the involvement of p54(nrb) in the DNA damage and repair response. The available genetic tools have not, so far, allowed us to draw conclusions whether PSF is required for DNA damage and repair in intact cells.

To our knowledge, there are as yet no reports of germ-line p54(nrb) deficiency in humans or other mammalian species. There has been a report of a translocation of PSF and p54(nrb) to the TFE3 helix-loop-helix transcription factor gene in human papillary renal cell carcinoma lines (35). A hybrid transcript is expressed, but its role in oncogenic transformation has not been established. There have also been reports describing two different gene trap alleles affecting the gene encoding TLS/FUS, a different tandem RRM protein. In one case, deficiency resulted in male sterility, modest radiosensitivity in fibroblasts, and severe radiosensitivity in whole mice (36). In the other, deficiency resulted in defective B-cell development, spontaneous chromosomal instability and perinatal death (37). These reports support a more general hypothesis that multifunctional RRM proteins affect both gene regulation and genome maintenance.

The phenotypic characterization of p54(nrb) knockdown in human cells, presented here, does not provide direct insight into how this protein influences DSB repair. However, earlier studies showed that PSF\textsuperscript{*}p54(nrb) binds DNA directly via DNA binding domain that overlaps the N-terminal region and RRMI of the PSF subunit (38,39). In our previous studies, PSF\textsuperscript{*}p54(nrb) and Ku bound linear DNA fragments independently, but cooperated to form a functional preligation complex (20). We suggest the same mechanism may apply *in vivo*. This is consistent with recent observations in our laboratory that PSF and p54(nrb) rapidly relocalize to sites of laser microbeam-induced DNA damage (Ha K. *et al.*, manuscript in preparation). It will be of interest to further investigate the repair defects in p54-deficient cells, including whether both NHEJ and HR are affected and whether the cells are sensitized to DNA damaging agents other than ionizing radiation.

Another potential effect of p54(nrb) knockdown, which is not mutually exclusive, is an effect on the synthesis and processing of mRNAs for other repair proteins. Many studies have shown that p54(nrb) affects synthesis and processing of specific mRNAs, although to our knowledge there is no direct evidence for an effect on levels of mRNAs for DNA damage response genes. Indeed, we show here that siRNA transfection does not change the levels of the known DSB repair proteins, Ku70, Ku80 and XRCC4, all of which participate in the rapid early phase of DSB repair that is affected by p54(nrb) deficiency.

Our clonogenic survival assay showed that attenuation of p54(nrb) expression largely eliminated the shoulder in the radiation survival curve. This has potential clinical significance, in that the shoulder region corresponds to the dose range used for single clinical radiation fractions...
(~2 Gy). Inhibition of p54(nrb) in tumor cells would be predicted to significantly increase therapeutic gain in radiotherapy. In this respect, it is of interest that a small, natural, virally encoded RNA has been shown to inhibit the binding of the PSF•p54(nrb) to DNA (39). Although this prior study dealt with promoter binding activity, not with DNA repair effects, the finding that there is cross-talk between DNA and RNA-binding sites in the PSF•p54(nrb) complex suggests a possible approach for the development of radiosensitizers based on RNAs or RNA analogs.

A potential concern regarding the survival data is that, although the difference between wild-type and knockout cells is highly significant (P < 0.001), the magnitude of this difference is relatively modest. It may be that the residual activity in knockout cells provides sufficient repair function to explain the observed levels of survival. Alternatively, other proteins may compensate for the absence of p54(nrb). Human cells encode a closely related protein, PSP1z. It could be that PSP1z partially compensates for p54(nrb) function in deficient cells, a possibility that has not yet been tested.

In addition to the role in DSB repair defined in the present study, PSF and p54(nrb) participate, separately or together, in diverse aspects of RNA biogenesis. Although more than 25 published studies address these functions, there is no consensus on their exact role: PSF or p54(nrb) have been implicated in regulation of transcriptional initiation, 3'end formation, splicing and nuclear-cytoplasmic transport. This apparent complexity may reflect interaction of PSF and p54(nrb) with diverse partner proteins. Androgen receptor (14,15,40), thyroid hormone receptor (13), retinoid X receptor (13), TORC1 (16) and RNA polymerase II (41) interactions contribute to effects of PSF•p54(nrb) on RNA synthesis or together, in diverse aspects of RNA biogenesis. These observations raise the question whether there are also specific partners involved in PSF•p54(nrb) repair function. Topoisomerase I interacts directly with PSF•p54(nrb) in vivo and in vitro (45,46) and has itself been shown to migrate to sites of laser-induced DNA damage (47). A more speculative candidate is PARP-1, which senses single-strand breaks and, under some conditions, DSBs. Like PSF•p54(nrb), PARP-1 is transiently recruited to sites of laser-induced DNA damage, suggesting a common mechanism (48). Further studies will be required to investigate these possibilities.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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