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Sequences in PSF/SFPQ mediate radioresistance and recruitment of PSF/SFPQ-containing complexes to DNA damage sites in human cells

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ABSTRACT

PSF (gene name SFPQ) is a member of a small family of proteins with dual functions in RNA biogenesis and DNA repair. PSF and PSF-containing complexes stimulate double-strand break repair in cell free systems, most likely via direct interaction with the repair substrate. Prior *in vitro* studies are, however, insufficient to demonstrate whether PSF contributes to DNA repair in living cells. Here, we investigate the effect of miRNA-mediated PSF knockdown in human (HeLa) cells. We find that PSF is essential for reproductive viability. To circumvent this and investigate the DNA damage sensitivity phenotype, we established a genetic rescue assay based on co-transfection of PSF miRNA and mutant PSF expression constructs. Mutational analysis suggests that sequences required for viability and radioresistance are partially separable, and that the latter requires a unique N-terminal PSF domain. As an independent means to investigate PSF sequences involved in DNA repair, we established an assay based on real-time relocalization of PSF-containing complexes to sites of dense, laser-induced DNA damage in living cells. We show that relocalization is driven by sequences in PSF, rather than its dimerization partner, $p54^{nrb}$/NONO, and that sequences required for relocalization reside in the same N-terminal domain that contributes to radioresistance. Further evidence for the importance of PSF sequences in mediating relocalization is provided by observations that PSF promotes relocalization of a third protein, PSPC1, under conditions where $p54^{nrb}$ is limiting. Together, these observations support the model derived from prior biochemical studies that PSF influences repair via direct, local, interaction with the DNA substrate.

1. Introduction

Three human proteins, polypyrimidine tract binding protein-associated splicing factor (PSF/SFPQ), 54 kDa RNA binding protein ($p54^{nrb}$/NONO), and paraspeckle component 1 (PSPC1) make up the *Drosophila* behavior human splicing (DBHS) family. All three share tandem RNA recognition motifs flanked by an additional region of homology. The additional region of homology, which is predicted to form a coiled-coil domain, promotes formation of heteromeric complexes between each protein and the other two family members [1–3].

All three mammalian DBHS proteins are components of nuclear paraspeckles. Paraspeckles regulate gene expression via retention of adenosine-to-inosine hyperedited mRNAs [4,5] (reviewed in [6]). In addition to their role in controlling expression of hyper-edited mRNAs, PSF and $p54^{nrb}$ have been reported to participate in a number of other processes relating to mRNA biogenesis, including pre-mRNA 3′-end formation, cyclic AMP signaling [7], and nuclear receptor-dependent transcriptional regulation [8–11]. Interestingly, PSF and $p54^{nrb}$ interact with DNA as well as with RNA. More than 15 years ago, Busch and coworkers purified and characterized a DNA-binding heterodimer of proteins that migrated at 52 kDa and 100 kDa in SDS-PAGE, almost certainly corresponding to the polypeptides later identified as $p54^{nrb}$ and PSF, respectively [12]. Subsequent work has shown that PSF binds directly to DNA, that it accelerates annealing of complementary single-stranded nucleic acids, and that it promotes invasion of supercoiled DNA by complementary oligonucleotides to form D-loops [13,14].

PSF binds directly to the homologous recombination protein, Rad51, and cooperates with it in DNA pairing and strand displacement assays [15]. A different PSF·$p54^{nrb}$ complex promotes nonhomologous end joining *in vitro*, suggesting its involvement in this other main pathway of DNA double-strand break (DSB) repair in vertebrates [16,17]. A recent report indicates that PSF and $p54^{nrb}$ are transiently recruited to DNA damage sites in human cells, and that release from these sites is regulated by a third protein, matrin 3 [18].

Abbreviations: RRM, RNA recognition motif; PSF, polypyrimidine tract binding protein-associated splicing factor; DSB, double-strand break; DBHS, *Drosophila* behavior human splicing.

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There are many proteins with dual functions in mRNA biogenesis and DNA repair. Examples include the TFIIH complex, which participates both in RNA polymerase II-mediated transcriptional initiation and in nucleotide excision repair (reviewed in [19]), PARP-1, which functions in both promoter/enhancer regulation and DNA single-strand break/base excision repair (reviewed in [20,21]), and Ku protein, which functions in both the control of mRNA expression and in the nonhomologous end joining (NHEJ) pathway of DNA double-strand break (DSB) repair (for examples [22,23], for reviews [24,25]). It appears that PSF and p54^{nrb} provide yet another example of this phenomenon.

Until recently, experimental support for the role of PSF and p54^{nrb} in DNA repair has derived principally from *in vitro* studies. We recently reported, however, that attenuation of p54^{nrb} expression in mammalian cells delays DSB repair and sensitizes the cells to ionizing radiation [26], which provides genetic evidence in support of the role of p54^{nrb} in DNA repair *in vivo*. Here we describe another, complementary set of experiments that implicate the PSF subunit in DNA repair *in vivo* in human cells. We find that PSF is essential in human cells, which necessitated a different approach toward establishing its function, using a genetic rescue assay. We find that the sequences required to rescue the radiosensitive phenotype correlate with those required for relocalization of PSF-containing protein complexes to sites of laser-induced DNA damage and with a previously defined DNA binding domain [27,28].

2. Materials and methods

2.1. Cells and immunostaining

HeLa cells were cultured at 37 °C in Dulbecco's minimal essential medium supplemented with 10% FBS, 2 mM glutamine and antibiotics. Plasmid DNAs were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen). Fixation and Immunostaining were performed as described in Supplementary Material.

2.2. Expression clones

Wild type and mutant human PSF, p54^{nrb}, and PSPC1 cDNAs were amplified by PCR and inserted in pDsRed-Monomer-N1 or pAcGFP-N1 (Clontech, Mountain View, CA) as described in Supplementary Material. An alignment highlighting the similarities in the coding sequences of these cDNA is shown in Supplementary Fig. 1. Human Ku80 and XRCC4 were inserted in pENTR/D-TOPO (Invitrogen, Carlsbad, CA). In-frame insertion of mCherry and or EYFP coding sequences was followed by lambda integrase-mediated transfer to pcDNA-DEST40 (Invitrogen).

2.3. miRNA treatment and protein expression detection

The p54^{nrb} miRNA knockdown and control vectors were constructed as previously described [26]. An analogous PSF miRNA knockdown vector was constructed using the oligonucleotides listed in Supplementary Table 1. Plasmids were transfected into HeLa cells and proteins were extracted and analyzed by immunoblotting using anti-p54^{nrb} mouse monoclonal antibody (Clone 3, BD Biosciences Pharmingen), anti-PSF mouse monoclonal antibody (Sigma, clone B92), or anti-actin rabbit antibody (BD Bioscences Pharmingen). Blots were developed using HRP-conjugated anti-mouse IgG antibody.

2.4. Phenotypic rescue of PSF-deficient cells

HeLa cells were co-transfected with PSF miRNA and with miRNA-resistant rescue plasmids expressing wild type or mutant PSF. After 24 h, cells were treated with spectinomycin (to select for miRNA expression) and G418 (to select for PSF rescue construct expression). After a further 24 h, 500 cells were seeded in 6-well plates. Some plates were exposed to ^{133}Cs γ-radiation (4 Gy). Plates were incubated for 10 d to allow colony growth, fixed with 80% ethanol for 15 min, and stained with 0.4% trypan blue. Colonies of >50 cells were counted, and surviving fraction was calculated relative to non-irradiated controls. Data are from two independent experiments.

2.5. Laser irradiation and imaging

Microirradiation was performed with a pulsed Ti:Sapphire laser (800 nm, Mira 900, Coherent Inc.). Laser power was optimized to produce localized DNA damage with minimal background outside the microbeam (Supplementary Fig. 2). Live-cell images were collected using a LSM510 META confocal microscope equipped with 543 nm HeNe and multi-line (458, 488, and 514 nm) argon lasers and a 40X C-Apochromat 1.2 N.A. water immersion lens (Carl Zeiss MicroImaging, Inc.). A heated stage (Carl Zeiss MicroImaging, Inc.) and an objective lens heater (Physitemp) were used to maintain cells at 37 °C. Fixed-cell images were collected using an Olympus BX60 microscope equipped with a 40X UPlanFl 0.75 N.A. lens.

For experiments with fixed cells, HeLa cells were grown on glass coverslips. After irradiation, cells were washed, fixed in 4% paraformaldehyde, 0.5% Triton X-100 for 10 min, washed again, and incubated in blocking solution (15% goat serum, 0.2% fish skin gelatin (Sigma) and 0.03% NaN_3 in PBS) containing 0.5% Triton X-100 for 10 min. Cells were rinsed and incubated with blocking solution without Triton X-100. Immunostaining was performed by incubating with rabbit anti-p54^{nrb} (Abcam, Cambridge MA), mouse monoclonal anti-PSF (Sigma, clone B92), rabbit anti-53BP1 (Novus Biologicals, Littleton, CO) or mouse monoclonal anti-γ-H2AX (Millipore) in blocking solution for 2 h at room temperature, washing, and incubating for 1.5 h with Alexa Fluor 350- or AlexaFluor 488-conjugated species specific secondary antibodies in blocking solution. After washing, a TUNEL reaction was performed using TAMRA-dUTP (Roche) and terminal deoxynucleotidyl transferase according to the manufacturer's protocol.

3. Results

3.1. PSF is required for reproductive viability and contributes to radioresistance in human cells

Prior work has shown that PSF stimulates homologous recombination and nonhomologous end joining reactions in cell-free systems. To determine whether PSF contributes to radioresistance *in vivo*, we designed a miRNA targeted to the sequences in the 3′ untranslated region of PSF mRNA (Supplementary Table 1). Constructs expressing PSF miRNA or a previously described p54^{nrb} miRNA [26] were transfected into HeLa cells. Transfection efficiency was >80% based on expression of a linked EmGFP transfection marker at 72 h post-transfection (data not shown). The miRNAs reduced PSF expression by about 90% and p54^{nrb} by about 70%, using actin as an internal standard (Fig. 1A and B). The effect was selective: PSF miRNA had little or no effect on p54^{nrb} expression and vice versa.

Ideally, radioresistance may be analyzed using stable knockdown cell lines, as we have previously done for p54^{nrb} [26]. In practice, however, we were not able to establish stable human knockdown cell lines using PSF miRNA, consistent with previous suggestions that attenuation of PSF expression may be deleterious in human cells [11]. We therefore established a rescue assay in which PSF miRNA and PSF expression constructs were co-transfected into HeLa cells, which were scored for clonogenic